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G Proteins Are Required for Spatial Orientation of Early Cell Cleavages in C. elegans Embryos

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Summary

Heterotrimeric G proteins are signal-transducing molecules activated by seven transmembrane domain receptors. In C. elegans, *gpb-1* encodes the sole Gβ subunit; therefore, its inactivation should affect all heterotrimeric G protein signaling. When maternal but no zygotic *gpb-1* protein (GPB-1) is present, development proceeds until the first larval stage, but these larvae show little muscle activity and die soon after hatching. When, however, the maternal contribution of GPB-1 is also reduced, spindle orientations in early cell divisions are randomized. Cell positions in these embryos are consequently abnormal, and the embryos die with the normal number of cells and well-differentiated but abnormally distributed tissues. These results indicate that maternal G proteins are important for orientation of early cell division axes, possibly by coupling a membrane signal to centrosome position.

Introduction

Heterotrimeric GTP-binding proteins (G proteins) are signal-transducing molecules that are found in all eukaryotes. The three subunits (α, β, and γ) form a complex that can couple signals from seven transmembrane receptors, which are activated by binding a variety of ligands, including hormones and neuropeptides. When inactive, the α subunit is in the GDP-bound state. Upon activation, GDP is exchanged for GTP, and the heterotrimer dissociates into an α monomer and a βγ dimer. Both can regulate a variety of enzymes and ion channels that change the levels of second messengers inside the cell (for reviews see Gilman, 1987; Birnbaumer, 1992; Clapham and Neer, 1993; Neer, 1995).

To study the function of G proteins in vivo, and to identify other elements acting in the signaling cascade, we have begun to explore G proteins in the nematode Caenorhabditis elegans. At present, nine α subunits have been identified in C. elegans, including members of the conserved families Gαo (Lochrie et al., 1991), Gαs (J.-H. Park et al., unpublished data), Gαq (Brundage et al., 1996), Gα12 (C. elegans genome sequencing consortium), and members of a novel family, designated GPA (Lochrie et al., 1991; Fino Silva and Plasterk, 1990). The Gαo protein has been shown to be involved in egg-laying and movement (Ségalat et al., 1995; Mendel et al., 1995), whereas *gpa-2* and *gpa-3* are involved in dauer formation, a pheromone-induced developmental program (Zwaal et al., submitted).

Different organisms contain either one or more Gβ subunits, and these typically interact with many different α subunits. Saccharomyces cerevisiae and Dictyostelium discoideum contain a single Gβ, but in mammals there are at least four different subtypes (Simon et al., 1991). In C. elegans, there appears to be a single β subunit (GPB-1, encoded by the *gpb-1* gene), which is 86% identical to mammalian Gβ subunits (van der Voorn et al., 1990). GPB-1 should, therefore, interact with all of the α subunits and be involved in all heterotrimeric G protein–regulated processes in the worm.

In this paper, we describe the function of Gβ in C. elegans development and behavior. Gβ is expressed in most or all cells, with the strongest expression in neurons. Gβ is also expressed in the germline and is provided maternally to the embryo, where it is found at the cell membrane and in the region of the asters. We find that Gβ is required during several stages of development and is involved in the regulation of behaviors such as egg-laying and locomotion. Furthermore, we identify a novel role for Gβ, namely in properly orienting division axes in early embryonic cells.

Results

Gβ Is Concentrated at the Membranes of Somatic and Germline Tissues

The GPB-1 protein has over 80% amino acid identity with mammalian Gβs. Because of the high conservation among Gβ genes, and because *gpb-1* is the only gene detected in low stringency hybridizations, *gpb-1* is thought to be the only Gβ gene in C. elegans (van der Voorn et al., 1990). Therefore, identifying the cells that express GPB-1 will show all sites where heterotrimeric G proteins may be active. We raised rabbit polyclonal antibodies against GPB-1 (see Experimental Procedures); with purified antisera, one prominent band of approximately 36 kDa is detected on a Western blot (data not shown).

In situ immunohistochemistry with this antiserum shows that GPB-1 is present in early embryos. Diffuse

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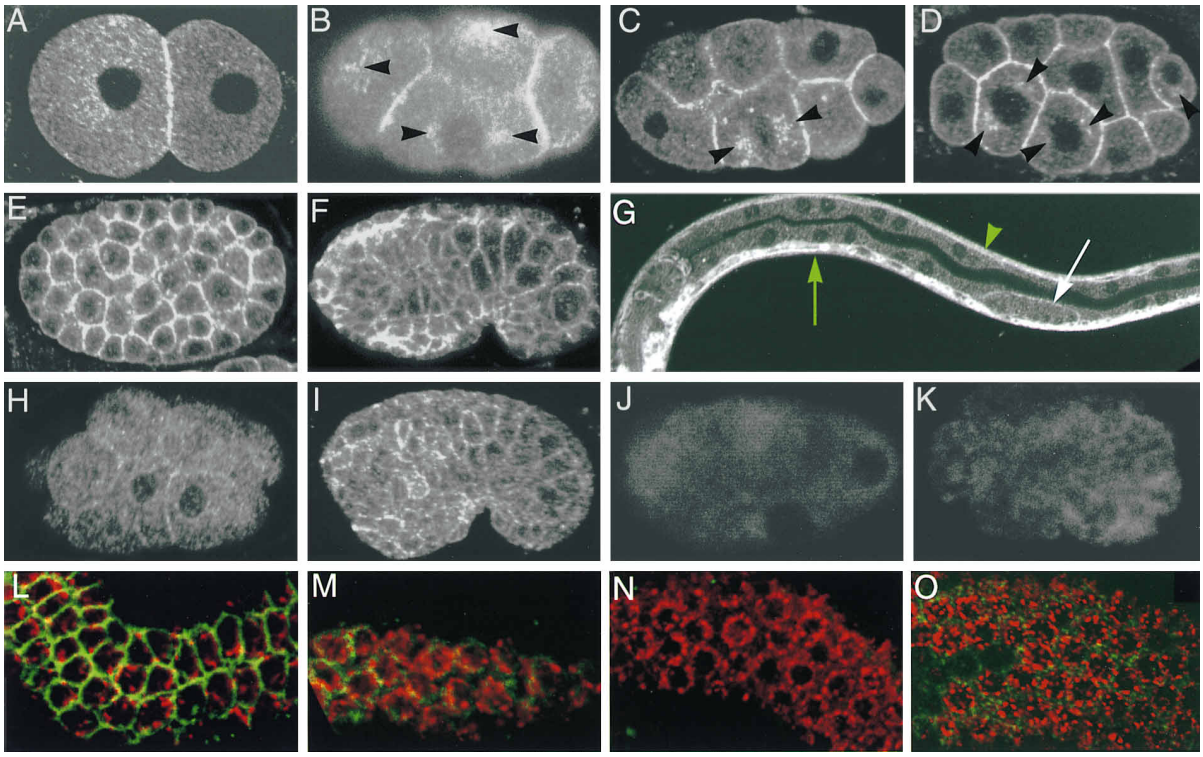


Figure 1. Expression of *gpb-1*

(A–G and L) Immunolocalization of anti-GPB-1 antiserum in wild-type animals. Shown are the following: 2-cell embryo, with staining concentrated at the region between the cells (A); 4-cell embryo (B); 12-cell embryo (C); 20-cell embryo (D); cleavage stage, about 200 cells (E); and a comma-stage embryo (F). In (A)–(F), staining is at the cell membrane and appears equally intense in all cells. In (B), (C), and (D), arrowheads mark staining that colocalizes with the asters.

