A genome-wide RNAi screen for enhancers of par mutants reveals new contributors to early embryonic polarity in Caenorhabditis elegans

Diane G. Morton¹, Wendy A. Hoose and Kenneth J. Kemphues

Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY 14853
D. G. Morton et al.

Running Title: RNAi screen for enhancers of par mutants

Key words: polarity, RNAi screen, par genes, nop-1, strd-1

1 Corresponding author:
Diane G. Morton
Department of Molecular Biology and Genetics,
Cornell University
433 Biotechnology Building
Ithaca, NY 14853-2703
E-mail: dgm7@cornell.edu

Telephone: (607) 254-4804
Fax: (607) 255-6249
The *par* genes of *Caenorhabditis elegans* are essential for establishment and maintenance of early embryo polarity and their homologs in other organisms are crucial polarity regulators in diverse cell types. Forward genetic screens and simple RNAi depletion screens have identified additional conserved regulators of polarity in *C. elegans*; genes with redundant functions, however, will be missed by these approaches. To identify such genes, we have performed a genome-wide RNAi screen for enhancers of lethality in conditional *par-1* and *par-4* mutants. We have identified eighteen genes for which depletion is synthetically lethal with *par-1* or *par-4*, or both, but produces little embryo lethality in wild type. Fifteen of the eighteen genes identified in our screen are not previously known to function in *C. elegans* embryo polarity and eleven of them also increase lethality in a *par-2* mutant. Among the strongest synthetic lethal genes, polarity defects are more apparent in *par-2* early embryos than in *par-1* or *par-4*, except for *strd-1(RNAi)*, which enhances early polarity phenotypes in all three mutants. One strong enhancer of *par-1* and *par-2* lethality, *F25B5.2*, corresponds to *nop-1*, a regulator of actomyosin contractility for which the molecular identity was previously unknown. Other putative polarity enhancers identified in our screen encode cytoskeletal and membrane proteins, kinases, chaperones, and sumoylation and deubiquitylation proteins. Further studies of these genes should give mechanistic insight into pathways regulating establishment and maintenance of cell polarity.
INTRODUCTION

The regulation of cell polarity is essential for development and function of diverse cell types. The six par genes, first identified for their role in regulating polarity in the one-cell Caenorhabditis elegans embryo (KEMPHUES et al. 1988; WATTS et al. 1996; MORTON et al. 2002) are central to the coordinated organization of polarity in a variety of cell types, including epithelial tissues, migrating cells, neurons and oocytes (reviewed in ST JOHNSTON and AHRINGER 2010; NANCE and ZALLEN 2011). In the C. elegans zygote, the par genes are required for segregation of cytoplasmic components including developmental determinants and cell timing regulators. Because of the high degree of conservation of the PAR proteins and their partners, C. elegans remains an excellent system for identifying key regulators of polarity.

The genes par-1 and par-4 encode conserved serine-threonine kinases. PAR-1 is related to mammalian MARK kinases; PAR-4 is the C. elegans homolog of LKB1, the upstream regulatory kinase of the AMPK kinase family, including MARKs (reviewed by ALESSI et al. 2006). LKB1 is a known tumor suppressor for which mutations lead to Peutz-Jegher’s syndrome and predisposition to cancer in epithelial tissues such as the intestine (HEMMINKI et al. 1998; JENNE et al. 1998). In the C. elegans embryo, PAR-1 and PAR-4 are essential for asymmetric localization of P granules and developmental regulators such as MEX-5 and PIE-1, and for asynchronous cell divisions of the early blastomeres (KEMPHUES et al. 1988; MORTON et al. 1992; GUO and KEMPHUES 1995; TENENHAUS et al. 1998; CUENCA et al. 2003; CEEKS et al. 2004; BUDIRAHARDJA and GONCZY 2008; RIVERS et al. 2008; TENLEN et al. 2008). However, mutations in either par-1 or par-4 do not strongly disrupt localization of the anterior PAR proteins (PAR-3, PAR-6 and PKC-3), or of the posteriorly-localized PAR-2 protein (ETEMAD-
While PAR-1 is itself localized to the posterior cortex during the first cell cycle, PAR-4 is not asymmetrically distributed in the early embryo (Guo and Kemphues 1995; Watts et al. 2000). That the anterior and posterior PAR proteins can become asymmetrically localized without PAR-1 or PAR-4 function suggests that PAR-1 and PAR-4 act downstream of the other PARs in the C. elegans embryo and are not essential for excluding the anterior PAR proteins, or that there are additional polarity components with redundant functions for these kinases.

Screens for maternal effect lethal mutations or RNAi knockdowns for phenotypes similar to the par mutants have enabled identification of many key polarity regulators and provided mechanistic insight into polarity establishment and maintenance (Cowan and Hyman 2007; St Johnston and Ahringer 2010). Yet there are likely to be additional gene products whose role in polarity is masked either by having redundant functions or by inefficient RNAi knockdown.

RNAi based screens for enhancers or suppressors of existing mutations provide a powerful means to identify additional genes with previously undetected roles in specific developmental processes. RNAi screens have identified suppressors of polarity mutants (Labbé et al. 2006; Spilker et al. 2009) and RNAi screens for synthetic lethality with conditional lethal mutants involved in morphogenesis (Sawyer et al. 2011), embryo elongation (Zahreddine et al. 2010) and neddylation (Dorfman et al. 2009) have also proved fruitful. (For a general discussion of RNAi screens, see Boutros and Ahringer 2008).

To uncover additional genes involved in early embryo polarity, we chose to perform an RNAi-based genome-wide screen for synthetic lethality with par-1 or par-4 conditional mutants. Such genes may give us insights into the functions of par-1 and par-4 as well as providing a more complete picture of the polarity system of the early embryo.
We have identified a set of 18 genes that when depleted by RNAi result in synthetic embryo lethality with par-1 or par-4 temperature-sensitive mutants under semi-permissive conditions, but have little effect in wild type. Several genes enhance lethality in par-1, par-4 and par-2 conditional mutants, suggesting that they may have polarity functions in C. elegans.

MATERIALS AND METHODS

Strains and culture: C. elegans strains were maintained by standard procedures (BRENNER 1974). Strains include N2 (Bristol) as wild type (WT), KK300 par-4(it57ts) V (MORTON et al. 1992), KK418 par-2(it5ts) III (KEMPHUES et al. 1988), KK196 lon-1(e185) par-3(e2074)III; sup-7(st5) X (CHENG et al. 1995), KK725 ncl-1(it142) III (ROSE et al. 1995), KK822 par-1(zu310ts) V, DH189 emb-9(b189ts) III (WOOD et al. 1980).

RNAi screen: Individual RNAi clones from the library of (KAMATH et al. 2003) were grown in 96-well trays overnight in LB broth containing 100 µg/ml ampicillin and 50 µg/ml tetracycline for RNAi by feeding (TIMMONS et al. 2001). The overnight cultures were diluted 1:100 in fresh media and grown for 3 hours, induced to express dsRNA by addition of IPTG (isopropyl β-D-thiogalactoside) to 100µM for 2 more hours, and concentrated five-fold for feeding.

To prepare worms for screening, KK300 par-4(it57ts) and KK822 par-1(zu310ts) worms were propagated at permissive temperature (16°), the embryos isolated by bleach treatment and hatched into M9 medium to synchronize populations. L1 larvae were transferred to NGM plates with OP50 bacteria to grow to L3-L4 stages. The larvae were washed and suspended in M9 containing 50 µg/ml ampicillin and approximately 10 worms in 60µl pipetted into wells of 96-well dishes. 30 µl of induced RNAi feeding bacteria from individual clones were added per well
and plates were incubated at semi-permissive temperature (19° for zu310 and 17° for it57). Plates were scored approximately four days later for production of unhatched embryos and results compared to those of (KAMATH et al. 2003) for N2, and to a parallel screen performed by Kelly Liu using emr-1 and lem-2 mutant strains, which do not affect embryo viability (unpublished). Positive controls included RNAi clones for par-2 and par-4. L4440 empty vector was the negative control (TIMMONS and FIRE 1998). In our hands, approximately 1-2% of the clones in the RNAi library did not grow, thus the number of genes that we tested represents about 16,500 genes, or 85% of the C. elegans genome.

For those RNAi clones that resulted in embryo lethality in par-1(zu310) or par-4(it57), RNAi tests were repeated in liquid as above, but viability of N2 embryos was assessed in parallel. RNAi clones causing lethality in par mutants but not in N2 were sequenced and compared to WormBase annotations (HARRIS et al. 2010) to verify identity and were retested by feeding L4 larvae on agar plates containing minimal medium with 12.5 ug/ml tetracycline and 50 ug/ml carbenicillin. Worms were transferred to fresh plates each day, and larvae and unhatched eggs counted to determine percent embryo lethality. Percent embryo lethality was determined from two or more independent RNAi inductions. Differences in lethality from L4440 vector-fed worms were computed by 1-tailed Student’s T-test, and a cut-off of p <0.05 was used to determine whether to include the RNAi clone in our set of enhancers. For RNAi in par-3(e2074); sup-7, 2-tailed T-tests were performed, since either suppression or enhancement of lethality might result. Some genes related to those in our screen were subsequently tested from the ORFeome-based RNAi library of (RUAL et al. 2004).

