INTRODUCTION

Kallmann syndrome (KS) is characterized by hypogonadotropic hypogonadism and inability to smell (anosmia), owing to impaired targeting and migration of olfactory axons and gonadotropin-releasing hormone (GnRH)-secreting neurons (Kallmann, 1944). During normal development, olfactory neurons project their axons through the cribriform plate and the meningeal tissue and enter the olfactory bulb. GnRH neurons migrate along the pathway of the olfactory nerve and across the olfactory bulb to eventually reach the hypothalamus (Schwandt-Fukuda and Pfaff, 1989; Wray et al., 1989). In KS, these migrations appear defective, and both olfactory axons and GnRH neurons are found bundled within the meninges above the cribriform plate (Schwandt-Fukuda et al., 1989). In addition, individuals with KS have severe hypoplasia or aplasia of olfactory bulbs and tracts, and, less commonly, unilateral renal aplasia and defects in closure of the lip and of the palate, which may reflect alterations in cell migration (Colquhoun-Kerr et al., 1999; Hermanussen and Sippell, 1985; Wegenke et al., 1975). Guidance defects of specific axonal tracts have been postulated, as several individuals with KS display other neurological symptoms, such as mirror movements, sensorineural deafness (White et al., 1983), eye-movement abnormalities, cerebellar ataxia and gaze-evoked horizontal nystagmus (Schwanhaus et al., 1989; Sunohara et al., 1986).

The gene responsible for the X-linked form of the disease (KAL-1) (Franco et al., 1991; Legouis et al., 1991) encodes an extracellular matrix protein, containing a putative protease inhibitor domain (WAP domain) followed by fibronectin type III (FNIII) repeats. KAL is expressed in the olfactory bulb, the central target of olfactory axons, at the time when innervation begins (Legouis et al., 1993; Rugarli et al., 1993). One study showed that KAL displays adhesive properties for a variety of neuronal and non-neuronal cell types, and modulates neurite outgrowth in vitro by modulating branching. Finally, we find that human KAL-1 cDNA can compensate for the loss of worm kal-1 and that overexpression of worm or human KAL-1 cDNAs in the nematode results in the same phenotypes. These data indicate functional conservation between the human and nematode proteins and establish C. elegans as a powerful animal in which to investigate KAL function in vivo. Our findings add a new player to the set of molecules, which appear to underlie both morphogenesis and axonal/neuronal navigation in vertebrates and invertebrates.

Key words: Kallmann syndrome, C. elegans, Morphogenesis, Neurite branching

SUMMARY

Kallmann syndrome is an inherited disorder defined by the association of anosmia and hypogonadism, owing to impaired targeting and migration of olfactory axons and gonadotropin-releasing hormone secreting neurons. The gene responsible for the X-linked form of Kallmann syndrome, KAL-1, encodes a secreted protein of still elusive function. It has been proposed that KAL-1 might be involved in some aspects of olfactory axon guidance. However, the unavailability of a mouse model, and the difficulties in studying cellular and axonal migration in vertebrates have hampered an understanding of its function. We have identified the C. elegans homolog, kal-1, and document its function in vivo. We show that kal-1 is part of a mechanism by which neurons influence migration and adhesion of epidermal cells undergoing morphogenesis during ventral enclosure and male tail formation. We also show that kal-1 affects neurite outgrowth in vivo by modulating branching. Finally, we find that human KAL-1 cDNA can compensate for the loss of worm kal-1 and that overexpression of worm or human KAL-1 cDNAs in the nematode results in the same phenotypes. These data indicate functional conservation between the human and nematode proteins and establish C. elegans as a powerful animal in which to investigate KAL function in vivo. Our findings add a new player to the set of molecules, which appear to underlie both morphogenesis and axonal/neuronal navigation in vertebrates and invertebrates.