(G) L2 larva. Staining is brightest in neuronal cells. Green arrow, ventral nerve cord; green arrowhead, dorsal nerve cord; white arrow, gonad. The circumpharyngeal nerve ring, which also shows strong staining, is out of this focal plane.

(L) A portion of the meiotic germline syncytium in a distal gonadal arm of a wild-type hermaphrodite. GPB-1 (green) is found at the membrane of developing gametes. Red staining shows P granules, a germline-specific marker used as a control.

(H, I, and M) Rescued *gpb-1*(*pk44*df); *pkEx179* animals.

(H) 20-cell embryo, showing weaker staining than wild-type (compare with [A]–[E]).

(I) Comma-stage embryo with wild-type level of staining (compare with [F]).

(M) Germline from adult hermaphrodite, most of whose progeny lived; staining is weaker than wild-type (compare with [L]).

(J, K, N, and O) *gpb-1*(*pk44*df); *pkEx179* probable mosaic animals.

(J) and (K) show a 4-cell and a 200-cell embryo, respectively, laid by *gpb-1*(*pk44*df); *pkEx179* probable germline mosaic mother, all of whose progeny died. There is no detectable GPB-1 staining in these embryos. The images were made very bright to show that there is no membrane-associated staining. The embryos were costained with an anti–P granule antibody as a control for permeability.

(N and O) Probable mosaic germlines from *gpb-1*(*pk44*df); *pkEx179* mothers (all progeny from these mothers died as embryos). Staining is undetectable in (N) and very weak in (O). In (L)–(O), GPB-1 is green and P granules are red.

All pictures are single-plane confocal images. Merging and false color in (L)–(O) was added using Adobe Photoshop software.

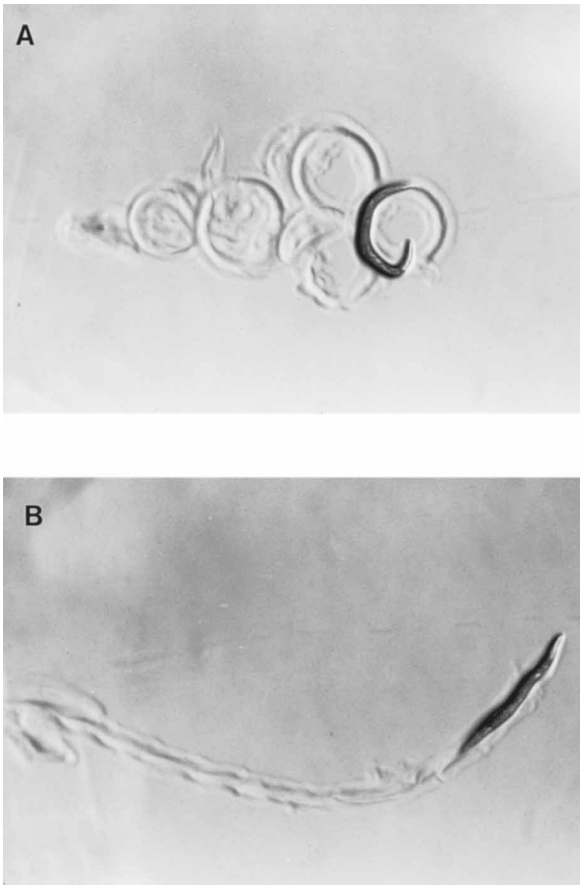
staining can be seen at the 1-cell stage (data not shown). From the 2-cell stage onward, GPB-1 is detected most strongly at the cell membrane, with staining concentrated at the contact between cells (Figures 1A–1F). Interestingly, staining also colocalizes with the asters (arrays of microtubules emanating from the centrosomes) just before and during early cell divisions (Figures 1B–1D). Up to the beginning of morphogenesis, staining continues at approximately equal levels in most or all cells (Figures 1E and 1F). Once tissue differentiation occurs, staining is brightest in neuronal cells (Figure 1G; data not shown). In larval and adult stages, GPB-1 expression is seen in most or all neurons, including the nerve ring, the dorsal and ventral nerve cords, and the preanal ganglion. In addition, the somatic gonad, vulva, and hypodermal seam cells have high expression (Figure 1G; data not shown). Other tissues, such as the intestine, pharynx, and body wall muscles, appear to have a low level of expression (Figure 1G; data not shown). The specificity of the antibody staining is indicated by the following controls: first, the staining is competed away by preincubation of the antibody with GPB-1 protein (see Experimental Procedures) and, second, *gpb-1* mutant embryos do not exhibit any staining (see below).

GPB-1 is also expressed in the germline. Figure 1L shows GPB-1 at the membranes of immature gametes in the dissected gonad of an adult hermaphrodite. This germline expression coupled with staining in early embryos suggests that GPB-1 is maternally contributed.

Table 1. GPB-1 Control of Locomotion

Animal	n	Wavelength (mm)	Amplitude (mm)	Waves (per minute)
NL441 (mosaic *gpb-1*)	8	0.32 ± 0.08	0.09 ± 0.01	10.0 ± 3.4
Wild type	8	0.37 ± 0.07	0.04 ± 0.01	21.0 ± 4.2
NL457 (*gpb-1* transgene)	8	0.38 ± 0.06	0.03 ± 0.01	0.9 ± 0.5
NL459 (*gpb-1* transgene)	8	0.44 ± 0.07	0.02 ± 0.01	2.5 ± 2.2

Tracks were measured that were made by L4 larvae in a bacterial lawn at 20°C. Strains are described in Experimental Procedures.



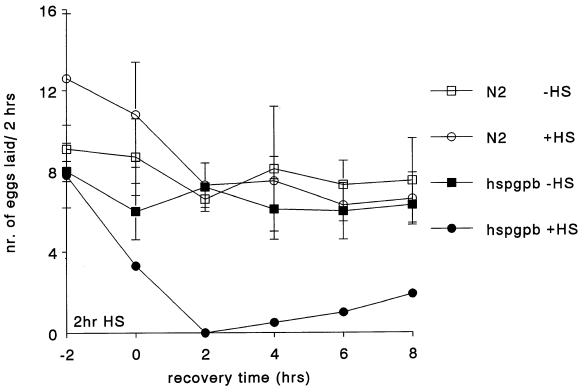


Figure 2. Transient Expression of *gpb-1* Affects Locomotion and Egg-Laying

(A and B) Wild-type animals transformed with the *gpb-1* gene fused to the heat shock promoter (*hsp*) and a cotransformation marker that causes animals to roll, making circular tracks (*rol-6(dm)*; Mello et al., 1991). (A) shows no heat shock. In (B), 2 hr after heat shock (2 hr at 30°C), animals move sluggishly and do not produce circular tracks.

