Supporting Information

Yu et al. 10.1073/pnas.1310468110

SI Methods

Worm Tracking. We applied the WormTracker to track *Caenorhabditis elegans* locomotion following a protocol described previously (1). To prepare recording plates, 10-cm Nematode Growth Medium (NGM) agar plates were equilibrated to 20 °C for 18–20 h and all subsequent procedures were conducted at 20 °C. Approximately 1 h before tracking, 1 mL of fresh overnight culture of the bacteria OP50 in L-broth was added to each recording plates and spread to cover the entire agar surface by fast swirling. Excess liquid was then withdrawn and discarded. The plates were covered with a sheet of Kimwipe to prevent dust accumulation and let dry. The recording plates had an even, thin lawn of bacteria on the entire surface.

Hermaphrodites were picked at the L4 larval stage, 18–20 h before tracking to control the age of the animals tracked. At the time of tracking, these animals became young adults. Individual animals were placed on recording plates. The recording plate was placed on a WormTracker. The worm was located under the microscope and recorded for 4 min. The entire process, including animal culture and recording, was performed at 20 °C.

To use the parallel worm tracker (2) for population assays, about 150 first-day adult worms were placed on each plate. Each plate was recorded at three nonoverlapping areas on the bacterial lawn. Each area was recorded for 30 s. The recording was performed at 20 °C. The average velocity of each area is used as a data point. Thus, one plate generates three data points.

RNAi. RNAi was performed as described (3). We used RNAi clones from the Vidal library (3), and from the Ahringer library (4) when the former was not available. Five to 10 L4 animals were placed on RNAi plates. They were transferred to another RNAi plate the next day and removed after 4 h when there were about 150 eggs on the plate. These eggs were cultured at 20 °C until they become first-day adults. The plates were then directly used for the parallel worm tracker analysis. For each gene, two trials of RNAi were performed and duplicates were used in each trial. For the WormTracker analysis of individual worms, RNAi animals were picked at L4 stage, moved to a new RNAi plate, and analyzed the next day following the WormTracker procedure.

Clustering. We performed hierarchical clustering on genes with locomotive phenotypes (Fig. S1). Average linkage hierarchical clustering was performed using uncentered correlations as a measure of distance between clusters. This method uses an agglomerative procedure to identify the most similar locomotion profiles and group them into clusters (for reviews see refs. 5 and 6). We used the software Cluster 3.0 (7) and Java Treeview (8) for calculation and visualization of the clusters.

Phorbol Ester Rescue Experiment. Phorbol 12-myristate 13-acetate (PMA, 5 μM) plates were prepared as described (9, 10). Briefly, 5 mM PMA stock (dissolved in 100% ethanol) was added to 55 °C molten NGM medium to a final concentration of 5 μM. The medium was poured into six-well plates in a fume hood, and allowed to solidify for 16 h. The control plates contained ethanol (EtOH) only. The plates were then seeded with 100 μL of fresh OP50 culture and kept at room temperature for 24 h.

First-day adults of wild-type N2 or *plc-3*(*tm1340*)/*mIn1; egl-8* (*n488*) were picked to NGM plates seeded with OP50 to lay eggs overnight. On the next day (day 1), hatched young stage-L1 larvae were transferred to 5 μM PMA plates or EtOH control plates. The homozygous *plc-3*(*tm1340*)*; egl-8*(*n488*) double-mutant larvae were picked under a SteREO Discovery V20 stereomicroscope (Carl Zeiss) by selecting against the *myo-2::GFP* carried by *mIn1*. We placed 50 stage-L1 animals in each well and used triplicates for each condition. Animal growth was monitored daily and the distribution of developmental stages was recorded when N2 animals started to reach adulthood. Specifically, the animals were recorded after 48 h (day 3) on EtOH control plates and 120 h (day 6) on PMA plates because animals grow slower on PMA plates (11). Missing and dead animals (due to early larval death or crawling off the media) were excluded in the final numbers.

Aldicarb and Levamisole Sensitivity. Aldicarb and levamisole acute paralysis assays were performed as previously described (10–12). Staged 1-d-old adult animals were used in both assays. For the aldicarb assay, NGM plates containing 2 mM aldicarb were made and seeded with bacteria the day before the assay. For each strain, 20 animals were placed on the plate and monitored every 10 min for 2 h. Animals were considered paralyzed if they show no movement after a gentle tap on the head using a worm pick. Three trials were performed for this assay. For the levamisole assay, 10 animals of each genotype were placed in a well of a 96-well microtiter plate containing 50 μL of 100 μM levamisole in M9 buffer. The number of paralyzed (not thrashing) animals was inspected every 10 min over a 60-min period. We performed six trials for this assay.