For RNAi of par-1, a clone containing nucleotides 1358-2952 (central linker domain) was used (HURD and KEMPHUES 2003). For RNAi of par-4, we used a feeding construct of par-4
D. G. Morton et al.

cDNA beginning at nucleotide 15 and extending through the gene into the 3’ UTR (gift of Daryl Hurd).

A fragment of F25B5.2 cDNA (nucleotides 1-425) was inserted into the L4440 vector and used for RNAi by feeding to confirm the Nop (no pseudocleavage) phenotype observed upon depletion of F25B5.2 from the RNAi feeding library (Kamath et al. 2003), which targets a different region of the gene.

Sequence analysis of nop-1: F25B5.2 cDNA was isolated from a C. elegans cDNA library (Gibco Proquest) by PCR amplification using iProof polymerase (Bio-Rad) with a forward primer containing ATGTCGGCGCCGAAGAAACGAAAAAG and reverse primer containing AGCTCAAATTTTAGCTCCAAACACAGTTG, cloned into a TOPO blunt vector (Invitrogen) and sequenced. This cDNA sequence was compared to the predicted transcript on Wormbase (Version WS231) by sequence alignment and has been deposited with Genbank (Accession JX293831). For genomic DNA sequencing, individual wild type and nop-1(it142) worms were lysed by Proteinase K treatment and their DNA amplified using primers to cover the F25B5.2 gene.

Immunofluorescence and microscopy: Primary antibodies included rabbit anti-PAR-1 (Guo and Kemphues 1995), monoclonal mouse anti-PAR-3 (Nance et al. 2003) and OIC1D4 monoclonal mouse anti-P granule (Strome 1986). Monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank, University of Iowa. Secondary antibodies included Alexa Fluor 594-labeled goat anti-mouse or anti-rabbit (Invitrogen) and Cy3-labeled donkey anti-mouse or anti-rabbit (Jackson ImmunoResearch). Fixations were done in methanol at -20° degrees for 15 minutes. For P granule staining, a post-methanol acetone step was included (Strome and Wood 1983). Slides were mounted with Vectashield containing DAPI.
D. G. Morton et al.

(Vector Laboratories). Images and time-lapse movies were acquired on a Leica DM RA2 microscope fitted with a Hamamatsu Orca-ER digital Camera using OpenLab software (Improvision). OpenLab software was used to measure length of PAR-3 anterior-posterior domain and overall length of embryos. Measurements were taken twice for each embryo. For live imaging, embryos were mounted on 2% agarose pads on microscope slides and covered with a coverslip. The temperature of the microscope room was controlled to the semipermissive temperature of the mutant being analyzed.

RESULTS

Identification of enhancers of conditional par-1 or par-4 mutants: To identify new components of the PAR polarity system in the C. elegans embryo, we screened a library of RNAi clones (Kamath et al. 2003) for those that increased embryo lethality in the weak par mutants, par-1(zu310ts) or par-4(it57ts). These par mutations each result in a single amino acid substitution in the mutant proteins (Watts et al. 2000; Spilker et al. 2009) and the mutant embryos show typical par phenotypes at the nonpermissive temperature of 25°. Under semipermissive conditions, 19° for par-1(zu310) and 17° for par-4(it57), approximately 10-20% of par-1(zu310) embryos and 20-35% of par-4(it57) embryos fail to hatch. Among the approximately 16,500 genes that we screened by RNAi (see Materials and Methods), we initially identified 709 candidate clones that appeared to increase embryo lethality of a par mutant, but had not been reported to cause embryo lethality in wild-type worms in the screen of (Kamath et al. 2003). Most of these 709 candidates were identified as par-4-enhancing clones, perhaps because our screen was done closer to the temperature-sensitivity threshold for par-4(it57), and this strain is more sensitive to temperature fluctuation than is par-1(zu310). We retested the candidate clones, comparing them side-by-side with embryo lethality in the wild-type N2 strain,
and narrowed our list to 105 that appeared to cause increased lethality in \textit{par-1} or \textit{par-4}, or both, but little embryo lethality in wild type (Table S1). We then used RNAi feeding of L4 stage worms on plates, where we could assess more accurately the percent lethality by counting embryos and larvae, to further test these RNAi clones for their effects on lethality in \textit{par-1(zu310)} and \textit{par-4(it57)} compared to wild-type worms. In performing RNAi tests with worms at this late larval stage, we may have eliminated some RNAi clones for which longer periods are required for depletion, but our goal was to identify the strongest enhancers of the \textit{par} mutants. Through this final step of screening we identified 19 RNAi clones that significantly increased lethality in \textit{par-1(zu310)} or \textit{par-4(it57)} compared to RNAi in wild-type worms (Table 1).

Seventeen out of nineteen RNAi clones significantly increase lethality in \textit{par-1(zu310)} under semipermissive conditions, and among these, seven also enhance lethality of \textit{par-4(it57)}. RNAi of two genes increased lethality in \textit{par-4(it57)} but not in \textit{par-1(zu310)}.

The putative functions of the lethality-enhancing genes identified in our screen include phosphorylation (\textit{strd-1, pig-1, ZK856.8, kin-10}), membrane or cytoskeletal proteins (\textit{mes-1, unc-59, T24F1.2, npp-22, str-43, ooc-3, lgl-1}), sumoylation and ubiquitination (\textit{gei-17, math-33, uba-2}), chaperones (\textit{hsp-2, sti-1}), and unknown (\textit{ntl-9, F25B5.2}) (Table 1). The RNAi effect of \textit{hsp-2}, a pseudogene with high sequence similarity to the \textit{C. elegans} HSP-70A family gene \textit{hsp-1} (HESCHL and BAIIIE 1989), is probably not specific for \textit{hsp-2}. We tested an \textit{hsp-1} RNAi clone, and as with \textit{hsp-2} RNAi, observed increased embryo lethality in \textit{par-4(it57)} (Table S2). Thus, the increased lethality in \textit{par-4(it57)} from \textit{hsp-2} RNAi is most likely due to knockdown of \textit{hsp-1}. We also see nonspecific RNAi effects from clone sjj_F23F12.2, which corresponds to an intronic region of \textit{F23F12.3} and has multiple gene targets for RNAi. We were unable to distinguish among these possible targets the source of the sjj_F23F12.2 RNAi effect. Eliminating that clone
leaves us with 18 genes identified by RNAi as enhancers of embryo lethality in \textit{par-1}, \textit{par-4}, or both mutant backgrounds (Table 1, Figure 1 A,B).

Three of the genes we identified, \textit{mes-1}, \textit{lgl-1} and \textit{ooc-3}, have known polarity functions in the early embryo, and their detection supports the idea that genes encoding new polarity components could be identified by our screen. \textit{mes-1} encodes a transmembrane protein required for asymmetric divisions of the germline precursor cells P2 and P3 (Berkowitz and Strome 2000; Bei et al. 2002) and for localizing PAR-2 at the four-cell stage (Arata et al. 2010). LGL-1 is a conserved polarity protein that has been shown to act redundantly with PAR-2 in the one-cell embryo (Beatty et al. 2010; Hoenge et al. 2010) and \textit{ooc-3} encodes an endoplasmic reticulum membrane protein required for proper localization of PAR proteins in the P1 cell of the two-cell embryo (Basham and Rose 1999; Pichler et al. 2000). While RNAi of \textit{mes-1} strongly enhanced embryo lethality of both \textit{par-1} and \textit{par-4} temperature-sensitive mutants, RNAi of \textit{ooc-3} only weakly increased lethality of \textit{par-1}(zu310) and did not increase lethality of \textit{par-4}(it57). RNAi depletion of \textit{lgl-1}, which has been shown to enhance lethality in weak \textit{par-2} mutants (Beatty et al. 2010; Hoenge et al. 2010) caused increased lethality in \textit{par-1}(zu310) but not in \textit{par-4}(it57).

Two genes identified in our screen, \textit{strd-1} and \textit{pig-1}, although not known previously to have roles in the early embryo, have been reported to act in neuronal cell polarity (Cordes et al. 2006; Kim et al. 2010) and in a caspase-independent programmed cell death pathway in \textit{C. elegans} (Denning et al. 2012). \textit{strd-1}, which encodes the \textit{C. elegans} ortholog of the mammalian STRAD$\alpha$ cofactor for LKB1, is a strong RNAi enhancer of embryo lethality in both \textit{par-1} and \textit{par-4}, consistent with a previous report of synthetic lethality between \textit{strd-1} mutants and \textit{par-1} or \textit{par-4} temperature-sensitive mutants at permissive temperature (Narbonne et al. 2010).
While in mammalian systems STRAD and LKB1 act together in polarization of neurons (Barnes et al. 2007; Shelley et al. 2007), in C. elegans, strd-1 has been shown to act independently of par-4 in regulating neuronal polarity (Kim et al. 2010). pig-1, a strong enhancer of par-1 lethality in our screen, encodes a serine threonine kinase with similar structure to PAR-1 and is important for polarized divisions of certain neuroblasts during C. elegans larval development (Cordes et al. 2006). pig-1 is also required for cell shedding in caspase-independent apoptosis of specific cells in the embryo and both par-4 and strd-1 appear to act upstream of pig-1 in this process (Denning et al. 2012). RNAi of another PAR-1-related kinase gene, sad-1, required for C. elegans neuronal cell polarity (Crump et al. 2001; Hung et al. 2007; Shelley and Poo 2011) that is not expressed in early embryos (Crump et al. 2001), did not enhance par-1(zu310) lethality. sad-1 is as similar in sequence to the par-1 gene as pig-1 and was used as a negative control in our RNAi experiments (Table 1).