(C) The effect of transient *gpb-1* expression on egg-laying. Wild-type N2 is depicted with open symbols and the transgenic *hsp/gpb-1* line with closed symbols. Non-heat-treated animals are depicted with squares and heat-treated animals (2 hr at 30°C) with circles. Each point represents the average number of eggs laid by 12 animals in a 2 hr time period. Every 2 hr, the animals were transferred to new plates and the number of eggs left on the plate was counted. For the heat-treated transgenic strain, standard errors were too small for the symbols used.

Overall, the expression pattern suggests multiple functions for *gpb-1*, in particular in some aspect of early embryogenesis and neuronal function.

Overexpression of Gβ Leads to Behavioral Defects

We examined the effect of *gpb-1* overexpression on C. elegans development and behavior by introducing multiple copies of wild-type *gpb-1* into wild-type animals. To confirm that these extra copies of *gpb-1* were correctly expressed, we fused the 5′ region of *gpb-1* to *lacZ* and *GFP* (Fire et al., 1990; Chalfie et al., 1994) and indeed found expression of these reporter constructs in a pattern similar to endogenous GPB-1 (see Experimental Procedures). Although animals with transgenic *gpb-1* grow somewhat slowly, most of them develop normally to adulthood. However, two behaviors are abnormal. First, the animals have defects in locomotion, moving sluggishly compared with wild type (Table 1). Second, adults are defective in egg-laying (Tables 2 and 3).

To determine whether these effects are due to abnormal development or abnormal cell function, we expressed *gpb-1* in wild-type adults under the control of a heat shock promoter. We found that sluggish movement and retention of eggs could both be induced transiently after exposure to heat (Figure 2). This indicates that overproduction of *gpb-1* results in the abnormal function of developmentally normal cells. Furthermore, this suggests that GPB-1 is normally a limiting component in signal transduction cascades involved in egg-laying and motility. Overexpression of *gpb-1* could result in a higher concentration of free βγ, which could directly regulate the activity of specific effector molecules, titrate away free α subunits, or both. That Gβ acts as a limiting component is also in seen in S. cerevisiae mating behavior, where overexpression of the G protein β subunit Ste4,

Table 2. GPB-1 Control of Egg-Laying: Developmental Stage of Newly Laid Eggs

Animal	n	1–8 Cells	9 Cells–Comma	Postcomma
NL441 (mosaic *gpb-1*)	40	35	5	0
Wild type	40	4	36	0
NL457 (*gpb-1* transgene)	40	0	9	31
NL459 (*gpb-1* transgene)	40	0	5	35

Newly laid eggs were examined for their stage of embryonic development. The stage was classified as follows: 1–8 cells, between the 9-cell stage and the comma stage, and later than comma stage. The first two lines show that rare mosaic animals, which had apparently lost their transgene in some somatic cells, lay their eggs while they are in an earlier developmental stage than the eggs from wild-type parents. The transgenic lines often were severely defective in egg-laying and became bloated with eggs. Hence, most eggs are in a late developmental stage.

which in a dimer with Ste18 directly activates downstream effector molecules, leads to constitutive activity of the mating pheromone pathway (Whiteway et al., 1989, 1990).

Zygotic Gβ Is Required for Development and Behavior

Using a transposon-based method (Zwaal et al., 1993), we isolated a loss-of-function mutant of *gpb-1*. As shown in Figure 3A, a 1.5 kb deletion removes four exons and causes a frameshift, resulting in an early stop codon. Animals homozygous for this allele develop into morphologically normal first stage (L1) larvae, but these show little body wall, pharyngeal, or defecation muscle activity. Soon after hatching, the internal tissues of these mutants shrink, and the animals die (Figures 3B and 3C). This resembles the phenotype of L1 larvae, in which the canal-associated neurons are ablated using laser surgery (J. Sulston, personal communication), suggesting that a defective osmoregulatory system might be the cause of death.

We rescued the larval lethal phenotype with a wild-type *gpb-1* transgene on an extrachromosomal array (Mello et al., 1991). Because extrachromosomal arrays are unstable at meiosis and mitosis (Stinchcomb et al., 1985; Mello and Fire, 1995), the rescued line consists of mosaic animals, in which not all cells that require *gpb-1* carry the transgene. These animals show a variety of developmental and behavioral defects, due to the absence of Gβ in different cells. For example, they can have a starved appearance, grow more slowly, or show growth arrest. Others show an increased amplitude in locomotion (Table 1) or are constitutive in egg-laying (Tables 2 and 3). As described above, overexpression of GPB-1 results in sluggish movement and reduced egg-laying. Thus, motility and egg-laying are oppositely affected by loss of function and overexpression of Gβ, strongly suggesting that these behaviors are under G protein control. As the β subunit presumably contributes to different G proteins, the *gpb-1* mosaic mutant phenotypes should correspond to phenotypes of different mutant α-subunit genes. Indeed, mutants of Gαo (Ségalat et al., 1995; Mendel et al., 1995), Gαq (Brundage et al., 1996), and Gαs (J.-H. Park et al., unpublished data) also affect motility and egg-laying behavior.

Maternal Gβ Is Required for Embryonic Development

In the germline of adults that are rescued by the transgene, *gpb-1* is expressed at a lower level than in wild type (Figure 1M). This is probably due to inefficient transcription of the transgene in the gonad (see Mello and Fire, 1995). Among these rescued animals, about 5% are sterile and have an abnormal germline (data not shown), suggesting that GPB-1 may play a role in germline development. Transgenic expression of *gpb-1* is also low during early embryogenesis (Figure 1H), but increases to a wild-type level after cell divisions are complete (Figure 1I). This suggests that zygotic expression of *gpb-1* starts at this time.

We observed that most fertile adults rescued by the transgene lay a small number of dead eggs, suggesting that maternal GPB-1 activity might be needed for early development. To investigate this possibility, we searched for rare animals that laid only dead eggs, which might occur if the germline was mosaic for, or had lost, the transgene. We found that about 1% of transgenic rescued adults lay only dead eggs; in the gonads of these animals, GPB-1 is either undetectable or expressed at a very low level (Figures 1N and 1O), consistent with the idea that these animals are mosaics that have lost the transgene in the germline. In addition, GPB-1 is undetectable in embryos laid by these mothers (Figures 1J and 1K). These results indicate that maternal GPB-1 has an essential function in early embryogenesis. We will refer to embryos derived from these germline mosaic mothers as *gpb-1* embryos.

