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C. elegans Brat homologs regulate PAR protein-dependent polarity and asymmetric cell division

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ABSTRACT

The evolutionary conserved PAR proteins control polarization and asymmetric division in many organisms. Recent work in *Caenorhabditis elegans* demonstrated that *nos-3* and *fbf-1/2* can suppress *par-2(it5ts)* lethality, suggesting that they participate in cell polarity by regulating the function of the anterior PAR-3/PAR-6/PKC-3 proteins. In *Drosophila* embryos, Nanos and Pumilio are homologous to NOS-3 and FBF-1/2 respectively and control cell polarity by forming a complex with the tumor suppressor Brat to inhibit *Hunchback* mRNA translation. In this study, we investigated the possibility that Brat could control cell polarity and asymmetric cell division in *C. elegans*. We found that disrupting four of the five *C. elegans* Brat homologs (*Cebrats*) individually results in suppression of *par-2(it5ts)* lethality, indicating that these genes are involved in embryonic polarity. Two of the *Cebrats*, *ncl-1* and *nhl-2*, partially restore the localization of PAR proteins at the cortex. While mutations in the four *Cebrat* genes do not severely impair polarity, they display polarity-associated defects. Surprisingly, these defects are absent from *nos-3* mutants. Similarly, while *nos-3* controls PAR-6 protein levels, this is not the case for any of the *Cebrats*. Our results, together with results from *Drosophila*, indicate that Brat family members function in generating cellular asymmetries and suggest that, in contrast to *Drosophila* embryos, the *C. elegans* homologs of Brat and Nanos could participate in embryonic polarity via distinct mechanisms.

Introduction

The PAR proteins (PAR-1 to -6 and PKC-3) play a central role during the establishment and maintenance of cell polarity in different cell types of many metazoans (Macara, 2004; Suzuki and Ohno, 2006). Their function is essential in cells where polarization is a prerequisite for asymmetric cell division. In these cells, such as stem cells, PAR-dependent cell polarization controls the asymmetric localization of the mitotic spindle as well as the asymmetric segregation of cell fate determinants along the polarity axis (Morrison and Kimble, 2006). In *Caenorhabditis elegans*, polarization of the zygote along the antero-posterior axis leads to a displacement of the mitotic spindle toward the posterior pole and to the polarized segregation of cell fate determinants, such as the germline markers P granules that localize posteriorly (Betschinger and Knoblich, 2004; Kemphues and Strome, 1997). As a consequence, asymmetric division of the *C. elegans* zygote results in the formation of two daughter cells with different sizes and fates: the larger anterior AB cell which generates most of the ectoderm, and the smaller posterior P_1 cell which gives rise to the endoderm and the germline. Loss of any of the 6 *par* genes or loss of *pkc-3* results in loss of polarity, subsequent abnormal symmetric cell division and, ultimately, in embryonic lethality (Kemphues and Strome, 1997). Although there is clear evidence for the essential requirement for PAR proteins in polarity, their precise mechanism of action remains poorly understood.

Maintenance of polarity in the *C. elegans* zygote is achieved by the mutual inhibition of two groups of PAR proteins localized at the cortex: The PAR-3/PAR-6/PKC-3 complex at the anterior cortex of the embryo and the PAR-1/PAR-2 group at the posterior cortex (Cuenca et al., 2003; Munro et al., 2004). Equilibrium between these two groups is essential for the maintenance of embryonic polarity. Depletion of *par-2* for instance, allows PAR-3/PAR-6/PKC to extend posteriorly and thus disrupts polarity, leading to embryonic lethality (Boyd et al., 1996; Cuenca et al., 2003). The lethality of *par-2* mutants can be suppressed by depletion of any member of the anterior PAR-3/PAR-6/PKC complex (Labbé et al., 2006; Watts et al., 1996), indicating that modulators of this complex can be identified for their capacity to suppress *par-2* lethality. Recently, a screen for *par-2(it5ts)* suppressors identified several new genes involved in PAR-dependent cell polarity (Labbé et al., 2006). This work showed that loss of function of *nos-3* or *fbf-1/2* restores polarity and viability in *par-2(it5ts)* mutant animals, possibly by regulating the function of the anterior PAR-3/PAR-6/PKC complex. Interestingly, *Drosophila* homologs of *nos-3* (Nanos) and *fbf-1/2* (Pumilio) are also involved in the control of cell polarity. Nanos and Pumilio, together with the protein Brain Tumor (Brat), inhibit the