Bleach Sensitivity. The bleach sensitivity assay protocol was modified from ref. 13. A diluted alkaline hypochlorite solution was used in our assay to allow more accurate timing. The solution was freshly prepared with 0.05 M NaOH and 2% (vol/vol) household bleach. Staged 1-d-old adult animals were used. Each animal was placed in a well of a 96-well microtiter plate containing 50 μL of alkaline hypochlorite solution. Both the time taken for the animal to stop moving and to start to break-up were recorded. Ten to 20 animals for each strain were tested.

SI Results

Locomotive Parameters. The WormTracker measures a total of 66 *C. elegans* locomotive parameters (Table S2). These parameters were arbitrarily chosen; therefore, it is necessary to use certain objective criteria to select the parameters that are most appropriate for our analysis. The large number of behavioral profiles we obtained enabled us to apply two criteria to select consistent and independent parameters.

We first used wild-type animal profiles to screen for parameters with low variance. We pooled all wild-type animals together and examined the coefficient of variance (ratio of the SD to the mean value) of each parameter (animal-to-animal variance, Table S3) and selected parameters with a coefficient of variance lower than 50%.

We also computed the mean values of wild-type animals tracked in each day and analyzed the day-to-day variation of the means (day-to-day variance, Table S3). Among the parameters that are highly variable among individual worms, two of them, duration of stopped time and number of reversals, showed more consistent day-to-day means. These data suggested that the intrinsic animal-to-animal variations of these parameters can themselves be used as new measurements. The other two parameters with high animal-to-animal variants, duration of forward movement and backward movement, are highly variable even for day-to-day means. It is possible that these parameters are

intrinsically random in *C. elegans* locomotion, or that our experimental conditions were not optimal for such measurements.

We then used mutant profiles to compute the Pearson correlation coefficient (PCC), a standard measurement of correlation, among all parameters (Table S4). There are three cases of highly correlated (PCC >0.7) parameters. (*i*) When the same parameter (e.g., flex) is measured for each of the 13 articulation points along the animal midline, the values for all points are highly correlated. We thus chose the parameter for the middle point to represent the entire group. (*ii*) The values for all parameters in forward movement showed strong correlations with those in backward movement (Table S4). Nevertheless, it is known that some mutants (e.g., *unc-4* mutants) have defects in only one type of movement (14). Therefore, we kept both forward and backward measurements. (*iii*) Velocity, centroid velocity, and frequency are highly correlated. We kept velocity and frequency because they measure two different aspects of worm movement.

After applying these two criteria, the remaining 10 locomotion parameters are velocity, flex, frequency, amplitude, and wavelength for both forward and backward locomotion.

Reproducibility of Locomotive Phenotypes. To evaluate the reliability of our genetic screen, we asked two questions: Can a phenotypic pattern detected in our screen be reproduced with a different set of animals? Can a phenotypic pattern be reproduced with different alleles?

We chose 30 strains with locomotive phenotypes and repeated the assay at least once with another set of animals on a different date. For each strain, the animals were split into two groups based on experimental dates. Both groups had at least five animals and were similar in size. A phenotypic profile was derived from each group and the PCC value between the two profiles was calculated. If the phenotypes are reproducible, the profile derived from earlier experiments should be highly correlated to that from later experiments. Indeed, the two phenotypic profiles are strongly correlated (PCC above 0.6) for 25 of the 30 strains (83%) and mildly correlated (PCC between 0.3 and 0.6) for three strains (10%) (Table S5). Only two strains showed inconsistent phenotypic patterns (PCC between −0.3 and 0.3) (Table S5). Because both of these strains were unoutcrossed, it is likely that the phenotypes were affected by unstable background mutations.

To evaluate the effects of background mutations, we chose 11 genes with locomotive phenotypes and tested them with at least two alleles. Comparing the locomotive profiles from different alleles of the same gene, we found that the phenotypic profiles are highly correlated (PCC above 0.6) in 10 out 12 cases (Table S6). Even the phenotypes of three out of four unoutcrossed strains were confirmed by another strain (Table S6). Altogether, these data showed that our screen results are reliable.