Two RNAi clones in our set, corresponding to uba-2 and kin-10, cause more than 20% embryo lethality in wild type C. elegans, but the lethality is significantly increased in combination with par mutations. For kin-10, which encodes a regulatory subunit of Casein Kinase II, this effect is stronger for par-4(it57). RNAi of the uba-2 gene, encoding a SUMO-activating protein, results in similar embryonic lethality levels in either par-1(zu310) or par-4(it57) mutants. To assess whether this was due to its role in the sumoylation pathway, we also tested RNAi of aos-1, which was not present in the RNAi library used for screening. AOS-1 forms a heterodimer with UBA-2 and both subunits act at the first step of protein sumoylation by activating SUMO (Johnson and Blobel 1997; Muller et al. 2001). We found that aos-1 RNAi also increases embryo lethality of par-1(zu310) and par-4(it57) (Table S2). gei-17, which
encodes a sumo ligase (HOLWAY et al. 2005), was also identified in our screen, suggesting that sumoylation may be important for polarity or PAR protein function in C. elegans.

ntl-9 encodes a protein with high similarity to Rcd1-like proteins, part of the CCR4-NOT complex which has diverse functions including transcription, deadenylation and ubiquitination (reviewed by PARKER and SHETH 2007; COLLART and PANASENKO 2012). Some C. elegans CCR4-NOT proteins are involved in RNA processing (SCHMID et al. 2009), but the function of ntl-9 has yet to be elucidated.

One gene of unknown function identified in our screen, F25B5.2, is has no obvious homologs outside of Nematodes and corresponds to nop-1, a gene previously identified by mutation (ROSE et al. 1995) that functions in cortical contractility of the one-cell embryo (see below).

Seven of the genes that we identified in our screen enhanced lethality of both par-1(zu310) and par-4(it57). However, one strong par-4-specific enhancer, corresponding to the HSP90/HSP70 co-chaperone sti-1, not only failed to enhance par-1(zu310) lethality but resulted in slightly higher viability of par-1(zu310) embryos relative to controls (Table 1, Figure 1A).

**Most of the enhancers of par-1 and par-4 embryo lethality also increase lethality in a weak par-2 mutant, but not in a par-3 mutant:** We tested whether the genes that we identified could also enhance another conditional par mutant, par-2(it5ts). par-2 encodes a RING-domain-containing ubiquitin ligase that associates with the posterior cell cortex of the zygote, is required to keep the anterior PAR proteins from reentering that domain, and for PAR-1 to localize to the posterior cortex (LEVITAN et al. 1994; BOYD et al. 1996; CUENCA et al. 2003; HAO et al. 2006). We found that RNAi of eleven of the eighteen genes identified in our screen also caused
increased embryo lethality in a *par-2* mutant background (Table 2, figure 1C); five of them (*strd-1, math-33, uba-2, gei-17, T24F1.2*) increase embryo lethality in all three *par* mutants (Figure 2).

Because several of the genes identified in our screen appear to act synergistically with two posterior domain protein-encoding genes, *par-1* and *par-2*, we wondered what their effect might be if an anterior *par* gene were compromised instead. We do not have temperature-sensitive mutants of *par-3, par-6* or *pkc-3* that would enable us to test this, but we were able to make use of a nonsense allele of *par-3* that can be partially suppressed by the *sup-7* tRNA amber suppressor (Cheng *et al.* 1995). In *par-3(e2074); sup-7(st5)*, approximately 45% of embryos are viable at 22°C. We determined the percent embryo viability when worms of this genotype were treated with RNAi for each of the genes that enhanced lethality in *par-1* or *par-4*. We found that RNAi of *uba-2, ntl-9, gei-17, nop-1, sti-1* and *kin-10* caused a significant increase in embryo lethality in the *par-3* mutant compared to control RNAi whereas others did not (Table 2, Figure 1D). The embryonic lethality observed for *kin-10* RNAi, however, is not significantly higher than that expected by adding the lethality produced in wild type (26%) to the lethality levels of *par-3(e2074); sup-7* controls (56.5%).

We also found that two RNAi clones, those corresponding to *lgl-1* and *math-33*, suppressed embryo lethality in the *par-3(e2074) sup-7* strain, suggesting that these two genes act antagonistically to *par-3*.

Several of the genes identified in our screen enhance lethality of multiple *par* mutants. Such genes may be acting in polarity processes or could be non-specific in their effect on conditional mutations. To address this, we tested whether the enhancers could also increase lethality in *emb-9(b189ts)*, which has a temperature-sensitive period late in embryogenesis (Wood *et al.* 1980). *emb-9* encodes the alpha chain of type IV collagen (Guo *et al.* 1991) and is
required for epidermal elongation during late embryogenesis as well as for gonadogenesis and larval development, but mutations do not compromise the early embryo (SCHIERENBERG et al. 1980; GUPTA et al. 1997). From *emb-9(b189)* worms shifted to the semipermissive temperature of 21° at the L4 stage, we found that 3% of *emb-9(b189)* embryos fail to hatch (n=2476), while young adults shifted to 25° produce 100% embryonic lethality (n=1244) and L4 worms shifted to 25° are sterile. Testing the effects of depleting our enhancer genes on *emb-9(b189)* at 21°, we found that only *gei-17* and *hsp-2* significantly increased embryo lethality (to 15% and 7% lethality respectively), though to a much lower extent than their effects on the *par* mutants (Supplementary Figure 1). Thus these two genes may be non-specific in their effect, or may act at multiple times in embryogenesis. Because RNAi of the remaining genes more specifically enhances lethality of weak *par* mutants they are good candidates for early functions in embryogenesis that influence processes mediated by the PAR proteins.

**F25B5.2 is nop-1.** One of the strongest enhancers of *par-1* and *par-2* conditional mutants to come out of our screen is *F25B5.2*. Most of the genes identified in our screen have homologs that suggest how they may function; *F25B5.2* does not. We observed that in *par; F25B5.2(RNAi)* one-cell embryos lack pseudocleavage, which is an actomyosin-driven constriction of the cell cortex early in the first cell cycle, at approximately the time of polarity establishment (HILL and STROME 1988; HIRD and WHITE 1993; MUNRO et al. 2004). Because *F25B5.2* is located in the same genetic region as a mutant lacking pseudocleavage, *nop-1(it142)* (ROSE et al. 1995), for which the molecular identity was unknown, we examined one-cell embryos produced by wild-type worms treated with *F25B5.2* RNAi, and found that even in the absence of a *par* mutation, depletion resulted in a Nop (no pseudocleavage) phenotype remarkably similar to the *nop-1* mutant (Figure 3, File S1).
A wild type cDNA clone corresponding to F25B5.2 shows a subtle splice variant resulting in two additional codons at the beginning of exon 6 compared to the predicted transcript for F25B5.2 in Wormbase (Version WS231). The coding region is the same in other respects and encodes a predicted 759 amino acid protein of unknown function, conserved among nematodes, but without obvious homologs in other animals. Sequence analysis of the F25B5.2 gene in the nop-1(it142) mutant revealed a G to A mutation predicted to change the codon for W698 (or W696 in the shorter form) to a UGA stop, to produce a truncated protein. Searches for conserved domains in the predicted NOP-1 protein by BLAST (Altschul et al. 1990), Pfam (Finn et al. 2010) or SMART (Letunic et al. 2009) database searches failed to identify any protein motifs that could suggest function.

It was previously reported that nop-1(it142) mutants have variable embryonic lethality (Rose et al. 1995) up to 23%; we observe only 5.3% embryo lethality (N=2548) in the KK725 nop-1(it142) strain, and this is increased to 15.3% embryonic lethality when nop-1(it142) worms are treated with nop-1(RNAi) (N=1680). A second RNAi construct representing a different portion of F25B5.2 than that used for our screen (see Materials and Methods) produces similar embryonic lethality: 1% in N2 (N=827) and 8% in nop-1(it142) (N= 752) and also results in embryos lacking a pseudocleavage furrow when knocked down in wild type (40/40). It seems likely that nop-1 is not absolutely essential for viability, but is important for actomyosin contractility in the zygote.