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Table 1
Mutations in *ncl-1*, *nhl-1*, *nhl-2* or *nhl-3* suppress *par-2* lethality

Genotype	20 °C (%)	25 °C (%)	*par-2* (RNAi) at 25 °C (%)
Wild-type	99.8±0.1[a]	99.3±0.5[a]	3.6±2.5
par-2(it5ts)	41.7±3.2	1.4±0.3	0±0
nos-3(q650); par-2(it5ts)	77.5±1.2[a]	49.5±5.0[a]	20.6±3.9[a]
par-2(it5ts) ncl-1(e1942)	94.2±1.0[a]	62.9±5.5[a]	11.2±4.9[a]
par-2(it5ts) nhl-1(gk15)	66.1±6.3[a]	11.5±2.6[a]	0.7±0.5
par-2(it5ts) nhl-2(ok818)	86.0±2.1[a]	64.7±5.6[a]	49.4±5.9[a]
nhl-3(tm2516); par-2(it5ts)	67.8±3.8[a]	12.0±2.1[a]	0.2±0.2
lin-41(ma104); par-2(it5ts)	53.3±3.8	3.7±1.7	0±0
par-2(it5ts) nhl-2(ok818) ncl-1(e1942) nhl-1(gk15)	63.8±7.6[a]	45.6±6.2[a]	ND

The value corresponds to embryonic viability, *i.e.* the average percentage of embryos that hatched over the total number of embryos±standard error of the mean over three independent assays.
[a] The value is significantly different ($p< 0.01$, Student *t*-test) from *par-2 (it5ts)* mutants. ND: not determined. See Materials and methods for details.

translation of *Hunchback* mRNA exclusively in the posterior part of the *Drosophila* embryo (Sonoda and Wharton, 2001). Brat has also been identified as a tumor suppressor in *Drosophila* brains where its depletion disrupts asymmetric neuroblasts division (Arama et al., 2000). In these stem cells, although Brat is asymmetrically localized, it is not involved in the PAR-dependent polarization process *per se*, but rather in the cell fate choice of the daughter cell committed to differentiation (Betschinger et al., 2006; Lee et al., 2006). These observations raised the possibility that a complex homologous to the *Drosophila* Nanos–Pumilio–Brat complex could control PAR-dependent embryonic polarity in *C. elegans* by translational inhibition.

Here we studied the involvement of *C. elegans* Brat homologs in embryonic polarity and their interaction with the *nos-3* pathway. Of the five Brat homologs identified in *C. elegans*, none of them have been implicated in cell polarity so far, and only two of them have previously been characterized: *lin-41* is a heterochronic gene involved in the larval-adult transition (Slack et al., 2000), whereas *ncl-1* functions as a cell growth inhibitor and was shown to be the functional ortholog of *Drosophila* Brat (Frank et al., 2002; Frank and Roth, 1998). Our results show that four of the five Brat homologs, including *ncl-1*, are involved in PAR-dependent embryonic polarity and asymmetric cell division and that they modulate PAR protein cortical localization. We found that embryos mutant for these Brat homologs displayed polarity-associated defects that were absent from *nos-3* mutants. Interestingly, we observed that while *nos-3* is required to maintain high levels of PAR-6 protein in the embryo, this was not the case for any of the animals mutant for Brat homologs. Taken together, our results indicate that Brat family members play conserved roles in regulating asymmetric cell division and suggest that, in contrast with the situation in *Drosophila* embryo, *C. elegans* Brat homologs could function independently of *nos-3* to control PAR-dependent embryonic polarity.