Table 3. GPB-1 Control of Egg-Laying: Rate

Animal	n	Number of Eggs in Uterus	n	Rate (eggs laid per hour)
NL441 (mosaic *gpb-1*)	10	3.1 ± 1.0	10	3.1 ± 1.0
Wild type	18	11.1 ± 2.5	8	6.0 ± 1.0
NL457 (*gpb-1* transgene)	12	24.7 ± 3.7		nd
NL459 (*gpb-1* transgene)	12	35.9 ± 9.0		nd

The first line shows that mosaic animals contain few eggs, apparently because they lay them prematurely (compare with Table 2). In contrast, strains that contain the *gpb-1* transgene retain their eggs in the uterus.

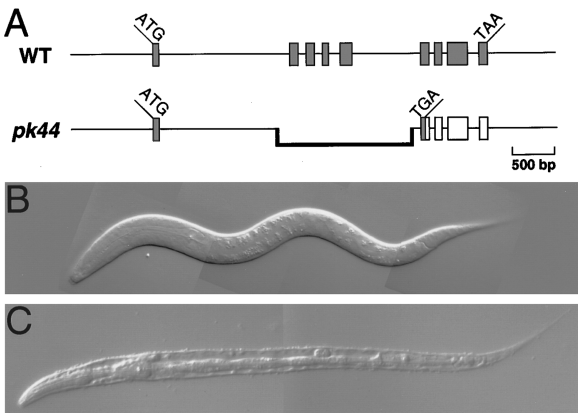


Figure 3. Inactivation of *gpb-1*

(A) Wild-type and deletion allele of *gpb-1*. Coding exons are depicted with stippled boxes, noncoding regions with a flat line. ATG represents the start codon; TAA and TGA represent two stop codons. *gpb-1*(*pk44*df) is a 1.5 kb deletion mutation that removes four exons and creates a putative new intron. Splicing of exon 1 to exon 6 would create a frameshift, resulting in a TGA stop codon after five amino acids. Open boxes represent the remaining *gpb-1* coding sequence that should not be translated in *pk44*df.

(B) and (C) show wild-type and *gpb-1* mutant L1 larvae, respectively. The *gpb-1* larva looks normal upon hatching, but has very little muscle activity. Body tissues then become progressively shrunken, as shown in (C), and the larva dies.

Maternal Gβ Is Required for Cleavage Spindle Orientation during Early Embryogenesis

Since GPB-1 is present in the early embryo, at cell membranes, and colocalized with asters, we looked for defects in the early cell divisions of *gpb-1* embryos. In wild-type embryos, cleavage planes follow a stereotypical pattern, resulting in cells with a fixed position relative to each other (Sulston et al., 1983). The first cleavage is asymmetric, resulting in a larger anterior cell (AB) and a smaller posterior cell (P_1). AB and P_1 divide in different orientations. The AB cell divides first, and AB and its descendants divide symmetrically and synchronously along particular orthogonal axes. The P_1 cell (and its smaller descendant P lineage cells) divides asymmetrically, after orientation of its cleavage plane by rotation of the nuclear–centrosomal complex (Hyman and White, 1987; Hyman 1989). After all cell divisions, centrosomes split and then migrate around the nucleus in opposite directions until they are 180° apart. In most cells, the plane of centrosome migration is invariant, suggesting this is a regulated process.

In *gpb-1* embryos, cleavage planes in early cell divisions are abnormal and apparently randomly oriented (Table 4). For example, ABa and ABp, which normally both divide along the left–right axis, can divide along many different axes (Table 4; Figures 4C and 4D). In addition, nuclear–centrosomal rotation of the P cells appears to be absent (Table 4). In general, divisions follow an unpredictable pattern rather than the precise stereotypical pattern of the wild type.

Although the orientation of cleavage axes is abnormal in *gpb-1* embryos, several other characteristics of early cell divisions are unaffected. First, mitotic spindles and the process of mitosis appear to be normal. Second, as in wild type, the first division is always asymmetric, with the larger daughter dividing before the smaller one. Third, germline P granules, which in wild type are segregated at the first four divisions to the germline precursor cell (Strome and Wood, 1983; Figure 4E), are mostly localized in *gpb-1* embryos, although stray P granules can be found (Figure 4F).

Because the cleavage axes are disoriented, early blastomeres in the developing embryo are found at abnormal positions, and some cell fates appear to be transformed (see legend to Figure 4). Dead *gpb-1* embryos have about the normal number of cells and well-differentiated tissues, including muscle, gut, epidermis, and pharynx (Figures 4B and 4H; data not shown). Taken together, the phenotype of *gpb-1* embryos indicates that Gβ is required to orient early cleavages properly. However, *gpb-1* appears not to be involved in cell cycle control,

Table 4. Spindle Orientations in Wild-Type and *gpb-1* Mutant Embryonic Cells

Cell	P_0	AB	P_1	ABa	ABp	EMS	P_2
Wild type	a/p	d/v	a/p	l/r	l/r	a/p	d/v[c]
gpb-1[a]	ns	ns	ns	d/v	d/v	da/vp	d/v[d]
gpb-1[a]	ns	d/v	l/r	l/r	d/v	a/p	d/v[d]
gpb-1[a]	ns	ns	ns	a/p	d/v	l/r	dl/vr[d]
gpb-1[a]	a/p	d/v	d/v	a/p	dr/vl	a/p	da/vp[d]
gpb-1[a]	ns	ns	ns	a/p	d/v	a/p	d/v[d]
gpb-1[a]	ns	d/v	a/p	a/p	l/r	a/p	d/v[d]
gpb-1[b]	d/v[e]	d/v	l/r	a/p	d/v	ar/pl	al/pr[d]
gpb-1[b]	ns	d/v	d/v	a/p	l/r	a/p	d/v[d]

Shown is the orientation of the initial spindle axis of the cells indicated in wild-type and eight different *gpb-1* mutant embryos. Data are taken from positions of centrosomes as the nuclear membrane broke down. Spindles often changed orientation as they elongated owing to constraints from the egg shell and other cells. In *gpb-1* mutant embryos, cells are named according to location in the embryo and cell division time. Later cell positions in *gpb-1* mutant embryos are increasingly abnormal and impossible to assign. Some *gpb-1* embryos have a more normal division pattern, possibly because they inherited a small amount of maternal *gpb-1* activity. a/p, anterior–posterior; d/v, dorsal–ventral; l/r, left–right; da/vp, dorsoanterior–ventroposterior; dl/vr, dorsoleft–ventroright; dr–vl, dorsoright–ventroleft; ar/pl, anterioright–posterioleft; ns, not scored.

[a] Embryo from NL361 hermaphrodite laying only dead eggs.

[b] Embryo from NL441 hermaphrodite laying only dead eggs.

[c] P_2 spindle oriented toward dividing EMS cell.

