Supporting information for Bülow *et al.* (2002) *Proc. Natl. Acad. Sci. USA* **99** (9), 6346–6351. (10.1073/pnas.092128099)

Supporting Methods

DNA Constructs. The *kal-1* coding region was amplified from the full-length cDNA clone *yk230c3* with primers containing *Kpn*I and *Xba*I sites at their 5' and 3' ends, respectively, and inserted into *pAIY.MCS* (1) yielding *pttx-3*::*kal-1*. *kal-1* expression constructs were generated by releasing the *kal-1cDNA/unc-54* 3' untranslated region (UTR) transcription unit from *pttx-3*::*kal-1* and replacing it for the *gfp/unc-54* 3' UTR cassette in the following plasmids:

pgcy-8::*gfp* → AFD neurons (2)

pmec-7::*gfp* → Touch neurons (3)

pBY103 (*punc-119.MCS*) → Panneuronal (4)

pBH21.10 → Enteric muscles (5)

pPD49.78 → Ubiquitous, heat-inducible (6)

punc-4::*gfp* → Ventral cord motor neurons (7)

plim-6int3::*gfp* → DVB motor neurons (and others) (8).

Transgenic Lines. The *kal-1*::*gfp* reporter construct was injected at 50 ng/μl into *pha-1* mutant animals with *pBX* as injection marker (9). One of the resulting extrachromosomal lines was chromosomally integrated to yield *otIs33IV*. *kal-1* cDNA driven by various different promoters was injected at 10 ng/μl with either *rol-6* (10) or *unc-122*::*gfp* (P. Loria and O.H., unpublished data) as injection marker. Extrachromosomal lines were chromosomally integrated (according to a protocol shown at http://cpmcnet.columbia.edu/dept/gsas/biochem/labs/hobert/protocols.html): *otIs35X*, *otIs76IV*, and *otIs77II* express *pttx-3*::*kal-1*; *otIs78, otIs79, otIs80,* and *otIs81II* express *punc-119*::*kal-1*,; *otIs124* expresses *pttx-3*::*kal-1mWAP*; and *otIs83V* expresses *pgcy-8*::*kal-1*. All integrants were outcrossed several times against wild-type N2.

Transgenic *gfp* Lines Used to Score Neuroanatomy.

AIY interneuron: *mgIs18*; *mgIs32* = integrated *Ex[ttx-3prom*::*gfp; plin-15]* (1)
AFD sensory neuron: *oyIs17* = integrated *Ex[gcy-8*::*gfp; plin-15]* (2) (a gift from P. Sengupta, Brandeis University)
Panneuronal marker: *otIs45* = integrated *Ex[unc-119*::*gfp]* (4)
ASE sensory neurons: *ntIs1* = integrated *Ex[pgcy-5*::*gfp; lin-15]* (2) (a gift from S. Lockery, Univ. of Oregon)
PVQ sensory neurons: *oyIs14* = integrated *Ex[psra-6*::*gfp; lin-15]* (a gift from P. Sengupta)
HSN motor neurons: *mgIs71* = integrated *Ex[ptph-1*::*gfp; rol-6]* (11)
D-type motor neurons: *oxIs12* = integrated *Ex[punc-47*::*gfp; lin-15]* (12)
DVB/RIS motor neuron: *otIs37* = integrated *Ex[pΔunc-47*::*gfp; lin-15]* [a gift from P. Loria and T. Boulin (Columbia University)]
AWA sensory neurons: *kyIs37* = integrated *Ex[podr-10*::*gfp; lin-15]* (13)
AWC sensory neurons: *kyIs140* = integrated *str-2*::*gfp* (14)
Mechanosensory neurons: *uIs25* = integrated *mec-18*::*gfp* (a gift from M. Chalfie, Columbia University)
VC motor neurons: *nIs106* = integrated *lin-11*::*gfp* (15).

Scoring Neuroanatomy. Neuroanatomy was scored with previously described reporter gene constructs expressed in transgenic animals. Axonal branches in AIY caused by *kal-1* over/misexpression (*otIs35*, *otIs76*, and *otIs77)* were scored with either of two chromosomally integrated *gfp* reporters (*mgIs18* and *mgIs32*) that exclusively label AIY in postembryonic animals. These branches usually emanated from the ventral portion of the axon and not the part of the axons that runs dorsally into the nerve ring. The branches are variable in length and can be up to 70 µm long, which roughly equals the length of the whole main axon. The branches are, unless otherwise noted, mostly unbranched themselves and have a strong preference to extend posteriorly. More than one process could often be seen to branch off the main axon. Because the AIY axon in wild-type animals thickens and displays various varicosities and small sprouts (<3 µm) at the entry point into the nerve ring, we scored only branches that emanated from this specific region if they were longer than 6 µm, an arbitrary number that happens to match the

approximate diameter of a cell body. Apparent cytoplasmic extensions from the cell bodies ("rabbit ears," "pointed tips") were not scored. Nevertheless, aberrant axon extensions from the cell body were often observed, but usually coexist with branches from the axon. Neurites that appear to emanate from the cell body may in fact have branched from the beginning of the axon shaft, extending posteriorly over and past the cell body, thus misleadingly appearing as a extension from the cell body.