**Most enhancers do not increase polarity phenotypes in early embryos of par-1 and par-4:** RNAi depletions that enhance lethality of weak par-1 and par-4 mutants may or may not do so by compromising PAR-1 and PAR-4 function in early embryonic polarity. Therefore, we investigated whether the enhancers affected the diagnostic early polarity phenotype of
mislocalization of P granules. P granules, non-membranous ribonucleoprotein organelles, are segregated to the posterior during the first cell cycle and subsequently to the germline lineage in *C. elegans* embryos (STROME and WOOD 1982), but are destabilized in embryos of strong *par-1* or *par-4* mutants and fail to localize during the first cell cycle (KEMPHUES et al. 1988; CHEEKS et al. 2004; GALLO et al. 2008). We examined P granules in 52 *par-1(zu310)* two-cell embryos at semi-permissive temperature by immunolocalization, and found that P granules were segregated properly to the P1 cell in all of them (Table 3, Figure 4). Choosing clones for RNAi treatment which resulted in greater than 70% lethality in *par-1(zu310)* at the semi-permissive temperature, we assessed whether the embryos exhibited P granule distribution defects similar to loss-of-function *par-1* mutations at the two-cell stage. Contrary to our expectations, we found that RNAi of most of the *par-1* enhancers had no effect on P granule distribution at this stage in the *par-1* sensitized background in spite of producing high rates of lethality (Table 3; left column). Only *strd-1* RNAi caused a high frequency of P granule mislocalization and loss of P granule aggregation in *par-1(zu310)* 2-cell embryos at 19° (39/43 embryos, Figure 4, Table 3).

In *par-4(it57)* at semipermissive temperature, none of the 53 two-cell embryos examined showed P granule mislocalization (Table 3). Consistent with our results with *par-1(zu310)*, we found that most of the enhancers of *par-4(it57)* lethality had little or no effect on P granule distribution. *strd-1* RNAi caused *par-4(it57)* embryos to have P granules that appeared much fainter than those of control embryos and that failed to localize to the posterior cell (Figure 4), similar to phenotypes produced by reduction of *par-1* or *par-4* by RNAi. The co-chaperone gene *sti-1* also produced an abnormal P granule phenotype in *par-4(it57)* two-cell embryos when knocked down by RNAi, with very faint P granules by immunofluorescence. These unaggregated granules were mostly localized, but some ectopic granules remained in the anterior
D. G. Morton et al.

(Figure 4G, Table 3; middle column). The relatively normal P granule localization observed in
par-1(zu310) and par-4(it57) mutants treated with RNAi for the remaining lethality-enhancing
genes suggests that the lethality is not due to loss of polarity in the first cell cycle.

The enhancement of P granule mislocalization defects in par-1 and par-4 embryos by
strd-1 RNAi is higher than that previously reported by Narbonne and colleagues reported for the
effects of strd-1 mutations on these par mutants. One possible explanation for this difference is
that our tests were carried out at the semi-permissive temperatures of 19° for par-1(zu310) and
17° for par-4(it57) rather than at 15°. We repeated strd-1(RNAi) on par-1(zu310) and par-
4(it57) at 15° and observed lethality and P granule phenotypes very similar to those reported by
Narbonne for strd-1;par double mutants: 82% embryonic lethality for strd-1(RNAi)par-1(zu310)
,(N= 745) and 84% lethality for strd-1(RNAi)par-4(it57), (N=640) and only 23/43 in strd-
1(RNAi); par-4(it57) and 0/62 in strd-1(RNAi); par-1(zu310) early embryos showed P granule
mislocalization. Thus there appears to be a threshold temperature for this polarity phenotype in
the par-1 and par-4 mutants.

We also asked whether two other aspects of early asymmetry, cell division asynchrony
and P1 spindle orientation at the two-cell stage, were affected by the enhancers. In par-1 mutant
embryos at semi-permissive temperature, we observed that cells of 2-cell embryos exhibited
closer cell division times than wild-type embryos (45 seconds on average, instead of two
minutes). This loss of asynchrony was further enhanced by RNAi knockdown of strd-1, and to a
lesser extent by pig-1 and mes-1 (Table 4). In par-4(it57), strd-1 or sti-1 RNAi increased cell
division timing defects, consistent with their enhancement of the P granule mislocalization
phenotype. These early alterations in cell division timing could result in defects in developmental
pattern, leading to the embryonic lethality caused by RNAi of these genes. We also examined P1
spindle orientation in par-1 and par-4 two-cell embryos treated with RNAi for the enhancing genes. In wild-type embryos, the spindle of the anterior AB cell orients transverse to the long axis of the embryo while the posterior P1 cell centrosome-nuclear complex rotates 90° to orient along the long axis, and P1 always divides later than AB. A transverse P1 spindle orientation is sometimes observed in stronger par-1 or par-4 mutants (Kemphues et al. 1988) or with par-1 RNAi, but we did not see this phenotype in either par-1(zu310) or par-4(it57) embryos at semipermissive temperatures. Although we noted a very low frequency of transverse P1 spindles in par-1(zu310) embryos following RNAi with some of the enhancers we did not observe this in par-4(it57) for any of them (Table 4).

**Strong enhancers of par-2 lethality contribute to par-2 polarity phenotypes.** In contrast to our findings for par-1 and par-4, the enhancers of par-2(it5ts) embryo lethality strongly enhance polarity defects in par-2(it5) early embryos. In par-2(it5) at 25°, P granules are generally segregated into the posterior cell at the first division, but are mislocalized at the four-cell stage (Kemphues et al. 1988). At 16°, 100% of par-2(it5) four-cell embryos contain P granules only in the P2 cell (N=59), like wild-type embryos. When treated with RNAi for the seven genes that caused more than 70% lethality in par-2(it5ts) at 16°, we observed a high percentage of four-cell embryos with mislocalized P granules present in two or more cells for all but uba-2 (Figure 4, Table 3; right column). The penetrance of the P granule mislocalization phenotype correlates with the degree of lethality produced by RNAi in par-2(it5), with lgl-1, math-33 and F25B5.2 producing the highest frequency of abnormal P granule mislocalization (Table 3). Thus the embryonic lethality of the par-2-enhancing genes, except for uba-2, is most likely due to their effects on early embryo polarity.
To assess whether the enhancers of \textit{par-2} lethality also enhanced other \textit{par-2} phenotypes, we examined spindle orientation and cell division timing at the two-to four-cell stage. In strong \textit{par-2} mutants the P1 rotation fails and both cells divide synchronously with spindles oriented transverse to the long axis of the embryo. In \textit{par-2(it5)} embryos treated with control RNAi, 96\% of embryos (N=45) showed a normal, longitudinal P1 spindle orientation at the permissive temperature of 16\°. However, when treated with RNAi for \textit{lgl-1}, \textit{math-33}, or \textit{F25B5.2}, 100\% of \textit{par-2(it5)} embryos had transverse spindle orientations in P1 (Figure 4, Table 4). RNAi of \textit{par-1} or \textit{par-4} in \textit{par-2(it5)} at 16\° also causes both cells of two-cell embryos to divide transversely. \textit{pig-1} or \textit{ntl-9} RNAi also caused a high incidence of transverse P1 spindle orientations, and about half of the \textit{strd-1} RNAi embryos showed this phenotype, but \textit{uba-2} showed only a slight increase in the penetrance of this phenotype (4/27 embryos). \textit{par-2(it5)} embryos treated with RNAi for the genes that result in transverse P1 spindle orientations also exhibited a greater degree of synchrony between AB and P1, as determined by DAPI staining of two-cell embryos and time-lapse videomicroscopy (Figure 4, Table 4).

\textit{par-2} is required for restricting PAR-3 the anterior domain in the zygote (ETEMAD-MOGHADAM \textit{et al.} 1995; CUENCA \textit{et al.} 2003) and for PAR-1 localization to the posterior cortex (BOYD \textit{et al.} 1996). We investigated whether the enhancers that increase lethality in \textit{par-2(it5)} increase mislocalization of PAR-3 and PAR-1 in the \textit{par-2} early embryos at permissive temperature. Consistent with the other polarity defects seen with these enhancers, all but \textit{uba-2(RNAi)} embryos exhibited abnormal extension of cortical PAR-3 and associated loss of PAR-1 in the posterior one-cell and two-cell \textit{par-2(it5)} embryos (Figure S1,Table 5).

Thus six enhancers of lethality in \textit{par-2(it5)} strongly enhance polarity defects in \textit{par-2} in spite of the fact that they were initially isolated as enhancers of lethality in \textit{par-1} or \textit{par-4}. Only
one of these six, *strd-1*, also causes early polarity phenotypes in *par-1* and *par-4*. We infer that the other five enhancers of *par-2* phenotypes, *nop-1, lgl-1, math-33, pig-1* and *ntl-9* have roles in early polarity that are redundant with *par-2*, but not with *par-1* or *par-4*, and that their depletion must enhance *par-1* or *par-4* lethality due to defects at or after the two-cell stage, perhaps by alterations of relative cell cycle rates in these mutants.

**DISCUSSION**

We have identified eighteen RNAi clones that strongly enhance embryo lethality of conditional mutations of *par-1* or *par-4* or both. Three clones correspond to genes with previously known roles in embryonic polarity (*lgl-1, mes-1, ooc-3*) and fifteen correspond to genes with previously unknown roles.