[d] P_2 spindle not oriented toward dividing EMS cell.

[e] Spindle was initially oriented along the short axis of the egg (d/v); it skewed as it grew and was oriented along the long axis of the egg (a/p) at the time of the first division.

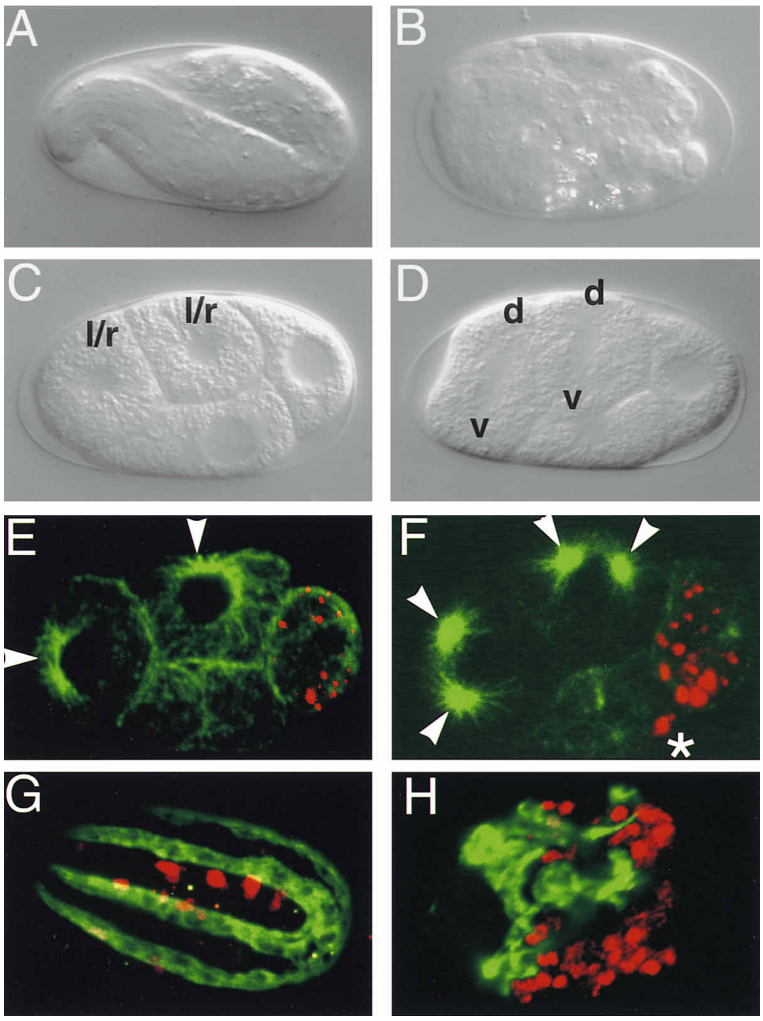


Figure 4. Development of Wild-Type and *gpb-1* Embryos

Wild-type (A, C, G, and E) and *gpb-1* mutant embryos (B, D, F, and H) are shown.

(A and B) Nomarski photomicrographs showing a wild-type embryo near hatching (A) and a terminal *gpb-1* mutant embryo of similar age (B), which has developed into a disorganized mass of tissues. Gut granules (light spots) are visible, indicating that gut differentiation has taken place.

(C and D) Nomarski photomicrographs of 4-cell embryos, in which the cells ABa and ABp (labeled as left–right [l/r] or dorsal [d] and ventral [v]) are dividing. In wild type (C), these cells always divide along the left–right axis, which is perpendicular to the paper. In this *gpb-1* embryo, ABa and ABp both divide along the dorsal–ventral axis (the clear area between d and v is the mitotic spindle). The ABa and ABp divisions occur along different axes in different *gpb-1* mutant embryos (see Table 4).

(E and F) 4-cell-stage embryos, in which ABa and ABp are dividing, stained for tubulin (green) and P granules (red), a localized germline-specific antigen (Strome and Wood, 1983). Anterior is left; dorsal is up. In wild type (E), only part of one aster can be seen in the dividing cells (arrowheads), since the spindle is along the left–right axis (perpendicular to the paper). The P granules (red) are found only in the posterior cell P_2. In the *gpb-1* embryo (F), both asters in each cell are visible in this plane (arrowheads). They have a normal appearance, but, instead of forming left–right axes, their orientations are anterior–posterior in one cell and dorsal–ventral in the other. Most of the P granules are found in the posterior cell P_2, but a few are found in the sister of P_2, called EMS (asterisk). This could occur if P granule localization was normal but an abnormal cleavage orientation failed to partition them correctly. Both embryos are a projection of four confocal sections taken at 5 μm intervals (15 μm total).

(G and H) Embryos stained for muscle (green) and hypodermis (red). Muscle staining in the wild-type embryo at the 2-fold stage (G) is seen in four bands along the body; hypodermal cells are also mostly found in rows. Muscle and hypodermal differentiation in the *gpb-1* embryo (H) occurs, but the tissues are disorganized. Different *gpb-1* mutant embryos contain different amounts of muscle, hypodermal, and pharyngeal tissue (data not shown), with some embryos having an apparent excess or reduction of some types. This suggests that cell fates may sometimes be transformed in *gpb-1* mutant embryos.

the generation of early asymmetries, cell survival, or tissue differentiation. This is reminiscent of what has been described for Dictyostelium: Gβ-deficient cells proliferate under growth conditions, but fail to aggregate in response to cAMP (Lilly et al., 1993).

Discussion

Signaling through heterotrimeric G proteins occurs in all eukaryotes. To learn more about the pathways of G protein signaling, we have been studying the role of G proteins in C. elegans. In this paper, we describe behavioral and developmental functions of *gpb-1*, the sole Gβ subunit gene, which should be involved in all G protein–mediated signaling in the animal. We find that G proteins are involved in the regulation of cell function, for example in processes such as egg-laying and locomotion. Moreover, G protein function is required during early embryogenesis for correct orientation of cleavage planes. Below we discuss these results and how they fit into our current knowledge of G protein function.

gpb-1 Expression

gpb-1 is expressed throughout development. It is expressed in the developing germline and may have a role there, and *gpb-1* activity is contributed as an essential maternal product to embryos. In embryos, GPB-1 is found at the membranes of all cells, particularly at the contact between cells. We also found that GPB-1 transiently localizes to the region of the asters during cell divisions. Zygotic expression of GPB-1 probably starts near the end of embryonic cell proliferation and thereafter is most prominent in neuronal cells, suggesting a

major function in neuronal activity. Significant somatic expression is also seen in other tissues, including the vulva and the somatic gonad. Low expression is seen in the intestine and body muscles. The membrane association of GPB-1 is consistent with known G protein function in transducing signals from seven transmembrane domain receptors.

