RNAi analysis of genes expressed in the ovary of *Caenorhabditis elegans*

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As a step towards comprehensive functional analysis of genomes, systematic gene knockout projects have been initiated in several organisms [1]. In metazoans like *C. elegans*, however, maternal contribution can mask the effects of gene knockouts on embryogenesis. RNA interference (RNAi) provides an alternative rapid approach to obtain loss-of-function information that can also reveal embryonic roles for the genes targeted [2,3]. We have used RNAi to analyze a random set of ovarian transcripts and have identified 81 genes with essential roles in embryogenesis. Surprisingly, none of them maps on the X chromosome. Of these 81 genes, 68 showed defects before the eight-cell stage and could be grouped into ten phenotypic classes. To archive and distribute these data we have developed a database system directly linked to the *C. elegans* database (Wormbase). We conclude that screening cDNA libraries by RNAi is an efficient way of obtaining *in vivo* function for a large group of genes. Furthermore, this approach is directly applicable to other organisms sensitive to RNAi and whose genomes have not yet been sequenced.

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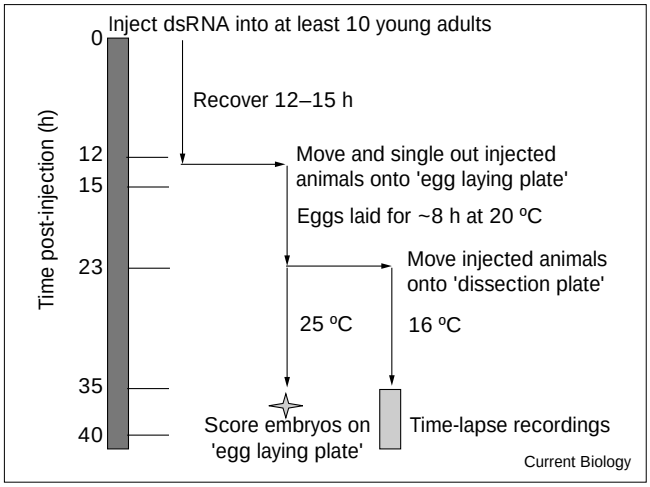
Figure 1



Strategy for phenotypic analysis. At least 10 animals were injected at time 0 with dsRNA derived from a single ovary clone. After recovery they were singled-out and left to lay eggs. The animals were removed from these plates and slowed down at 16°C, while their progeny were moved to 25°C to score for embryonic lethality. If more than 40% of embryos laid in the 'egg laying plate' did not hatch then injected animals were dissected to analyze the early embryo. If dissected animals contained no embryos (RNAi-induced sterility) then the injection was repeated and embryos recorded around 24 h post-injection.

Results and discussion

Embryogenesis in *C. elegans* provides a window for viewing fundamental cellular [4–6] and developmental [7,8] processes, and RNAi provides a way of discovering genes important in these stages. To increase the chance of finding genes important in early embryos we used RNAi to test genes expressed in the ovary. To detect the earliest defects resulting from RNAi we used a two-step strategy (Figure 1, and see Supplementary material). We first identified animals that laid more than 20% dead embryos between 15 and 24 hours post-injection (embryonic lethals). We next collected time-lapse recordings of the cellular events in early embryos derived from those same animals about 35 hours after injection. When genes were found to be also required for egg production, we reinjected the same dsRNA into new animals and made time-lapse recordings of early embryos before egg production ceased, usually 24 hours post-injection.

From a total of 350 ovarian cDNAs tested by RNAi, 101 were found to cause embryonic lethality. These 101 cDNAs encoded 81 different genes; RNAi of 36 of these also led to egg production defects in the injected animal (Table 1). This suggests that more than a quarter of the genes transcribed in the ovary are required during embryogenesis or oogenesis. When we compared these 81 genes with those predicted in the *C. elegans* proteome (WORMPEP [9]), all but three had sequence in common with predicted genes. Splice predictions and gene boundaries were, however, not always consistent between our cDNAs and WORMPEP. This observation agrees with the results of a large-scale comparison that found differences

Table 1

Summary of screen.

Clones analyzed	350
Clones showing embryonic lethal phenotypes by RNAi	101
Genes required for embryogenesis	81
Genes required for oogenesis	36
Genes that function before eight-cell stage	68
Percentage of genes required for embryogenesis showing a BLAST hit with *P* values < e-35 against organism shown	
Saccharomyces cerevisiae	64
Drosophila melanogaster	72
Homo sapiens	81
Chromosome distribution of genes required for embryogenesis (expected/observed)*	
Chromosome 1	11/22
Chromosome 2	14/15
Chromosome 3	10/16
Chromosome 4	13/15
Chromosome 5	20/12
Chromosome X	12/0

*One gene is omitted from this analysis as it is within unfinished genome sequence.

between cDNA data and the predicted *C. elegans* proteome in over half of the cases (D. Thierry-Mieg, J. Thierry-Mieg and Y. Kohara, personal communcation).