Key words: Kallmann syndrome, C. elegans, Morphogenesis, Neurite branching

INTRODUCTION

The Kallmann syndrome gene homolog in C. elegans is involved in epidermal morphogenesis and neurite branching

Elena I. Rugarli1,*†, Elia Di Schiavi2,* Massimo A. Hilliard2, Salvatore Arbucci2, Cristina Ghezzi1, Anna Facciolli2, Giuseppe Coppola2, Andrea Ballabio1,3 and Paolo Bazzicalupo2,†

1Telethon Institute of Genetics and Medicine (TIGEM), via P. Castellino III, 80131 Naples, Italy
2International Institute of Genetics and Biophysics (IIGB), via P. Castellino III, 80131 Naples, Italy
3Faculty of Medicine, II University of Naples, Naples, Italy
*These two authors contributed equally to this work
†Authors for correspondence (e-mail: rugarli@tigem.it and bazzical@iigb.na.cnr.it)

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attributed to the lack of an anatomical connection between the olfactory epithelium and the brain (Ballabio and Rugarli, 2001; Rugarli, 1999). However, the mechanism by which lack of KAL leads to the olfactory and other neurological phenotypes and to the abnormalities of renal and palate development is completely unknown.

One of the biggest limitations in exploring KAL function has been the unavailability of an animal model. In fact, a KAL murine homolog has not been identified so far, despite intensive effort. In recent years, the invertebrate Caenorhabditis elegans has become very attractive as an animal in which to study human disease genes (Ahringer, 1997; Culetto and Sattelle, 2000). Moreover, cell migration and axon guidance cues in invertebrates and mammals have been found to share structural and functional homologies (Bielloch et al., 1999; Branda and Stern, 1999; Brose and Tessier-Lavigne, 2000; Chisholm and Tessier-Lavigne, 1999; Montell, 1999).

We have identified kal-1, the C. elegans ortholog of the human KAL gene, and provide the first evidence of its function in vivo. We show that kal-1 is expressed by a subset of neurons beginning in embryogenesis, and is involved in neurite branching and in epithelial morphogenesis. Furthermore, we suggest significant functional conservation between vertebrates and invertebrates, as human KAL cDNA can rescue the phenotype of a nematode loss-of-function mutation and overexpression of worm or human KAL cDNAs in the nematode results in the same phenotypes. These data shed new light on pathogenesis of KS and support the use of C. elegans as a powerful animal for further functional studies of the human KAL gene.

**MATERIALS AND METHODS**

**Nematodes**

Culture, maintenance and genetic crossing procedures for nematodes were described as usual (Sulston and Hodgkin, 1988). All strains that were not isolated in our laboratory were obtained from the C. elegans Genetics Center, care of T. Stiernagle (The University of Minnesota), with the exception of the strain carrying evls82a [unc-12 9;:::GFP; dpy-20(+)], which was a kind gift from J. G. Culotti (Toronto, Canada). The following mutant strains were used: CB2769, which was a kind gift from J. G. Culotti (Toronto, Canada). Sequences of diagnostic primers are available upon request. The fragment was directionally cloned between the Sphl and BamHI sites of vectors pPD95.75 and pPD21.28 for plasmids GB102 and GB105, respectively (http://www.ciwemb.edu/pages/firelab.html) (Fire et al., 1990). Plasmid CeKAL was obtained by substituting in GB102 the GFP coding sequence with that of kal-1 cDNA from the ATG to the stop codon. Plasmid CeHuKAL was obtained by substituting, in CeKAL, the sequences of the C. elegans cDNA from position 156 to the end with the corresponding region of human cDNA. This construct leads to the translation of a protein containing the first 52 amino acids of the nematode cDNA fused in frame with amino acids 77 to 680 of human KAL. Further details of plasmids construction can be obtained on request.