GPB-1 in Larval Development and Nematode Behavior

Inactivation of the *gpb-1* gene leads to zygotic death at the first larval stage. These larvae appear fairly normal at hatching, but have little muscle activity, and internal tissues progressively become shrunken. This phenotype is suggestive of a defect in the excretory system. This probably regulates osmotic balance, and ablation of the excretory canal–associated neurons leads to a phenotype similar to that caused by the deletion of *gpb-1* (J. Sulston, personal communication). In *vab-8* mutants, misplacement of the canal-associated neuron cells results in a thin, pale, and uncoordinated posterior half, and all tissues in the posterior half appear starved (Manser and Wood, 1990). Interestingly, mutants in some ras pathway genes have an appearance similar to that of *gpb-1* mutants, suggesting a possible link in function. Indeed, the lethality of *let-23* epidermal growth factor receptor mutants correlates with its absence in the excretory system, suggesting this lethality is due to a defect in osmoregulation (Koga and Ohshima, 1995; Simske and Kim, 1995).

Overexpression of *gpb-1* and mosaic rescue of *gpb-1* mutants reveal later functions of Gβ. Overexpression of *gpb-1* results in egg-laying deficiency and sluggish movement, whereas loss of *gpb-1* function in mosaic animals results in the opposite effects: increased amplitudes in locomotion and premature egg-laying. This indicates that these behaviors are under G protein control. Previously, different α subunits have been implicated in these behaviors as well. Egg-laying deficiency correlates with up-regulation of Gαo and Gβ, but with down-regulation of Gαq and Gαs. Similarly, hyperactivity correlates with down-regulation of Gαo and Gβ, but with up-regulation of Gαs and Gαq (Ségalat et al., 1995; Mendel et al., 1995; Brundage et al., 1996; R. Korswagen and R. H. A. P., unpublished data). At present, we cannot yet distinguish whether the effects of GPB-1 are direct, by regulation of effector molecules by the βγ dimer, or indirect, through regulation of Gα activity (or both).

It is likely that *gpb-1* is involved in processes other than those described here. For example, Gα subunits involved in odorant response (K. Roayaie, J. G. Crump, and C. I. Bargmann, personal communication) and dauer pheromone response (Zwaal et al., submitted) have been identified.

Maternal GPB-1 Is Required for Proper Spindle Orientation

In wild-type C. elegans embryos, cell division planes follow a fixed pattern, and this results in the invariant position of embryonic cells relative to each other (Sulston et al., 1983; Strome, 1993; Rhyu and Knoblich, 1995; White and Strome, 1996). *gpb-1* activity is maternally provided, but embryos with no detectable GPB-1 can be obtained from transgenically rescued homozygous mothers that have little or no expression in the germline (presumably these are germline mosaics). In these embryos, mitotic spindles in early cell divisions are formed properly, but their orientations are abnormal and apparently random. As a result, early blastomeres are incorrectly positioned, and although tissues differentiate, they are disorganized.

The phenotype of *gpb-1* embryos is different from mutants in the *par* genes (for partitioning defective), which also affect cleavage plane orientation. The *par* genes are required for several aspects of asymmetry in early embryonic cells, and are involved in the localization of cytoplasmic components (Kemphues et al., 1988; Levitan et al., 1994; Guo and Kemphues, 1995; Etamad-Moghadam et al., 1995; for review see White and Strome, 1996). *par* mutants have some abnormally oriented cleavage axes, but in addition have defects in other early events such as the segregation of germline P granules and the asymmetric first cleavage. In *gpb-1* embryos, the only defect we have observed is in the orientation of cleavage axes; early asymmetries appear to be normal, and P granules are segregated normally. Therefore, heterotrimeric G proteins are not generally involved in early cell polarity, but may specifically regulate centrosome position. This could occur by affecting the plane of centrosome migration after duplication or through involvement in centrosomal rotation after migration in P cells. The involvement of the Gβ subunit in cleavage plane orientation suggests that a Gα subunit should also be involved in this process, but none of the α subunit mutants examined thus far has a maternal effect.

The process of cell polarization in other organisms shows some similarity to GPB-1 function in cleavage plane orientation. G proteins transduce external signals in mating pheromone–induced projection formation in S. cerevisiae, and polarized chemotaxis toward an attractant in neutrophils and Dictyostelium (Devreotes and Zigmund, 1988; Chenevert, 1994; Downey, 1994). In yeast budding, establishment of polarity requires Cdc42, a small GTPase of the Rho/Rac family, for the control of actin organization (for reviews see Chant, 1994; Chenevert, 1994; Drubin and Nelson, 1996).

How might GPB-1 act in orienting C. elegans cleavage axes? The effect we see may be indirect, with G proteins affecting some process required for spindle orientation. However, an attractive possibility is suggested by the protein distribution in early embryos. GPB-1 is found at cell membranes from the 2-cell stage onward, but appears to be concentrated at the contact between cells. This suggests that certain, as yet unidentified G protein–coupled receptors might be activated at the point of cell–cell contact, resulting in a localized source of activated βγ. This could regulate some component(s) of the cytoskeleton involved in directing centrosome position. Cell contact–induced cleavage plane orientation is not unprecedented in the C. elegans embryo; at least two cell divisions (of EMS and E) were shown to require a specific cell contact for proper orientation, although other cleavage planes are thought to be cell-autonomously determined (Goldstein, 1995).

It is also possible that G protein activation is independent of a receptor. For example, it has been shown that GTP-bound tubulin can activate G proteins by transferring GTP to Gα (Roychowdhury et al., 1993; Roychowdhury and Rasenick, 1994). In this scenario, a component of the cytoskeleton could activate G proteins, which in turn could regulate other cytoskeletal components.

Just before and during cell divisions, GPB-1 is associated with the region of the asters. This raises the possibility that the βγ dimer of G proteins provides a direct link between some component of the asters and a localized site on the cell cortex. Astral localization of Gβ has been observed in mammalian cells (Lin et al., 1992), so it is conceivable that it has a similar role in the cells of other animals.

Experimental Procedures

General Maintenance

The methods used for cultivation of C. elegans have been described by Brenner (1974). Strains used in this study were wild-type N2, NL310 (*mut-2(r459); gpb-1(pk13::Tc1)*), CB1362 (*dpy-20(e1362)*), CB128 (*dpy-10(e128)*), SP127 (*unc-4(e120)/mnC1(dpy-10[e128] unc-52[e444])*), and CB1489 (*him-8(e1489)*), described by Brenner (1974), Herman (1978), Hodgkin et al. (1979), and Zwaal et al. (1993). Animals were transformed according to Mello et al. (1991).

Construction of Plasmids

The plasmid pRP403 contains the wild-type *gpb-1* gene on a 6.9 kb SacI fragment, with 2.0 kb upstream of the ATG start codon and 1.1 kb downstream sequence of the TAA termination codon, cloned into a derivative of pK18 (van der Voorn et al., 1990).