Surprisingly, although the 81 genes are distributed randomly among the autosomes and show no evidence of clustering (data not shown), none is on the X chromosome (Table 1). On the basis of known gene distribution in the *C. elegans* genome and our sample size, we would have expected 12 genes to map on the X chromosome. This result leads us to speculate that there must be a strong selection against maintaining genes important for oogenesis or embryogenesis on the X chromosome. A paucity of X-linked genes has also been noted among a set of genes with enriched germline expression identified by microarray analysis [10].

Our results show that *C. elegans* ovary-expressed genes required for embryogenesis encode mostly conserved proteins. Sequence comparison of the 81 predicted genes and their corresponding expressed sequence tags (ESTs) with other genomes showed that the majority have significant BLAST scores (Table 1). The fraction of proteins conserved at levels reported here is at least twice that seen when the whole genome is analyzed. A similar trend has been observed among genes with visible phenotypes in *Drosophila melanogaster* [11]. In addition, because our library was not normalized it is likely that our sample set is enriched for abundantly expressed genes, which have also shown a higher likelihood of being conserved [12,13]. Our results thus lend support to the idea that high expression level, essential function and broad sequence conservation are all positively correlated.

We used the earliest deviation from wild type to classify the genes we found. The penetrance of RNAi-induced embryonic lethality differed among the 81 genes. Because of practical considerations, the six genes giving rise to 20–40% lethality were not analyzed in detail. Of the remaining 75, 68 (90%) showed defects during the first 50 minutes of development. Most genes also show additional phenotypes, however, which led us to build a database system to distribute the raw data (described below). These 75 genes fell into ten phenotypic classes (Figure 2). The first and largest category (39 genes) cause arrest as abnormal one-cell stage embryos (class 1, Figure 2). In addition, for 36 of the 39 genes in this class, the injected mothers ceased egg production prematurely. Significantly, all of the 29/81 genes predicted to encode proteins involved in protein synthesis or turnover fell into this class. This result underscores the importance for dynamic control of protein production during the earliest stages of *C. elegans* development.

In the second class we placed genes that have a role in forming the polar bodies or the pronuclei (Figure 2i). Most of these genes would be expected to have a role in meiosis or cytokinesis. Indeed, in this class we found genes already known to be required for meiosis, including the katanin p60 homolog *mei-1* [14] and dynein heavy chain (*dhc-1*) [15], and seven more genes including a kinesin-like gene (*klp-15*) [16] and a cyclin-like gene (H31G24.4).

The third class is defined by a single gene (Y49E10.19) that is required for an early event in the one-cell stage termed 'pseudocleavage' (Figure 2j). This gene is similar to *D. melanogaster* anillin, an actin-binding protein involved in cytokinetic furrow formation [17]. In the next four classes we found genes required to position the mitotic spindle or genes which are involved in different aspects of cell division (class 4–7, Figure 2l–n). Two additional classes (8 and 9) included genes affecting general properties of the embryo. Even in these classes, the phenotypes correlated with the molecular classification of the genes: RNAi analysis of genes predicted to be involved in RNA splicing resulted in granular cytoplasm (class 8, Figure 2o), and those predicted to be involved in energy production led to a general slowdown in embryogenesis (class 9). Finally, the last class (class 10) was made up of genes whose function is required to complete embryogenesis but for which no defects in the early embryo were observed.

We next wondered how the RNAi phenotype data compared with previously reported mutant phenotypes. We found that only seven genes whose functions we identified through RNAi had been previously identified through classic genetic analysis. Notably, three of these (*mex-3*, *mex-6* and *mei-1*) were identified in screens for maternal-effect lethal mutations [14,18,19]. When we compared the known genetic phenotypes with the RNAi phenotypes we found that our RNAi data agreed with the reported phenotype for

Figure 2

Gene classification according to first defects observed in the early embryo. **(a–g)** Wild-type embryo; **(h–o)** RNAi phenotypes derived from ovarian cDNA clones. (h) *sp4h2*(*RNAi*), which matches a predicted polyubiquitin homolog, shows catastrophic one-cell arrest with vacuoles coalescing near center. (i) *sp7h7*(*RNAi*), matching *mei-1*, a microtubule-binding protein required in meiosis shows multiple small polar bodies (arrows). (j) *sp11d5*(*RNAi*), which matches Y49E10.19, a predicted gene with sequence similarity to *D. melanogaster* and human actin-binding protein anillin, lacks a pseudocleavage furrow seen in the wild type at this stage (compare with (c) arrow). (k) *sp12h8*(*RNAi*), matching *sur-6*, a putative regulatory subunit of protein phosphatase 2A, shows super-asymmetric positioning of the first mitotic spindle, a phenotype associated with microtubule-based defects (arrows). (l) *sp4d1*(*RNAi*), matching the predicted gene K12D12.1, a member of the DNA topoisomerase II family, does not reform nuclei after first division. (m) *sp15f8*(*RNAi*), matching the predicted gene C39E9.13, a possible subunit of DNA replication factor C, shows delayed cell-cycle timing in the P1 cell (compare with nuclear breakdown in (f) arrow). (n) *sp5A8*(*RNAi*), matching the predicted gene F55F8.4, shows aberrant cell–cell contacts. (o) *sp8F11*(*RNAi*), matching the predicted gene W07E6.4 which is a putative mRNA splicing factor, shows cytoplasm containing large granules (arrows). Note that of the genes shown, only *mei-1* has been previously reported by mutant analysis [14].