**Reporter and overexpression constructs**

The kal-1 regulatory region fragment of 4.3 kb present in all constructs was generated using PCR on genomic DNA. The primer sequences are available upon request. The fragment was directionally cloned between the Sphl and BamHI sites of vectors pPD95.75 and pPD21.28 for plasmids GB102 and GB105, respectively (http://www.ciwemb.edu/pages/firelab.html) (Fire et al., 1990). Plasmid CeKAL was obtained by substituting in GB102 the GFP coding sequence with that of kal-1 cDNA from the ATG to the stop codon. Plasmid CeHuKAL was obtained by substituting, in CeKAL, the sequences of the C. elegans cDNA from position 156 to the end with the corresponding region of human cDNA. This construct leads to the translation of a protein containing the first 52 amino acids of the nematode cDNA fused in frame with amino acids 77 to 680 of human KAL. Further details of plasmids construction can be obtained on request.

**Transgenic lines**

Germline transformation was accomplished as described (Mello and Fire, 1995). The following co-injection markers were used: pRF4 [rol-6 (su1006)] (Mello and Fire, 1995); plin-15(+), a gift from S. Emmons (NY); pJM67 (elt-2::GFP), a gift from J. McGhee, Calgary; and GB110 (Fe65::GFP), a gift from M. Bimonte, Naples. In general, the test plasmids were co-injected with the marker DNA at a 1:1 ratio. Rescue with the genomic region was assayed by co-injecting, in kal-1(gb503) worms, cosmid K03D10 and plasmid pJM67 (elt-2::GFP), at 20 and 20 ng/µl, respectively. HuKAL was injected at 100 ng/µl, while marker DNA was injected at 20 ng/µl. Lower ratio produced less penetrant phenotypes. For each construct, several independent transgenic lines were obtained and analyzed. Embryonic lethality is reported for three lines for each construct: gbEx13a, b, c, for CeKAL and gbEx17a, b, c, for HuKAL (Table 1).

**Microscopy**

Live animals were anesthetized and mounted on 2% agarose pads containing 3 mM Na-Azide. They were observed with a Zeiss Axioshot using DIC (Differential Interference Contrast) optics or epifluorescence or with a BioRad MRC600 confocal microscope. Staining of embryos with rhodamine-conjugated phalloidin was as previously described (Priess and Hirsh, 1986). For whole-mount immunolocalization, embryos, larvae or L4 males were permeabilized and fixed with methanol-acetone and stained with mABMH27 antibodies (Baird et al., 1991; Francis and Waterston, 1991).

**Embryonic phenotype**

Embryonic lethality was observed in strains with different genotypes and carrying different extrachromosomal arrays. gbEx12 is an extrachromosomal array containing plasmid GB110 (Fe65::GFP) in which GFP is expressed in embryos in several cells. gbEx13 is an
extrachromosomal array containing plasmid CeKAL and, as selectable markers, plasmid GB110 and plasmid pRF4, which confers a roller phenotype. Results were scored from three independent lines. gbEx16 is an extrachromosomal array containing plasmid pJM67 (elt-2::GFP) and the pln-15(+). gbEx17 is an extrachromosomal array containing plasmid HuKAL and, as selectable markers, plasmid pJM67 (elt-2::GFP) and the pln-15(+). Results from three independent lines were scored.

Embryonic lethality was determined by picking 5-10 L4 animals of different genotype to several separate plates, allowing them to lay eggs and transferring them every 6-12 hours. Laid eggs were counted just after removing the mother, and larvae that had hatched were counted after 24 hours: the difference between these values was scored as embryonic arrest. In transgenic strains, only transformed animals, recognizable because of the expression of GFP from a marker plasmid, were counted. The stages when mutant embryos arrested were determined by following the development of embryos using DIC optics. Embryos were followed from 1.5 hours after fertilization until either development arrested or the embryo hatched.

Male tail defects
The tail defects were observed in males of different genotypes and carrying different extrachromosomal arrays. gbEx10 is an extrachromosomal array containing cosmid K03D10 and the pJM67 (elt-2::GFP) plasmid as a selection marker (other independent lines obtained with the same DNA mixture gave similar results); gbEx13b and gbEx17a are extrachromosomal arrays described above, each transferred both to a kal-1; him-8 and to a him-5 background.