RNA Interference (RNAi). RNAi was performed with both injection and feeding techniques (16). The double-stranded (ds) RNA was delivered to transgenic animals that express a variety of *gfp* markers (*mgIs18*, *oxIs12*, and *mgIs71*) to allow monitoring neuroanatomy. Neither neuroanatomical defects nor defects in gross morphology or simple behaviors (coordinated locomotion) could be observed. Production and delivery of the RNAi was found to be functional, because both feeding (less consistent) and injection techniques yielded an almost complete suppression of the branching defects induced by *kal-1* expression in AIY. We cannot, however, conclude that *kal-1* expression is completely abrogated and thus cannot interpret the observation that we do not see neuroanatomical defects in *kal-1*(*RNAi*) animals. It should also be noted that AIY contains small axonal branches that can be seen only on the electron microscopical level (17) (D. Hall and O.H., unpublished data) and whose alteration in *kal-1*(*RNAi*) animals we would have been unable to observe with light microscopical techniques.

Immunohistochemistry. We purified two protein fusions of glutathione *S*-transferase (GST) with amino acids 372-522 of *Caenorhabditis elegans KAL-1* (comprising FnIII repeat 3) or amino acids 522-700 (FnIII repeat 4), respectively. These fusion proteins were used in combination to raise a polyclonal Ab in rats and the Ab was purified from crude serum by affinity chromatography with the antigen. For immunostaining, worms were fixed with Bouins' fixative (18) and incubated with the primary Ab at a concentration of 1:25. As a secondary Ab, an Alexa 546-labeled goat anti-rat Ab (Molecular Probes) was used at 1:300, and neuroanatomy was evaluated with epifluorescence microscopy.

Stainings on *otIs35* (*Is[pttx-3*::*kal-1]*) (Fig. 2*A*) or two (*pttx-3*::*kal-1mWAP*) and three (*pttx-3*::*kal-1* and *pttx-3*::*kal-1S241K*) independent extrachromosomal lines, respectively (Fig. 6), were indistinguishable.

Note that KAL-1 localizes along the whole length of the axons of the AIY interneurons. Although Fig. 2*A* in the article clearly shows the KAL-1-induced branches, Fig. 6 demonstrates the even distribution of KAL-1 protein along the axon more clearly. Because of the small axon diameter we cannot conclusively determine whether the visualized protein is located on the axonal cell surface. As shown in Fig. 6*A*, the protein is localized to the cell surface (*Inset*), which could indicate secretion of KAL-1. This notion is also corroborated by the fact that *C. elegans* KAL-1, expressed in vertebrate tissue culture cells, is secreted into the medium (possibly because of the absence of an intact extracellular matrix) and that vertebrate tissue sections reveal KAL-1 to be immobilized in the extracellular matrix (19).

Modifier Screen and Mapping of Modifier Mutants. Modifier mutants were isolated with a clonal F1 screen of 1,310 haploid genomes with ethyl methanesulfonate (EMS) as a mutagen. Briefly, *mgIs18; otIs35* animals that showed a 100% penetrant branching phenotype in AIY interneurons were mutagenized and the F1 progeny singled onto plates. Four to five days later, the mutant F2 progeny were mounted onto slides and the percentage of suppression/modification was scored under a high-power fluorescence microscope in at least 20 animals. Animals were singled out from populations showing a modified phenotype and their progeny rescored for the phenotype. To exclude possible array effects, all mutants were crossed into the independently integrated line *otIs76 mgIs18* and the phenotype verified. Subsequently, all mutants were backcrossed twice with N2 in the *otIs76 mgIs18* background except for *ot17*, which was backcrossed after linking it to *lon-2(e678)*.

KAL-1 Protein Binding Experiments. The different *C. elegans* KAL-1::Fc fusion proteins were generated by PCR and cloned upstream from the hinge region of human IgG1::Fc. The *C. elegans* KAL-1::Fc fusion protein contains amino acids 1-679; *C.*

elegans WAP::Fc contained only the WAP domain (amino acids 1-155). *C. elegans* mWAP::Fc is identical to *C. elegans* KAL-1::Fc except two point mutations that disrupt two disulfide bonds in its WAP domain (C134S and C135S). Fusion proteins were transiently expressed in HEK-293T and collected in DMEM-containing 2% ultralow IgG FCS (GIBCO/BRL). Expression of the Fc-fusion proteins in the supernatant was determined by immunoblotting with anti-human Fc Abs. Binding experiments were performed as described (20). In brief, confluent monolayers of cells in 24-well dishes (in triplicates) were incubated for 1 h at 4°C with blocking medium (0.1% BSA in DMEM/F12), followed by a 1-h incubation with conditioned medium containing 0.25–0.5 mg/ml Fc-fusion proteins. Bound proteins were detected by additional incubation with ^{125}I-Protein A. High salt washes were done by washing bound Fc-fusions twice with phosphate buffer containing 0.8 M NaCl.

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