Surprisingly, the only enhancer identified in our screen that clearly functions with *par-1* and *par-4* in polarity during the first cell cycle is *strd-1*. *strd-1* encodes an ortholog of STRADα, a pseudokinase cofactor of the mammalian ortholog of PAR-4, LKB1. Binding of STRAD has been shown to increase activity of LKB1 in mammalian systems (BAAS et al. 2003; BOUDEAU et al. 2003a; HAWLEY et al. 2003; reviewed by ALESSI et al. 2006). In *C. elegans* STRD-1 can be immunoprecipitated with PAR-4, and colocalizes with PAR-4 at the cortex of early embryos (NARBONNE et al. 2010). However, it is unclear whether STRD-1 acts in the early embryo to increase activity of PAR-4. *strd-1* mutations do not on their own result in embryonic lethality, but do result in increased lethality when combined with conditional *par-1* and *par-4* mutants (NARBONNE et al. 2010). By examining phenotypes at semipermissive temperatures, we have found that *strd-1* does contribute to polarity processes regulated by *par-1* and *par-4* in the zygote such as asymmetric P granule localization and cell cycle timing. The high lethality but
normal early polarity observed in \textit{par-1(zu310); strd-1(RNAi)} embryos at the lower temperature of 15° suggests that \textit{strd-1} synergizes with \textit{par-1} in other processes of embryogenesis that are more sensitive to reductions in PAR-1 activity, which may or may not be occurring at the one-cell stage. Recently Kim \textit{et al.} have reported that \textit{strd-1} functions independently of \textit{par-4} in establishing \textit{C. elegans} neuronal polarity, by acting in a complex with SAD-1, a kinase with high structural similarity to PAR-1, but which does not appear to act in the early embryo (Kim \textit{et al.} 2010). Whether the enhancing effects of \textit{strd-1} depletion in the early embryo are due to effects on activities of PAR-4, PAR-1, or another kinase, will be of future interest.

Besides \textit{strd-1}, the only other RNAi enhancer for which lethality correlated with early polarity defects in \textit{par-4(it57)} was \textit{sti-1}. STI-1 is a highly conserved stress-induced phosphoprotein that can interact directly with HSP-70 or HSP-90 in \textit{C. elegans} (Gaiser \textit{et al.} 2009; Song \textit{et al.} 2009) to act as a co-chaperone for target proteins. HSP-90 has been shown to act with the co-chaperone CDC-37 to regulate stability and activity of LKB1 (Boudeau \textit{et al.} 2003b; Nony \textit{et al.} 2003; Gaudef \textit{et al.} 2012). In \textit{C. elegans}, CDC-37 has been shown to affect steady-state levels of PKC-3 and phosphorylated PAR-4, and is essential for regulating the mutual restriction of the anterior and posterior PAR proteins during polarity establishment (Beers and Kemphues 2006). The fact that RNAi of either \textit{hsp-1}, which encodes an HSP-70A type protein, or \textit{sti-1} reduces viability of weak \textit{par-4} mutants could mean that HSP-70 and STI-1 chaperones are also important for PAR-4 stability, phosphorylation status or function. \textit{sti-1} RNAi did not increase polarity defects in \textit{par-1(zu310)}; indeed \textit{sti-1} RNAi even suppressed lethality. Thus, while \textit{sti-1} may be acting through another protein to affect PAR-4 function, it seems unlikely that it is acting through PAR-1.
Most of the par-1 and par-4 enhancing genes did not exhibit strong early polarity defects when depleted in par-1 and par-4 backgrounds in spite of high levels of embryonic lethality. There are two possible explanations for this. First, their effects on polarity might be too subtle to detect in our assays, but sufficient to lead to cascading defects in later stages. For example, the slight loss of asynchrony at the two-cell stage could result in a development defect only detectable in later stage embryos. Alternatively, the lethality may reflect a role for these genes with PAR-1 and PAR-4 in a later essential function of the two kinases. For example, the absence of intestine in par-4 and par-1 mutants could reflect a role in specification of the EMS or E cell fates. MES-1, for example, is known to have an important function in polarity and intercellular signaling between EMS and P2 at the four-cell stage (Berkowitz and Strome 2000; Bei et al. 2002; Arata et al. 2010); other enhancers in our set may also be acting at this stage or later.

It is noteworthy that genes we initially identified as enhancers of par-1 or par-4 or both, but that do not show increased polarity phenotypes in those mutants when depleted by RNAi, nonetheless very effectively enhance par-2 early polarity phenotypes. One interpretation of this result is that modest reductions in efficiency of processes that par-2 mediates can enhance lethality in sensitized par-1 and par-4 backgrounds. This interpretation is supported by the example of lgl-1, which has been shown to act redundantly with PAR-2 in early polarity (Beatty et al. 2010; Hoeg et al. 2010).

Another possible explanation for the differences we see in polarity phenotypes among the par mutants depleted of the enhancers could be due to differences in the par mutations used for our studies. While each of the mutants shows strong early polarity phenotypes at nonpermissive temperature, they may have different sensitivities at lower temperatures for their early polarity functions. We note that strd-1 RNAi in par-1(zu310) and par-4(it57) produced strikingly
different frequency of P granule localization defects depending on temperature, despite producing similar levels of lethality. The enhancers may affect localization of some components of the cell that we did not assay, independent of P granule localization, in these mutants.

Nonmuscle myosin is critical for driving cytoplasmic and cortical flows that transport the PAR proteins and other components to their polarized domains shortly after fertilization (Munro et al. 2004). After this establishment phase, PAR-2 is essential for maintaining the polarized domains (Cuenca et al. 2003). It has recently been shown, however, that when myosin-directed cortical flows are compromised, PAR-2 is able to direct polarity establishment via a microtubule-directed process (Zonies et al. 2010; Motegi et al. 2011); the redundant genes that we have identified that increase polarity phenotypes in the par-2 mutant may be particularly important for polarity maintenance or for robustness of actomyosin flows.

One of the enhancers with the highest levels of lethality and polarity phenotypes in the par-2 background is nop-1. In addition to lacking pseudocleavage, nop-1(RNAi) or nop-1(it142) embryos have reduced ruffling and cortical flows (Supplemental Movie File S1). The effects of nop-1(RNAi) on actomyosin contractility may explain its synthetic lethality with the par mutants, particularly par-1 and par-2. Since PAR-1 and PAR-2 act together in PAR-2’s redundant role with myosin contractility in polarity establishment (Motegi et al. 2011), nop-1 would be expected to enhance polarity defects and embryo lethality in both par-1 and par-2 mutants, as we observe.

The strong par-1 enhancer pig-1, (par-1-like gene), encodes a kinase in the AMP kinase family with very similar structure to PAR-1 (Cordes et al. 2006). The closest mammalian homolog to PIG-1 is MELK/KP38, a kinase for which the RNA is highly enriched in mouse oocytes and very early embryos (Heyer et al. 1997; Heyer et al. 1999) and is also related to the
egg-enriched Xenopus pEg3 (BLOT et al. 2002). PIG-1 in C. elegans is important for asymmetric division of certain neuroblasts in the Q lineage (Cordes et al. 2006), and its enhancement of embryo lethality in par mutants suggests that it is also playing a role in early embryo polarity. Like nop-1, pig-1 RNAi increases embryonic lethality in both par-1(zu310) and par-2(it5) at semipermissive temperatures but has little effect on viability in par-4(it57), and early polarity defects are seen only when par-2 is compromised. Although we did not see effects on P granule localization in early par-1 embryos treated with pig-1 RNAi, one possibility, currently under investigation, is that PIG-1 acts redundantly with PAR-1 in the early C. elegans embryo in other aspects of embryo polarity. It will also be of interest to learn whether the enriched MELK in vertebrate embryos regulates cellular asymmetries.

Among the other enhancers identified in our screen is the de-ubiquitylase gene math-33. MATH-33 is closely related to mammalian USP7 (HAUSP), which has multiple targets for its deubiquitylating activity that include the tumor-suppressor p53 and its ubiquitin E3 ligase, MDM2, as well as FOXO4, PTEN, and other proteins important for oncogenesis and stem cell maintenance (Reviewed by Nicholson and Suresh Kumar 2011). Changes in ubiquitylation status may regulate signaling pathways either by directing proteins for degradation by the proteasome, or by altering protein conformation and activity (Haglund and Dikic 2005; Mukhopadhyay and Riezman 2007). RNAi of several proteasome components has been shown to suppress embryonic lethality in par-2(it5) (Labbe et al. 2006) suggesting that proteasomal function is indeed important for polarity in C. elegans embryos. par-2 itself encodes a ubiquitin E3 ligase (Hao et al. 2006), but reducing activity of math-33 by RNAi enhances, rather than suppresses, the lethality of the par-2 mutant, suggesting that MATH-33 does not act in
opposition to PAR-2 activity. Determining the targets of the MATH-33 deubiquitylase in *C. elegans* will be important for understanding its function in the early embryo.

We identified two sumoylation genes in our screen, encoding the SUMO-activating protein subunit UBA-2 and the SUMO ligase GEI-17. RNAi of these genes causes low levels of embryonic lethality in wild type, and each synergistically enhances embryonic lethality in each of the four *par* mutant strains tested. However, we did not observe striking polarity phenotypes in early embryos, suggesting that the essential role for sumoylation in *par*-mediated processes may be occurring after the two-cell stage. Because cytoskeletal proteins made up 7% of sumoylated *C. elegans* proteins identified in a proteomic study (Kaminsky *et al.* 2009), we speculate that one or more of these cytoskeletal proteins could be the relevant target responsible for the enhancing phenotype. We also note that *gei-17* increases lethality in *emb-9(b189)* embryos, which have a temperature-sensitive period late in embryogenesis, suggesting that the function of *gei-17* is not specific to the early embryo.