To further eliminate possible noise from background mutations in unoutcrossed strains, we verified the phenotypes of all unoutcrossed strains with RNAi. Among the 119 mutants that showed significant locomotive phenotypes, 36 strains were unoutcrossed. To perform RNAi on these 36 genes, we used the strain TU3401, a strain that is sensitized to RNAi in neurons and desensitized to RNAi in other tissues (15). We first used a different system, the parallel worm tracker (2), to examine whether RNAi can reproduce the velocity phenotype of the mutants. The parallel worm tracker analyzes a population of worms simultaneously by reducing each worm into one dot and measuring its velocity. We eliminated 13 genes that showed significant velocity phenotypes in mutants but normal velocity in RNAi animals. We then used the WormTracker to analyze at least 10 RNAi animals for each of the remaining 23 genes and derived the locomotive profiles from the RNAi data. Twelve genes showed positively correlated (PCC above 0.3) RNAi and mutant locomotive profiles (Table S6) and were kept in our final list. Among them was *nlp-1*, where the two unoutcrossed alleles showed different phenotypes. RNAi confirmed the phenotype of one allele (*ok1469*), suggesting that the phenotypes in the other allele (*ok1470*) are affected by background mutations. Among the remaining 11 genes whose RNAi locomotive profiles are inconsistent with mutant profiles, most of them showed no RNAi phenotype, with the only exception of the gene *nlp-17*. *nlp-17* RNAi showed significant phenotypes with locomotive parameter values more than 10% deviant from wild-type values. The discrepancy between *nlp-17* RNAi and mutant phenotypes might be caused by background mutations in the mutant strain. It should be noted that genes with no RNAi phenotypes may still be locomotive genes because of low RNAi penetrance.

After the genetic screen and RNAi verification process, we concluded that 87 neuronal signaling genes are involved in regulating locomotive behaviors. This is based on phenotypic profiles from 94 mutants. The phenotypic profiles from 19 genes have been validated by additional alleles or RNAi results, including all 14 unoutcrossed strains.

Clustering of Locomotive Behavioral Profiles. We first attempted to identify interacting genes by clustering their behavioral profiles. Hierarchical clustering of the 94 mutant behavioral profiles revealed a couple of functional groups corresponding to known genetic pathways (Fig. S1). For example, five of six genes (*dop-3*, *dgk-1*, *eat-16, gpb-2*, and *goa-1*) in one cluster (Fig. S1) encode proteins that inhibit ACh release (16, 17). Another cluster of eight genes (Fig. S1) has five genes (*egl-8*, *unc-2*, *unc-13*, *unc-31*, and *unc-73*) that function at least in part, in the Gαo–Gαq network that promotes ACh release (16, 17). We were, however, unable to recognize more functional modules from the cluster data. Therefore, we sought a new computational approach to construct the locomotive gene networks by using |PCC| values and GeneOrienteer scores.

Drug Sensitivity of *plc-3* Mutants. Two drugs, the acetocholinesterase inhibitor aldicarb and the ACh receptor agonist levamisole, have been commonly used to examine defects in ACh signaling. Previous findings have shown that *egl-30*, *egl-8* mutants are highly resistant to aldicarb, no different from wild-type in response to levamisole, and that *unc-73* mutants are mildly resistant to aldicarb and hypersensitive to levamisole (11, 18, 19). We found that *plc-3* mutant animals showed hypersensitivity to both aldicarb and levamisole (Fig. S2 *A* and *B*). However, the *plc-3* drug sensitivity might be caused by defects in cuticle integrity instead of ACh signaling, because *plc-3* mutants displayed increased sensitivity to alkaline hypochlorite treatment (Fig. S2*C*). When exposed to alkaline hypochlorite, *plc-3* mutant animals stopped moving and started to disintegrate significantly faster than wild-type worms (Fig. S2*C*). Because *egl-8* mutants showed normal bleach sensitivity (Fig. S2*C*), the cuticle defect is likely *plc-3* specific. The complication of *plc-3* cuticle defects makes drug assay results less conclusive.

1. Cronin CJ, et al. (2005) An automated system for measuring parameters of nematode sinusoidal movement. *BMC Genet* 6:5–23.
2. Ramot D, Johnson BE, Berry TL, Jr., Carnell L, Goodman MB (2008) The Parallel Worm Tracker: A platform for measuring average speed and drug-induced paralysis in nematodes. *PLoS ONE* 3(5):e2208.
3. Rual JF, et al. (2004) Toward improving Caenorhabditis elegans phenome mapping with an ORFeome-based RNAi library. *Genome Res* 14(10B):2162–2168.
4. Kamath RS, et al. (2003) Systematic functional analysis of the Caenorhabditis elegans genome using RNAi. *Nature* 421(6920):231–237.
5. Fielding A (2007) *Cluster and Classification Techniques for the Biosciences* (Cambridge Univ Press, Cambridge, UK), p 246.
6. Everitt B, Landau S, Leese M (2001) *Cluster Analysis* (Oxford Univ Press, London), 4th Ed, p 237.
7. de Hoon MJ, Imoto S, Nolan J, Miyano S (2004) Open source clustering software. *Bioinformatics* 20(9):1453–1454.