Materials and methods

Strains and alleles

All strains were maintained as described by Brenner (1974) and were grown at 15 °C unless otherwise stated. The wild-type strain was the N2 Bristol strain. The alleles used in this study were LGI: *lin-41(ma104)*; LGII: *nos-3(q650)*, *nhl-3(tm2516)*; LGIII: *par-2(it5ts)*, *nhl-2(ok818)*, *ncl-1 (e1942)*, *nhl-1(gk15)*. The *nhl-2(ok818)* deletion allele and *ncl-1(e1942)* point mutation allele can be considered as null alleles since they induce a strong decrease in their respective protein expression levels (Frank et al., 2002; Frank and Roth, 1998 and this study Fig. 4C). *nhl-1(gk15)* and *nhl-3(tm2516)* are predicted to result in the truncation of NHL domains and *lin-41(ma104)* has not been characterized molecularly. At 25 °C, *par-2(it5ts)* represents a strong loss of function but not a null allele of *par-2* (Table 1 and Figs. 4D and E).

Mutant strains were generated by genetic crosses. The presence of *par-2(it5ts)* mutation in each double, triple and quadruple mutant strain was determined by non-complementation assay at 25 °C. The presence of mutant alleles for *nos-3(q650)*, *nhl-3(tm2516)*, *nhl-2 (ok818)* and *nhl-1(gk15)* in each mutant strains was assessed by PCR using pairs of primers specific for each locus. The presence of *ncl-1 (e1942)* mutant allele in each strain was assessed by microscopy with the observation of an enlarged nucleoli in early embryos and further confirmed by genotyping. The presence of *lin-41(ma104)* mutant allele in *lin-41(ma104)*; *par-2(it5ts)* double mutants was confirmed by the presence of the linked dumpy phenotype.

Protein analysis and phylogenetic tree

Drosophila Brat protein sequence was retrieved using *Ensembl* (release 49). Sequences of *C. elegans* Brat homologs were retrieved from *Wormbase* (release WS190). Protein domains were identified using SMART (Letunic et al., 2006; Schultz et al., 1998). A phylogenetic tree was generated by aligning Brat homolog sequences from *C. elegans*, *Drosophila melanogaster* and *Homo sapiens* using ClustalW and phylogenetic reconstruction was performed using PHYML with the maximum likelihood method and the JTT substitution model.

Suppression assays

For mutant animals, L4 animals from each strain were shifted to semi-restrictive (20 °C) or fully restrictive (25 °C) temperature for 24h. Nine worms were transferred to three plates (three worms per plate) and allowed to lay eggs at the same temperature for 16h before being removed. The viability of the progeny was determined after incubating another 24h at the same temperature, by dividing the number of hatched embryos by the total number of progeny.

For *par-2(RNAi)* and *fem-3(RNAi)* assays, the viability of the progeny was determined as described above except that animals were maintained on plates containing bacteria expressing dsRNA for *par-2* or *fem-3* (Kamath et al., 2003).

Time-lapse analysis of embryonic development

For the visualization of early embryonic development in live specimens, embryos were obtained by cutting open gravid hermaphrodites using two 25-gauge needles and individually mounted on a coverslip coated with 0,1% poly-L-lysine in 2µl of egg buffer. The coverslip was placed on a 2% agarose pad and the edge was sealed with petroleum jelly. Time-lapse images were acquired by a Zeiss HRM camera (Carl Zeiss Canada Ltd., Toronto, Canada) mounted on a Zeiss Axio-Imager Z1 microscope, and the acquisition system was controlled by Axiovision software. Images were acquired using Plan Apochromat 63×/1.4 NA or 100×/1.4 NA objectives, at 10s intervals, except to determine the timing between AB and P_1 divisions in experiments described in Table 2 where images were acquired at 2s intervals. Image analysis was performed using ImageJ software.

Indirect immunofluorescence

For immunofluorescence analysis, embryos were fixed in methanol and stained as described previously (Labbé et al., 2004). The following primary antibodies were used: rabbit anti-PAR-1 (1:500, generated as described in Guo and Kemphues, 1995), rabbit anti-PAR-6 (1:150, generated as described in Hung and Kemphues, 1999), rabbit anti-PAR-2 (1:20, Labbé et al., 2006), goat anti-PAR-2 (1:150, Santa Cruz Biotechnology sc-9277), mouse P4A1 anti-PAR-3 (1:150) and mouse OIC1D4 anti-P granules (1:300) (Developmental Studies Hybridoma Bank, University of Iowa). Polyclonal antisera recognizing the GST-NCL-1(25–377) or GST-NHL-2(153–500) fusion proteins were respectively produced in two rabbits (GN-19) by Sigma Genosys (Woodlands,