To obtain a fusion protein consisting of the entire GPB-1 protein fused to the maltose-binding protein (MBP), a fragment containing the entire open reading frame (ORF) of *gpb-1* was amplified from 1 μg of poly(A)$^+$ RNA. The RNA was reverse transcribed with a poly(dT) primer, followed by polymerase chain reaction (PCR) amplification with primers corresponding to the first and the last 20 nt of *gpb-1* that were extended with an EcoRI and an HindIII site, respectively. The 1 kb band was gel purified and ligated into the pMAL-c vector (New England Biolabs) using the EcoRI and HindIII sites, yielding pRP414. From pRP414, the KpnI–HindIII fragment containing the *gpb-1* ORF was recloned into pGEX-3X (Pharmacia) for fusion of GPB-1 to glutathione S-transferase (GST), yielding pRP422.

gpb-1/lacZ and *GFP* reporter genes were constructed as follows. The 6.9 kb fragment of pRP403 was first recloned in pUC18 (pRP418). The 4.8 kb SphI–NarI of pRP418 (containing all the 5′ sequence and ending in intron 5) was cloned in front of *lacZ* using the SphI and BstBI sites of pPD22.11 to yield pRP427. The 5.2 kb SphI–EcoRV fragment of pRP418 (containing the same 5′ end and ending in exon 7) was cloned in front of *lacZ* using the SphI and SmaI sites of pPD22.11 to yield pRP428, or in front of *GFP* using the same sites of pPD95.81 to yield pRP437.

The plasmid pRP417 contains the promoter of *hsp16-48* (Stringham et al., 1992) fused exactly to the start codon of *gpb-1*. In a first PCR, two fragments were amplified with primers containing extensions that facilitate cloning. hspKpn (5′-GATC**GGTACC**GCTG GACGGAAATAGTGG; KpnI site is in bold) and βhsp (5′-**CAAGTTCG CTCAT**TCTTGAAGTTTAGAG) were used to amplify the 0.4 kb heat shock promoter (*hsp*) from pC31, such that it becomes flanked on one side by a KpnI site and on the other side by the first 13 bp of *gpb-1* (shown in bold). To amplify the first 700 bp of *gpb-1* from pRP403 flanked by the last 10 bp of the *hsp*, we used hspβ (5′-AACTTCAAGA**ATG**AGCGAACTTGACCAAC; start codon in bold) and 721 (5′-CACTACGACACGAGTGGAGG). This latter fragment contains a unique SacII site in the first intron of *gpb-1*. Both fragments were isolated from gel and mixed for reamplification. In the first three cycles (without any primer) the annealing conditions were 3 min at 37°C, allowing the overlapping flanks of the two fragments to anneal (underlined in βhsp and hspβ). Subsequently, the outer primers (hspKpn and 721) were added, and the total 1.1 kb fragment was amplified in 30 additional cycles with annealing conditions of 1 min at 55°C. This fragment was gel purified, digested with KpnI and SacII, and substituted for the KpnI–SacII fragment of pRP403 (swapping all sequences upstream of the start codon).

Other plasmids used in this study were pPD22.11 (containing *lacZ*; Fire et al., 1990); pPD95.81 (containing *GFP*; A. Fire, personal communication); pC31 (containing *hsp16-48*; Stringham et al., 1992); pRF4 (containing the dominant *rol-6(su1006)* marker; Kramer et al., 1990); and pMH86 (containing the wild-type *dpy-20* gene; Han and Sternberg, 1991).

Production of GPB-1 Antisera

GPB-1 was fused to MBP or GST as described above. JM101 transformed with pRP414 or pRP422 were grown in TB and induced with IPTG. MBP–GPB-1 and GST–GPB-1 fusion proteins were extracted from sonicated pelleted bacteria, purified over an amylose column and a GST column, respectively, and mixed with Freund's adjuvant. Two rabbits were injected at 4 week intervals, three times with MBP–GPB-1 at a dose of 200 μg/ml per injection and then once with GST–GPB-1 at the same dose. The antisera were purified over a protein A column and tested on Western blots. The antiserum 68088 used in this study specifically recognized a band of approximately 36 kDa in wild-type N2 extracts, the typical size of G protein β subunits (data not shown). In extracts of a *gpb-1* deletion strain rescued by the extrachromosomal transgene (NL361; see below), the same band was detected, indicating that the transgene produced a GPB-1 protein of normal size. The antibody has virtually no cross reactivity, as assayed by in situ immunohistochemistry on *gpb-1* embryos (see below; Figures 1J and 1K).

Antibody Staining

Embryos, larvae, and adults were prepared for antibody stainings as follows (essentially as in Albertson, 1984). Animals were placed on a poly-L-lysine-coated slide in water, a coverslip was applied, and the liquid was wicked away until they were slightly squashed. This was then frozen on dry ice for 10 min, the coverslip was flicked off, and the slides were put immediately into −20°C methanol for at least 10 min and −20°C acetone for at least 10 min. The tissues were then rehydrated quickly through an acetone series, placed in PBS with 0.1% Tween 20 (PBST) for 10 min, in PBST plus 1% nonfat milk for 30 min, and then the primary antibody was applied. Incubations of the primary antibody were typically overnight at 4°C and of the secondary antibody at room temperature for 2 hr. All antibodies were diluted in PBST before use. The antibodies used are as follows: anti-GPB-1, 1:1000 dilution of protein A–purified serum, as described above; anti–P granule monoclonal cell supernatant (0IC1D4; Strome and Wood, 1983); anti-LIN-26 rabbit polyclonal serum (a hypodermis-specific antibody; M. Labouesse, personal communication); monoclonal antibody 5.8.1 against body wall myosin (Miller et al., 1983); rat monoclonal antibody YL1/2 against tubulin (Kilmartin et al., 1982). To examine carefully the colocalization of anti-GPB-1 and the astral region, we performed double staining with anti-tubulin antibodies. In all cases where a concentrated region on anti-GPB-1 staining was seen, anti-tubulin antibodies also stained in that region.

The specificity of staining with the anti-GPB-1 antibody was tested in a competition experiment. Protein A–purified antibody diluted 1:10,000 was mixed with anti–P granule antibody OIC1D4 (as an internal control) and no or 170 μg/ml GPB-1 fusion protein (fused to MBP). This was left overnight at 4°C, and then embryos were stained as described above. Without competition, staining was as bright as with the dilution of 1:1000 used for characterization of the expression pattern. Inclusion of GPB-1 fusion protein abolished both astral and membrane-associated staining by anti-GPB-1 antibody, as well as the staining of neurons in later developmental stages, but did not affect anti–P granule staining.