8. Saldanha AJ (2004) Java Treeview—extensible visualization of microarray data. *Bioinformatics* 20(17):3246–3248.
9. Reynolds NK, Schade MA, Miller KG (2005) Convergent, RIC-8-dependent Galpha signaling pathways in the Caenorhabditis elegans synaptic signaling network. *Genetics* 169(2):651–670.
10. Schade MA, Reynolds NK, Dollins CM, Miller KG (2005) Mutations that rescue the paralysis of Caenorhabditis elegans ric-8 (synembryn) mutants activate the G alpha(s) pathway and define a third major branch of the synaptic signaling network. *Genetics* 169(2):631–649.
11. Miller KG, Emerson MD, Rand JB (1999) Goalpha and diacylglycerol kinase negatively regulate the Gqalpha pathway in C. elegans. *Neuron* 24(2):323–333.
12. Nurrish S, Ségalat L, Kaplan JM (1999) Serotonin inhibition of synaptic transmission: Galpha(0) decreases the abundance of UNC-13 at release sites. *Neuron* 24(1):231–242.
13. Gravato-Nobre MJ, et al. (2005) Multiple genes affect sensitivity of Caenorhabditis elegans to the bacterial pathogen Microbacterium nematophilum. *Genetics* 171(3):1033–1045.
14. White JG, Southgate E, Thomson JN (1992) Mutations in the Caenorhabditis elegans unc-4 gene alter the synaptic input to ventral cord motor neurons. *Nature* 355(6363): 838–841.
15. Calixto A, Chelur D, Topalidou I, Chen X, Chalfie M (2010) Enhanced neuronal RNAi in C. elegans using SID-1. *Nat Methods* 7(7):554–559.
16. Perez-Mansilla B, Nurrish S (2009) A network of G-protein signaling pathways control neuronal activity in C. elegans. *Adv Genet* 65:145–192.
17. Bastiani C, Mendel J (2006) Heterotrimeric G proteins in *C. elegans*. *WormBook* 2006: 1–25.
18. Williams SL, et al. (2007) Trio's Rho-specific GEF domain is the missing Galpha q effector in C. elegans. *Genes Dev* 21(21):2731–2746.
19. Lackner MR, Nurrish SJ, Kaplan JM (1999) Facilitation of synaptic transmission by EGL-30 Gqalpha and EGL-8 PLCbeta: DAG binding to UNC-13 is required to stimulate acetylcholine release. *Neuron* 24(2):335–346.

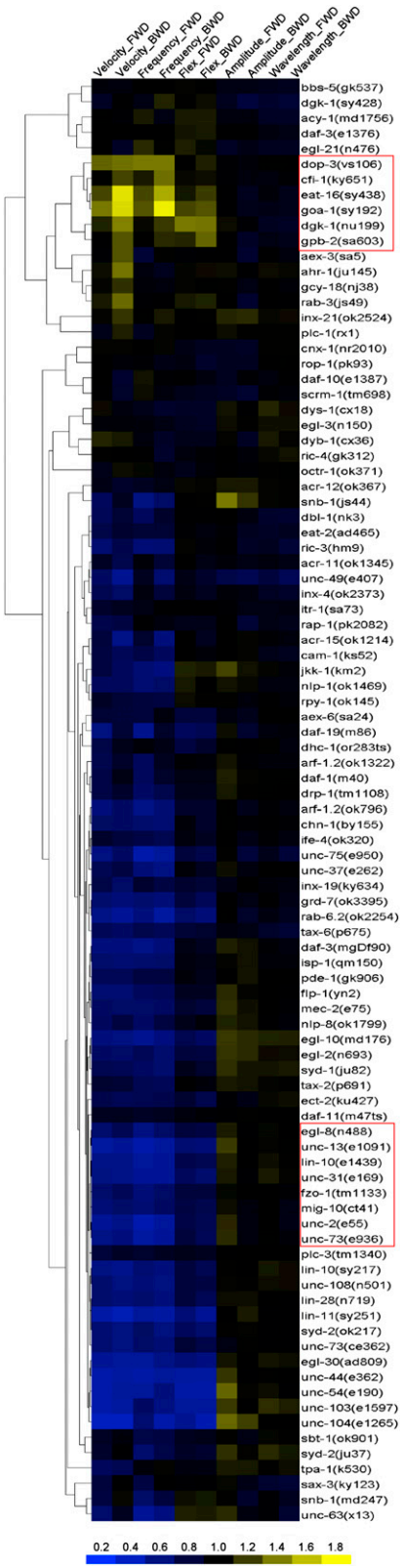

Fig. S1. Hierarchical clustering of behavioral profiles. Two clusters labeled in red highlight two opposite functional groups in the Gαo and Gαq signaling network.