Three of the genes identified in our study, *math-33*, *ntl-9* and *pig-1*, are also among 14 genes found by RNAi to increase lethality in a weak *vab-10A/Plakin* mutant (Zahreddine *et al.* 2010). VAB-10A is required for attachment of intermediate filaments to membranous organelles to form hemidesmosomes during *C. elegans* embryo elongation (Bosher *et al.* 2003). We have shown that RNAi of *math-33*, *ntl-9* or *pig-1* strongly increases early embryo phenotypes in a weak *par-2* mutant; their enhancement of lethality during morphogenesis suggests that they might also function in epithelial polarity. However, none of our 18 *par*-enhancing genes overlap with a set of recently identified *C. elegans* genes acting redundantly in gastrulation (Sawyer *et al.* 2011).
Our set of 18 genes that enhance lethality of par-1, par-4, or both is certainly not an exhaustive set of genes with roles in embryonic cell polarity. Our screen was biased toward genes with no or very weak RNAi phenotypes on their own. Many genes with important roles in early embryonic polarity will have strong lethal phenotypes including pleiotropic effects that could obscure a role in polarity. Furthermore, because our goal was to identify genes with strong effects on the par system, we performed our screens in a way that would eliminate genes with subtle effects or for which RNAi depletion must begin earlier in larval development to see an effect. There are also likely to be genes that we missed due to incomplete coverage of the genome in the set of RNAi genes that we used. Indeed, aos-1 and unc-61, the heterodimeric partners of enhancers uba-2 and unc-59 respectively (JOHNSON and BLOBEL 1997; JONES et al. 2002; JOHN et al. 2007), were missing from the library, but are effective enhancers of par-1 and par-4 (Supplementary Table 2). Nevertheless, our screen has identified a number of candidates for polarity function that have not previously been known. Most of these genes are conserved, and may function redundantly with key polarity regulators, ensuring the robustness of polarity establishment and maintenance. Investigating their roles in C. elegans embryogenesis and in polarity in other animals will enable us to develop a more complete understanding of this highly complex process.

ACKNOWLEDGMENTS

We thank Jun Kelly Liu for sharing unpublished results from a parallel RNAi enhancer screen, Rich McCloskey, Alex Beatty, Jun Kelly Liu, and the Cornell Worm Group for helpful discussions, Daryl Hurd for par-1 and par-4 RNAi feeding constructs and Mona Hassab for
D. G. Morton \textit{et al.}

media preparation. This research was supported by National Institutes of Health grants HD27689 and GM079112 to K.J.K.
TABLES

TABLE 1. Percent embryonic lethality in conditional par mutants treated with RNAi of screen positives

<table>
<thead>
<tr>
<th>RNAi gene target</th>
<th>Function</th>
<th>N2</th>
<th>par-1(zu310)</th>
<th>par-4(lt57)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Genotype</td>
<td>(19° C)</td>
<td>(19° C)</td>
<td>(17° C)</td>
</tr>
<tr>
<td>L4440 (negative control)</td>
<td>RNAi feeding vector</td>
<td>0.8 ± 0.6</td>
<td>14.0 ± 1.6</td>
<td>26.7 ± 13.8</td>
</tr>
<tr>
<td>sad-1 (negative control)</td>
<td>AMPK-related kinase, neuronal polarity</td>
<td>0.6 ± 0.2</td>
<td>19.0 ± 7.2</td>
<td>21.0 ± 9.5</td>
</tr>
<tr>
<td>par-4 (positive control)</td>
<td>PAR-4 kinase, embryo cell polarity</td>
<td>26.2 ± 8.9**</td>
<td>84.8 ± 5.2**</td>
<td>87.1 ± 1.9***</td>
</tr>
<tr>
<td><strong>PHOSPHORYLATION:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>strd-1</td>
<td>PAR-4 kinase cofactor</td>
<td>0.6 ± 0.4</td>
<td>98.4 ± 1.6**</td>
<td>90.2 ± 6.2**</td>
</tr>
<tr>
<td>pig-1</td>
<td>AMPK-related kinase, neuronal polarity</td>
<td>1.8 ± 0.4*</td>
<td>95.7 ± 3.6**</td>
<td>40.8 ± 7.2</td>
</tr>
<tr>
<td>kin-10</td>
<td>Casein Kinase II regulatory subunit</td>
<td>26.3 ± 8.3**</td>
<td>36.8 ± 2.4 a</td>
<td>68.8 ± 4.5*</td>
</tr>
<tr>
<td>ZK856.8</td>
<td>Calcium-binding phosphatase-like</td>
<td>0.4 ± 0.2</td>
<td>38.1 ± 9.1*</td>
<td>11.3 ± 3.8</td>
</tr>
<tr>
<td><strong>CYTOSKELETAL &amp;</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>MEMBRANE PROTEINS:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mes-1</td>
<td>Membrane protein, embryo cell polarity</td>
<td>0.7 ± 0.1</td>
<td>88.0 ± 2.6**</td>
<td>64.0 ± 17.5*</td>
</tr>
<tr>
<td>unc-59</td>
<td>Septin GTPase</td>
<td>2.2 ± 0.3*</td>
<td>54.0 ± 6.2**</td>
<td>43.7 ± 9.7</td>
</tr>
<tr>
<td>lgl-1</td>
<td>Cell polarity</td>
<td>0.7 ± 0.4</td>
<td>51.1 ± 2.2*</td>
<td>23.5 ± 4.0</td>
</tr>
<tr>
<td>Gene</td>
<td>Description</td>
<td>Percent Embryo Lethality ± Standard Deviation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>-----------------------------------------------------------</td>
<td>-----------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T24F1.2</td>
<td>Membrane protein</td>
<td>1.6 ± 1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>npp-22</td>
<td>Nucleoporin</td>
<td>0.9 ± 0.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>str-43</td>
<td>Membrane protein</td>
<td>0.8 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ooc-3</td>
<td>ER membrane protein, cell polarity</td>
<td>0.8 ± 0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>UBIQUITINATION, SUMOYLATION:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>math-33</td>
<td>USP7/HAUSP ubiquitin hydrolase</td>
<td>1.9 ± 1.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>uba-2</td>
<td>SUMO-activating protein</td>
<td>24.4 ± 2.6**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gei-17</td>
<td>SUMO ligase</td>
<td>5.0 ± 1.2**</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>CHAPERONES:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sti-1</td>
<td>HSP90/HSP70 co-chaperone</td>
<td>0.9 ± 0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hsp-2</td>
<td>Pseudogene, also targets HSP-1</td>
<td>2.8 ± 1.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>UNKNOWN FUNCTION:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ntl-9</td>
<td>Rcd1 superfamily</td>
<td>1.0 ± 0.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F25B5.2 (nop-1)</td>
<td>Unknown; nematode-specific</td>
<td>0.8 ± 0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F23F12.3 intron</td>
<td>Unknown; secondary targets</td>
<td>0.6 ± 0.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Percent embryo lethality ± standard deviation from mean following RNAi feeding of clones corresponding to the indicated genes

* enhanced lethality, p < 0.05 compared to L4440 control in the same genetic background

** enhanced lethality, p < 0.001 compared to L4440 control

a enhanced lethality compared to L4440 control but not significantly different from result in N2 (p > 0.05)
D. G. Morton et al.

b suppression of lethality, p < 0.01 compared to L4440 control
**TABLE 2.** Percent embryo lethality in *par-2(it5ts)* and *par-3(e2074); sup-7* treated with RNAi of enhancers of *par-1* and *par-4* embryo lethality