Transient Overexpression of GPB-1

The plasmid pRP417 contains the promoter of *hsp16-48* fused exactly to the start codon of *gpb-1*, as described above. We injected

50 ng/μl pRP417 along with 150 ng/μl of pRF4 (containing the *rol-6(dm)* marker) into the wild-type strain N2 to create an extrachromosomal array (NL349; *pkEx169*). The transgene was integrated into the genome upon irradiation with 40 Gy from a ^{137}Cs source (see Mello and Fire, 1995) and backcrossed twice against N2 to produce strain NL451 containing the transgene *pkIs181*. Transgenic animals were grown at 15°C to prevent leakage expression of the promoter and were placed at 30°C for 2 hr to induce promoter activity. To assess the effects on egg-laying, six young adult N2 or NL451 hermaphrodites were first transferred to a fresh NGM plate seeded with OP50 and allowed to lay eggs at 15°C for 2 hr. After transfer to new plates, the animals were placed at 30°C for 2 hr and then transferred to fresh plates at 15°C every 2 hr. The number of eggs laid was counted at the end of every 2 hr. Control non-heat-shocked N2 and NL451 hermaphrodites were treated in the same way, except for omitting the 2 hr at 30°C.

Overexpression of *gpb-1* and Expression of Reporter Constructs

The plasmid pRPP03, containing the wild-type *gpb-1* gene, was injected into N2 and *dpy-20(e1362)* at different concentrations: at 10 ng/μl along with 10 ng/μl pMH86 (*dpy-20(+)* marker) and 180 ng/μl carrier pGEM5 in *dpy-20(e1362)* to produce NL411 (*dpy-20(e1362); pkEx178*); at 20 ng/μl along with 10 ng/μl pMH86 (*dpy-20(+)* marker) and 170 ng/μl carrier pGEM5 in *dpy-20(e1362)* to produce NL457, NL458, and NL459 (*dpy-20(e1362); pkEx179*, *pkEx180*, and *pkEx185*, respectively); and at 50 ng/μl along with 150 ng/μl pRF4 (*rol-6(dm)* marker) in N2 to produce NL350 (*pkEx170*). All strains obtained showed defects in egg-laying and locomotion, which roughly progressed with increasing concentration of injected pRP403.

To confirm that pRP403 was correctly expressed, transgenic lines with *gpb-1/lacZ* and *GFP* reporter genes (see above) were made in both N2 and *dpy-20(e1362)* backgrounds. The *lacZ* lines obtained showed only intense staining of neuronal tissue and pharyngeal muscle, whereas the *GFP* lines obtained showed additional staining in other tissues, including body wall muscle, gut, vulva, and somatic gonad (unpublished data). This closely resembles the expression pattern seen with the antiserum.

Inactivation of *gpb-1*

A mutant in which the transposon Tc1 was inserted between positions 2918 and 2919 in intron 5 of *gpb-1* was isolated from a frozen mutant library (*pk13*; see Zwaal et al., 1993). With the outer set of primers 3581 and 3906 (5′-GCGTTGATGTCTGACTCGTG and 5′-GGTGAAGCTCTCGTGGTGCTG) and the nested set of primers 3582 and 3907 (5′-CACATGCCGTCTCGGATATC and 5′-AACATTAT GGAAGTGGAAG) spanning a genomic region of 2.2 kb, a 1.5 kb deletion derivative of *pk13::Tc1* was detected (*pk44*df; df denotes deficiency). *gpb-1*(*pk44*df) was isolated using a sib-selection protocol (Rushforth et al., 1993).

The *gpb-1*(*pk44*df) deletion allele could not be maintained as a homozygote, indicating it was either lethal or linked to a lethal mutation. After seven backcrosses against *dpy-10(e128)*, homozygotes still could not be maintained. The strain NL344 contains the *gpb-1*(*pk44*df) allele balanced with mnC1 (Herman, 1978). A quarter of the progeny of NL344 arrest at the L1 stage, and these are homozygous for *gpb-1*(*pk44*df), as determined by PCR (data not shown). The *gpb-1*(*pk44*df) PCR product was isolated from gel and sequenced in a cycle-sequence protocol to determine the deletion end points (Craxton, 1991). *pk44*df contains the following sequence around the site of the deletion: AAGTTGTAAA-gtatttatgttgt-ATCACTTTTT. The sequence in capitals is present in the wild-type *gpb-1* gene; the sequence in lowercase is not present elsewhere in *gpb-1* or in Tc1 and was apparently introduced when the deletion was formed. On Southern blots, a wild-type 4 kb BamHI band as well as a 1.5 kb smaller band of nearly equal intensity were observed in NL344, confirming that the detected PCR fragment represents a true deletion that is heterozygous in this strain (data not shown).

Rescue of *gpb-1*(*pk44*df)

Two of the transgenic arrays described above were used to rescue the lethal phenotype of *gpb-1*(*pk44*df): *pkEx170*, containing pRP403 (*gpb-1(+)*) and pRF4 (*rol-6(dm)*), and *pkEx179*, containing pRP403 (*gpb-1(+)*) and pMH86 (*dpy-20(+)*). Rescuing strains necessarily show the phenotype of the visible marker present in the array (*rol-6(dm)* and *dpy-20(+)*, respectively), since loss of the transgene would result in L1 lethality. Rescue by the transgenes confirms that the lethality of *gpb-1*(*pk44*df) is due to removal of *gpb-1*. Moreover, it shows that the 6.9 kb SacI fragment of the *gpb-1* region probably contains most or all of the *gpb-1* regulatory sequences. The strain NL361 (*gpb-1*(*pk44*df); *pkEx170*) was obtained by crossing *gpb-1*(*pk44*df)/mnC1; *him-8(e1489)* males with *pkEx170* transgenic hermaphrodites and finding a F2 rolling animal that segregated only rolling animals and dead larvae. NL441 (*dpy-20(e1362)*; *gpb-1*(*pk44*df); *pkEx179*) was obtained by crossing rare spontaneous *pkEx179* transgenic males with *dpy-20(e1362)*; *gpb-1*(*pk44*df)/+ hermaphrodites and picking obligatory non-Dpy segregating F2.

Generation and Analyses of Embryos from Probable Germline Mosaics

Two strains were used for generating probable germline mosaics: NL361 and NL441 (described above). Single L4 or young adult hermaphrodites were picked to individual plates, and these were scored the next day for the production of only dead embryos; 7 of 694 NL361 and 8 of 1870 NL441 produced only dead embryos. To examine early development, we obtained young embryos from these hermaphrodites by cutting them in half in egg salts (5 mM HEPES [pH 7.2], 110 mM NaCl, 4 mM KCl, 5 mM $MgCl_2$). The embryos were then mounted for Nomarski microscopy on poly-L-lysine-coated coverslips over agar pads and sealed with petroleum jelly. A 4D-video recording system (Hird and White, 1993) was used to record early cell divisions in multiple focal planes, and these were played back to analyze early cleavages. The orientation of cell divisions was noted when the asters grew large but before a spindle was set up, because the spindle often skewed as it grew owing to constraints from the egg shell and other cells. Some of these embryos were stained with antibodies as described above.

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