Class	Wild type	RNAi phenotype	Description of class	Number of genes
1	(a)	(h)	Egg production and activation	39
2	(b)	(i)	Formation of polar bodies and pronuclei	9
3	(c)	(j)	Pseudocleavage	1
4	(d)	(k)	Nuclear migration and spindle positioning	2
5	(e)	(l)	Division of P0 (cytokinesis; chromosome separation; nuclear membrane formation in daughters)	5
6	(f)	(m)	Division of AB and P1 (including relative timing)	4
7	(g)	(n)	Cell position and contacts	1
8		(o)	Appearance of cytoplasm	2
9			Rate of early embryogenesis	5
10			No defects detected in first 50 min	7

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mei-1 (Figure 2i) and for the fragment of *mex-6* used, which also targets *mex-5* [19]. However, our *mex-3* transcript gave a phenotype in the early embryo that was not previously reported, and which could be due to the fact that we have analyzed a splicing variant for this gene, as shown by our EST sequence. The other four genes (*daf-21*, *emb-5*, *sur-6* and *act-2*) [20–23] are known to have pleiotropic effects, including an embryonic requirement. The only gene in this group to have been analyzed genetically in the early embryo is *emb-5*, where only one allele showed subtle defects [24]. These were not seen in our RNAi analysis. It is noteworthy, however, that the other three genes show embryonic RNAi defects before the eight-cell stage that provide clues into their function in the early embryo.

In order to distribute the data described here, we developed a new database on top of the ACeDB database management system [25], using the AcePerl and AceBrowser software modules [26]. This database is freely accessible to the public at http://www.rnai.org and the software will also be made available on an Open Source basis at http://stein.cshl.org. Searching the database can be accomplished using accession numbers (GenBank or Wormbase) for the gene used in the interference experiment as well as by using plain text. Each record includes links to Wormbase and GenBank, a brief description of the observed phenotype, and a set of fields that use a controlled vocabulary to describe common phenotypic features. These records are accompanied by the raw data in the form of compressed digital video segments (Figure 3).

We have shown that RNAi testing of ovary-expressed genes coupled with phenotypic analysis of the embryo is an efficient way of quickly obtaining *in vivo* functional data for a large number of genes in the well defined context of early embryogenesis in *C. elegans*. Moreover, the use of a cDNA library as starting material does not require a sequenced genome and can be used in other organisms sensitive to RNAi. We have found a high level of conserved genes along with several 'novel' genes whose developmental role can be assigned in the early embryo. As many other nematode species cause disease and economic damage around the globe [27], nematode-specific genes found here may also provide starting candidates for developing anti-nematode agents.

Supplementary material

Supplementary material including full methodological detail is available at http://current-biology.com/supmat/supmatin.htm.

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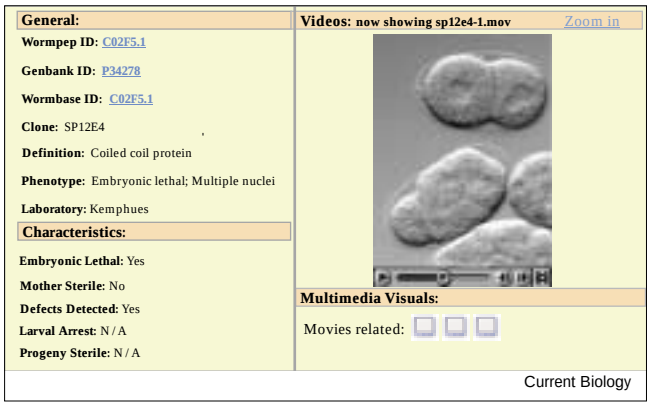
Figure 3



Screen shot of one example from the RNAi database. Result of searching for C02F5.1. The left side shows links to other databases and RNAi-derived phenotypic descriptions. The right side shows the raw data as time-lapse digital movies (single frame shown) of embryos from mothers injected with dsRNA. Other movies from the same RNAi test are linked.

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