<table>
<thead>
<tr>
<th>RNAi gene target</th>
<th><em>par-2(it5)</em></th>
<th><em>par-3(e2074); sup-7</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>L4440 (negative control)</td>
<td>9.0 ± 1.9</td>
<td>56.5 ± 11.1</td>
</tr>
<tr>
<td>sad-1 (negative control)</td>
<td>16.0 ± 5.8</td>
<td>58.5 ± 15.1</td>
</tr>
<tr>
<td><em>par-4</em> (positive control)</td>
<td>61.8 ± 11.3**</td>
<td>97.5 ± 1.4**</td>
</tr>
<tr>
<td><em>strd-1</em></td>
<td>79.6 ± 1.3**</td>
<td>65.8 ± 9.3</td>
</tr>
<tr>
<td><em>pig-1</em></td>
<td>96.5 ± 0.1**</td>
<td>69.5 ± 15.8</td>
</tr>
<tr>
<td><em>mes-1</em></td>
<td>28.7 ± 19.3</td>
<td>70.5 ± 13.0</td>
</tr>
<tr>
<td><em>ntl-9</em></td>
<td>81.0 ± 12.5**</td>
<td>89.4 ± 4.6**</td>
</tr>
<tr>
<td><em>gei-17</em></td>
<td>42.1 ± 3.7*</td>
<td>81.0 ± 7.5*</td>
</tr>
<tr>
<td><em>F25B5.2</em> (<strong>nop-1</strong>)</td>
<td>99.4 ± 0.4**</td>
<td>79.8 ± 3.5*</td>
</tr>
<tr>
<td><em>F23F12.3</em> intron</td>
<td>45.9 ± 15.1*</td>
<td>69.0 ± 5.3</td>
</tr>
<tr>
<td><em>math-33</em></td>
<td>99.1 ± 1.3**</td>
<td>33.2 ± 7.6d</td>
</tr>
<tr>
<td><em>uba-2</em></td>
<td>71.9 ± 6.9*</td>
<td>94.2 ± 5.3**</td>
</tr>
<tr>
<td><em>unc-59</em></td>
<td>15.2 ± 7.1</td>
<td>62.5 ± 16.5</td>
</tr>
<tr>
<td><em>lgl-1</em></td>
<td>98.9 ± 0.1**</td>
<td>25.9 ± 13.2d</td>
</tr>
<tr>
<td><em>ZK856.8</em></td>
<td>23.4 ± 6.4</td>
<td>66.2 ± 13.0</td>
</tr>
<tr>
<td><em>kin-10</em></td>
<td>23.9 ± 12.0</td>
<td>84.4 ± 39.0c</td>
</tr>
<tr>
<td><em>T24F1.2</em></td>
<td>50.6 ± 5.6*</td>
<td>62.0 ± 5.0</td>
</tr>
<tr>
<td><em>npp-22</em></td>
<td>57.0 ± 8.6*</td>
<td>54.3 ± 14.1</td>
</tr>
<tr>
<td><em>str-43</em></td>
<td>11.1 ± 5.6</td>
<td>58.1 ± 17.2</td>
</tr>
<tr>
<td><em>ooc-3</em></td>
<td>28.7 ± 15.6</td>
<td>64.9 ± 14.2</td>
</tr>
<tr>
<td><em>hsp-2</em></td>
<td>21.1 ± 9.3</td>
<td>66.8 ± 7.5</td>
</tr>
<tr>
<td><em>sti-1</em></td>
<td>29.3 ± 12.7*</td>
<td>77.5 ± 9.4*</td>
</tr>
</tbody>
</table>
Percent embryo lethality ± standard deviation from mean following RNAi feeding of clones corresponding to the indicated genes, ordered by degree of lethality produced in par-1(zu310).

*enhanced lethality, p < 0.05 compared to L4440 control in the same genetic background

** enhanced lethality, p < 0.001 compared to L4440 control

a 16°

b 22°

c not significantly different from expected lethality produced by RNAi in wild type added to control levels of lethality

d suppression of lethality, p < 0.05 compared to L4440 control.
### TABLE 3. P Granule mislocalization in early par embryos treated with RNAi of enhancers of lethality

<table>
<thead>
<tr>
<th>Genotype</th>
<th>par-1(zu310,) 19° (2-cell)</th>
<th>par-4(it57), 17° (2-cell)</th>
<th>par-2(it55), 16° (4-cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L4440 control</td>
<td>0 (52)</td>
<td>0 (53)</td>
<td>0 (59)</td>
</tr>
<tr>
<td>par-1</td>
<td>100% (18)</td>
<td>100% (27)</td>
<td>100% (22)</td>
</tr>
<tr>
<td>par-4</td>
<td>100% (20)</td>
<td>91% (12)</td>
<td>100% (16)</td>
</tr>
<tr>
<td>strd-1</td>
<td>91% (43)</td>
<td>88% (24)</td>
<td>54% (24)</td>
</tr>
<tr>
<td>pig-1</td>
<td>3% (36)</td>
<td>--</td>
<td>70% (37)</td>
</tr>
<tr>
<td>mes-1</td>
<td>0 (50)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>ntl-9</td>
<td>0 (22)</td>
<td>--</td>
<td>62% (13)</td>
</tr>
<tr>
<td>gei-17</td>
<td>0 (16)</td>
<td>0 (22)</td>
<td>--</td>
</tr>
<tr>
<td>nop-1</td>
<td>6% (33)</td>
<td>--</td>
<td>90% (30)</td>
</tr>
<tr>
<td>math-33</td>
<td>0 (43)</td>
<td>0 (17)</td>
<td>100% (18)</td>
</tr>
<tr>
<td>uba-2</td>
<td>4% (27)</td>
<td>0 (18)</td>
<td>0 (15)</td>
</tr>
<tr>
<td>lgl-1</td>
<td>--</td>
<td>--</td>
<td>100% (23)</td>
</tr>
<tr>
<td>sti-1</td>
<td>--</td>
<td>48% (44)</td>
<td>--</td>
</tr>
</tbody>
</table>

Percent of embryos with mislocalized P granules is indicated, followed by (N).

RNAi clones producing more than 70% embryonic lethality in the par mutant at semipermissive temperature were used for this analysis.

"--" indicates that lethality was lower than 70%.
TABLE 4. Spindle orientation and cell cycle phenotypes of two-cell *par* embryos treated with RNAi for the indicated genes at semipermissive temperatures.

<table>
<thead>
<tr>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>par-1(zu310), 19°</td>
</tr>
<tr>
<td>Percent with P1 Transverse Spindle (N)</td>
</tr>
<tr>
<td>RNAi</td>
</tr>
<tr>
<td>L4440</td>
</tr>
<tr>
<td>par-1</td>
</tr>
<tr>
<td>par-4</td>
</tr>
<tr>
<td>strd-1</td>
</tr>
<tr>
<td>pig-1</td>
</tr>
<tr>
<td>mes-1</td>
</tr>
<tr>
<td>ntl-9</td>
</tr>
<tr>
<td>gei-17</td>
</tr>
<tr>
<td>nop-1</td>
</tr>
<tr>
<td>math-33</td>
</tr>
<tr>
<td>uba-2</td>
</tr>
<tr>
<td>sti-1</td>
</tr>
</tbody>
</table>
RNAi clones producing more than 60% embryonic lethality in the *par* mutant at semipermissive temperature were used for this analysis.

```
<table>
<thead>
<tr>
<th></th>
<th>--</th>
<th>--</th>
<th>0 (27)</th>
<th>11 (27)</th>
<th>--</th>
<th>--</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>kin-10</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>lgl-1</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100** (23)</td>
<td>96** (25)</td>
</tr>
</tbody>
</table>
```

"--" indicates that lethality was lower than 60%. Percentages are rounded to the nearest whole number.

* p < 0.05, chi-square test

** p < 0.0001, chi-square test
D. G. Morton et al.

**TABLE 5. PAR-3 posterior extension in par-2(it5); enhancer(RNAi) one-cell and two-cell embryos (16°)**

<table>
<thead>
<tr>
<th></th>
<th>One-cell stage</th>
<th>Two-cell stage</th>
<th>Frequency of mislocalization in P1ᵇ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>prophase-metaphaseᵃ</td>
<td>anaphaseᵃ</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>50.5±5.3% (28)</td>
<td>57.6±5.0% (10)</td>
<td>0 (41)</td>
</tr>
<tr>
<td><strong>strd-1</strong></td>
<td>62.8±6.4%* (18)</td>
<td>58.0±7.2% (8)</td>
<td>71% (25)</td>
</tr>
<tr>
<td><strong>pig-1</strong></td>
<td>70.0±5.8%* (14)</td>
<td>71.2±6.7%* (7)</td>
<td>96% (26)</td>
</tr>
<tr>
<td><strong>ntl-9</strong></td>
<td>61.7±8.3%* (17)</td>
<td>71.3±4.9%* (9)</td>
<td>93% (46)</td>
</tr>
<tr>
<td><strong>nop-1</strong></td>
<td>65.4±7.6%* (24)</td>
<td>68.1±7.6%* (8)</td>
<td>94% (50)</td>
</tr>
<tr>
<td><strong>math-33</strong></td>
<td>62.4±6.0%* (18)</td>
<td>74.7±6.6%* (7)</td>
<td>83% (40)</td>
</tr>
<tr>
<td><strong>lgl-1</strong></td>
<td>55.9±2.7%* (10)</td>
<td>63.7±6.9%* (7)</td>
<td>89% (35)</td>
</tr>
<tr>
<td><strong>uba-2</strong></td>
<td>50.2±5.4% (18)</td>
<td>57.0±8.5% (3)</td>
<td>0 (53)</td>
</tr>
<tr>
<td>Control, 25°</td>
<td>62.8±6.4%* (11)</td>
<td>8.0±11%* (2)</td>
<td>100% (22)</td>
</tr>
</tbody>
</table>

ᵃAverage size of PAR-3 domain along the anterior-posterior axis of the embryo as percent of embryo length followed by (N), determined by immunostaining. Stage was determined by DAPI staining. Genes producing more than 70% embryonic lethality by RNAi in par -2(it5) at 16° were used for this analysis.

ᵇPercentage of embryos exhibiting abnormal PAR-3 extension in the posterior P1 cell (N).