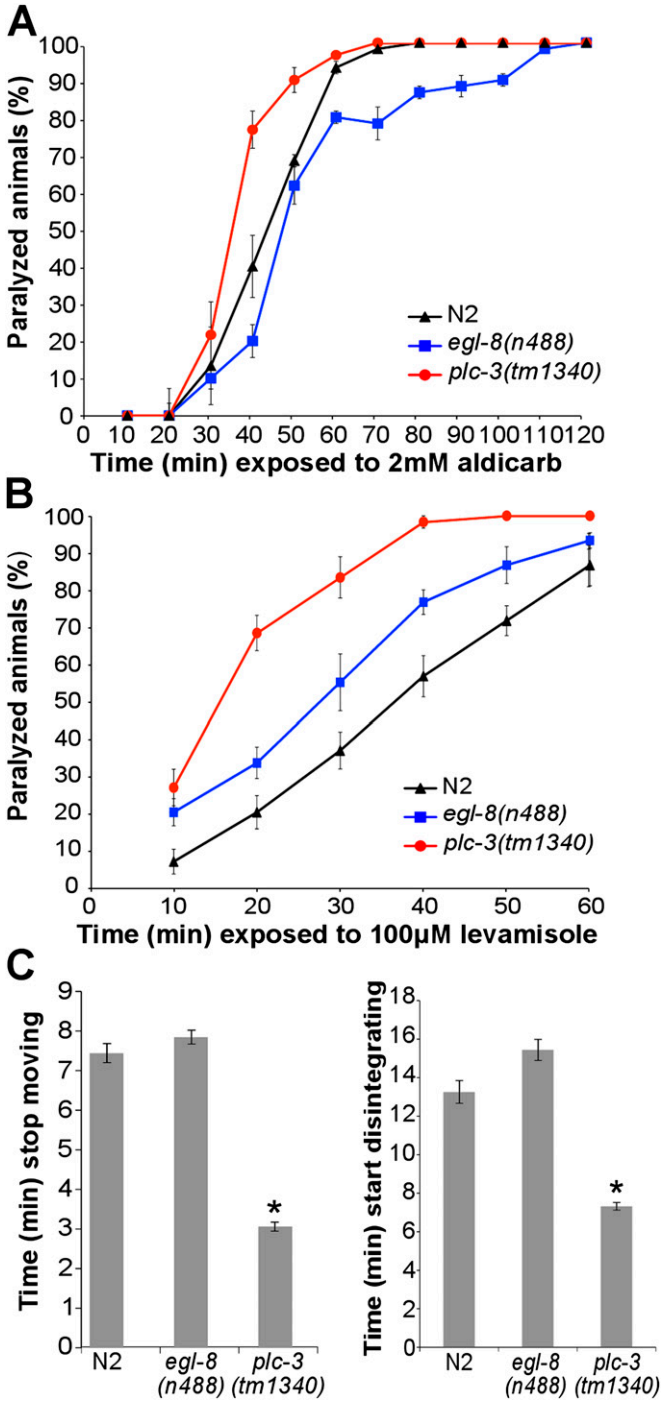

Fig. S2. Drug sensitivity of *plc-3* animals. *plc-3* mutant animals are hypersensitive to aldicarb (*A*), levamisole (*B*), and alkaline hypochlorite (*C*). Data are means and SE. *$P < 0.01$ compared with wild-type N2 animals in Student *t* test.

Table S1. Mutants of neuronal signaling genes tested

Table S1

Table S2. Locomotion parameters

Table S2

Table S3. Coefficient of variance (CV) of parameters (green indicates CV less than 0.5)

Table S3

Table S4. PCC of parameters (green indicates PCC less than 0.7)

Table S4

Table S5. PCC of locomotive profiles derived from different animals (red indicates PCC less than 0.3)

Table S5

Table S6. PCC of locomotive profiles derived from different alleles (red indicates PCC less than 0.3)

Table S6

Table S7. Worm genes with locomotive phenotypes [ortholog data were obtained from InParanoid and WormBase (WS230); human disease information was obtained from www.omim.org]

Table S7