* Value significantly different from control RNAi at 16°, p <0.01, Student’s T test.
FIGURE LEGENDS

FIGURE 1. Embryonic lethality produced by RNAi of screen positives in weak par mutants.
Percent embryonic lethality upon RNAi of the indicated genes in A) par-1(zu310); B) par-4(it57); C) par-2(it5); D) par-3(e2074); sup-7, at the temperature shown for each genotype. Red bars indicated enhanced lethality compared to L4440 control RNAi (p <0.05), while those in light gray are not significantly different from the control. Blue bars indicate suppression of embryo lethality compared to L4440 control RNAi (p <0.05). Genes depleted by RNAi are ordered along the X axis for each par mutant by level of embryo lethality produced. Error bars indicate standard deviation from the mean. sad-1 RNAi is an additional negative control, while par-4 RNAi is a positive control.

FIGURE 2. RNAi enhancers of embryonic lethality affect multiple par genes. Venn diagram showing the genes identified by RNAi that significantly increase lethality (p < 0.05) in par-1, par-2 and par-4 conditional mutants.

FIGURE 3. F25B5.2(RNAi) embryos exhibit a Nop (No Pseudocleavage) phenotype like that of nop-1(it142) mutant embryos. The top row shows a wild-type one-cell embryo at A) the time of ruffling, about 6.5 min prior to nuclear envelope breakdown (NEBD); B) pseudocleavage (arrowheads), approximately 2.5 minutes prior to NEBD; C) NEBD; and D) the two-cell stage. E-H) nop-1(it142) embryo at the same times relative to NEBD (G) as those in A-D. I-L) F25B5.2(RNAi) embryo at the same intervals. Cortical ruffling and pseudocleavage are not observed in either nop-1(it142) or F25B5.2(RNAi) embryos. Anterior is to the left for all embryos. Time-lapse movies of F25B5.2(RNAi) and wild-type embryos from which these images are taken can be viewed in File S1 and File S2.

FIGURE 4. P granule phenotypes and AB/P1 cell cycle synchrony in par mutants treated with RNAi for screen positives. Embryos are immunostained with anti-P granule antibodies (green) and the DNA costained with DAPI (blue). A-D) par-1(zu310) two-cell embryos from worms at semipermissive
D. G. Morton et al.

temperature (19°) after the indicated RNAi treatment. L4440 is empty vector control RNAi. E-F) par-4(it57) two-cell embryos from worms at semipermissive temperature (17°) after the indicated RNAi treatment. I-K) par-2(it5) two-cell embryos and M-O) four-cell embryos from worms at semipermissive temperature (16°) after the indicated RNAi treatment. L, P) Two-cell and four-cell par-2(it5) embryos grown at the nonpermissive temperature, 25°. Q, R) Wild-type two-cell and four-cell embryos are shown for comparison.
LITERATURE CITED


D. G. Morton et al.


D. G. Morton et al.

BOUDEAU, J., A. F. BAAS, M. DEAK, N. A. MORRICE, A. KIELOCH et al., 2003a
MO25alpha/beta interact with STRADalpha/beta enhancing their ability
to bind, activate and localize LKB1 in the cytoplasm. Embo J 22: 5102-
5114.

BOUDEAU, J., M. DEAK, M. A. LAWLOR, N. A. MORRICE and D. R. ALESSI, 2003b Heat-
shock protein 90 and Cdc37 interact with LKB1 and regulate its

BOUTROS, M., and J. AHRINGER, 2008 The art and design of genetic screens: RNA

BOYD, L., S. GUO, D. LEVITAN, D. T. STINCHCOMB and K. J. KEMPHUES, 1996 PAR-2 is
asymmetrically distributed and promotes association of P granules and
PAR-1 with the cortex in C. elegans embryos. Development 122: 3075-
3084.


BUDIRAHARDJA, Y., and P. GONCZY, 2008 PLK-1 asymmetry contributes to
asynchronous cell division of C. elegans embryos. Development 135:
1303-1313.

CHEEKS, R. J., J. C. CANMAN, W. N. GABRIEL, N. MEYER, S. STROME et al., 2004 C.
elegans PAR proteins function by mobilizing and stabilizing
D. G. Morton et al.


DORFMAN, M., J. E. GOMES, S. O’ROURKE and B. BOWERMAN, 2009 Using RNA interference to identify specific modifiers of a temperature-sensitive,
embryonic-lethal mutation in the *Caenorhabditis elegans* ubiquitin-like
Nedd8 protein modification pathway E1-activating gene *rfl-1*. Genetics
**182**: 1035-1049.

Etemad-Moghadam, B., S. Guo and K. J. Kemphues, 1995 Asymmetrically
distributed PAR-3 protein contributes to cell polarity and spindle

Finn, R. D., J. Mistry, J. Tate, P. Coggill, A. Heger et al., 2010 The Pfam protein
families database. Nucleic Acids Res **38**: D211-222.

Gaiser, A. M., F. Brandt and K. Richter, 2009 The non-canonical Hop protein
from *Caenorhabditis elegans* exerts essential functions and forms binary
complexes with either Hsc70 or Hsp90. J Mol Biol **391**: 621-634.

Gallo, C. M., E. Munro, D. Rasoloson, C. Merritt and G. Seydoux, 2008
Processing bodies and germ granules are distinct RNA granules that
interact in *C. elegans* embryos. Dev Biol **323**: 76-87.

Gauda, H., N. Aznar, A. Delay, A. Bres, K. Buchet-Poyau et al., 2012 Molecular
chaperone complexes with antagonizing activities regulate stability and
activity of the tumor suppressor LKB1. Oncogene **31**: 1582-1591.

Guo, S., and K. J. Kemphues, 1995 *par-1*, a gene required for establishing
polarity in *C. elegans* embryos, encodes a putative Ser/Thr kinase that is
asymmetrically distributed. Cell **81**: 611-620.
D. G. Morton *et al.*


**Hao, Y., L. Boyd and G. Seydoux,** 2006 Stabilization of cell polarity by the *C. elegans* RING protein PAR-2. Dev Cell **10:** 199-208.

**Harris, T. W., I. Antoshechkin, T. Bieri, D. Blasiar, J. Chan *et al.,* 2010**

WormBase: a comprehensive resource for nematode research. Nucleic Acids Res **38:** D463-467.

**Hawley, S. A., J. Boudeau, J. L. Reid, K. J. Mustard, L. Udd *et al.,* 2003** Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. J Biol **2:** 28.


D. G. Morton et al.


D. G. Morton et al.


D. G. Morton et al.


D. G. Morton et al.


D. G. Morton et al.


D. G. Morton et al.


FIGURE 1. Embryonic lethality produced by RNAi of screen positives in weak par mutants.

Percent embryonic lethality upon RNAi of the indicated genes in A) par-1(zu310), B) par-4(it57), C) par-2(it5), D) par-3(e2074); sup-7, at the temperature shown for each genotype. Red bars indicated enhanced lethality compared to L4440 control RNAi (p <0.05), while those in light gray are not significantly different from the control. Blue bars indicate suppression of embryo lethality compared to L4440 control RNAi (p <0.05). Genes depleted by RNAi are ordered along the X axis for each par mutant by level of embryo lethality produced. Error bars indicate standard deviation from the mean. sad-1 RNAi is an additional negative control, while par-4 RNAi is a positive control.
FIGURE 2. RNAi enhancers of embryonic lethality affect multiple par genes. Venn diagram showing the genes identified by RNAi that significantly increase lethality (p < 0.05) in par-1, par-2 and par-4 conditional mutants.
FIGURE 3. *F25B5.2(RNAi)* embryos exhibit a Nop (No Pseudocleavage) phenotype like that of *nop-1(it142)* mutant embryos. The top row shows a wild-type one-cell embryo at A) the time of ruffling, about 6.5 min prior to nuclear envelope breakdown (NEBD); B) pseudocleavage (arrowheads), approximately 2.5 minutes prior to NEBD; C) NEBD; and D) the two-cell stage. E-H) *nop-1(it142)* embryo at the same times relative to NEBD (G) as those in A-D. I-L) *F25B5.2(RNAi)* embryo at the same intervals. Cortical ruffling and pseudocleavage are not observed in either *nop-1(it142)* or *F25B5.2(RNAi)* embryos. Anterior is to the left for all embryos. Time-lapse movies of *F25B5.2(RNAi)* and wild-type embryos from which these images are taken can be viewed in File S1 and File S2.
FIGURE 4. P granule phenotypes and AB/P1 cell cycle synchrony in par mutants treated with RNAi for screen positives. Embryos are immunostained with anti-P granule antibodies (green) and the DNA costained with DAPI (blue). A-D) par-1(zu310) two-cell embryos from worms at semipermissive temperature (19°) after the indicated RNAi treatment. L4440 is empty vector control RNAi. E-F) par-4(it57) two-cell embryos from worms at semipermissive temperature (17°) after the indicated RNAi treatment. I-K) par-2(it5) two-cell embryos and M-O) four-cell embryos from worms at semipermissive temperature (16°) after the indicated RNAi treatment. L, P) Two-cell and four-cell par-2(it5) embryos grown at the nonpermissive temperature, 25°. Q, R) Wild-type two-cell and four-cell embryos are shown for comparison.