Male tails were observed using DIC optics (40x magnification) and only clearly visible sides were scored. In transgenic lines only transforming worms were mounted and observed. In the rescue experiment, adult male worms were chosen randomly from strains carrying the extra-chromosomal arrays gbEx10, gbEx13b and gbEx17a. These males were first scored for tail defects and then for the presence of the GFP marker. kal-1 RNA interference (Fire, 1998) was carried out by injecting dsRNA from the fifth exon in him-5(e1490) hermaphrodites. Tails of F1 males were scored and about 30% of them showed ray abnormalities.

Statistics
P values for different experiments were calculated using both chi-square and z statistics.

RESULTS
Identification of the C. elegans KAL-1 homolog
TBLASTN searches of public databases with the human KAL protein sequence identified a homologous gene within the C. elegans cosmid, K03D10. A corresponding almost full-length cDNA clone was completely sequenced. Comparison of the cDNA and genomic sequences revealed that the gene was composed of six exons; we refer to the gene as kal-1 (for X-linked Kallmann syndrome gene homolog, number 1) and to the predicted 700 amino acids protein as CeKAL-1. RT-PCR analysis did not show alternatively spliced forms of RNA from this locus (not shown).

Similar to its vertebrate counterpart, CeKAL-1 contains a hydrophobic signal peptide at the N terminus followed by a cysteine-rich region, a whey acidic protein domain (WAP), and three fibronectin type III domains (FNIII) (Fig. 1A,B). The presence and topological organization of these domains is conserved between nematode and human, and is unique to KAL proteins. The CeKAL-1 protein contains a putative GPI anchoring site at the C-terminus that is absent in the other species. No other predicted C. elegans ORF contains both WAP and FNIII domains. The identification of kal-1 indicates that an ancestral gene with sequence and domain organization similar to KAL-1 was already present before the invertebrate-vertebrate separation.

Generation of kal-1 mutants
To study the function of kal-1 in C. elegans, we generated the deletion mutant strain, gb503, after chemical mutagenesis and screening by PCR. The deletion spans from the middle of the fourth exon to the middle of the fifth intron (Fig. 2A), thus introducing a stop codon that eliminates the 440 terminal amino acids of CeKAL-1. The deleted gene still has, in principle, the potential to encode a 263 amino acid protein. However, two criteria indicate that gb503 is a null rather than a hypomorph mutant: (1) RT-PCR analysis of the RNA from mutant worms shows that no stable mRNA (<2% of wild-type) is made from the gb503 locus (not shown); and (2) the tail defects (see below) of males heterozygous for gb503 and edD3, a deletion that covers the kal-1 locus, are indistinguishable from the tail defects of homozygous gb503 males.

In addition to the loss-of-function mutant, we also generated mutants overexpressing KAL proteins by transforming wild-type worms with constructs in which the 5’ regulatory region of kal-1 drove expression of the kal-1 cDNA (construct CeKAL of Fig. 2B) or of human KAL-1 cDNA (construct HuKAL of Fig. 2B).

Mutant animals with reduced and with increased kal-1 function showed similar defects albeit with different penetrance: embryonic lethality, abnormalities of larval morphology and, in adult worms, defects of the male tail and neurite outgrowth defects. These phenotypes are described separately below.

Embryonic ventral enclosure is defective in kal-1 mutants
Reduction or increase of kal-1 function results in embryonic lethality and morphological abnormalities of newly hatched larvae. Worms transformed with CeKAL show variably penetrant embryonic lethality, which ranges from 45 to 73%, depending on the extrachromosomal array. The embryonic lethality of the loss-of-function mutant gb503 is lower than that of overexpressing mutants but also in this case the difference from controls is statistically significant (Table 1).

Three main morphogenetic processes occur during C. elegans embryogenesis: gastrulation, ventral enclosure and elongation. C. elegans gastrulation involves the ingestion of gut, germline and mesoderm precursors, and leaves a depression, called gastrulation cleft, on the ventral side of the embryo. The gastrulation cleft is then closed by a short-range movement of ventral neuroblasts flanking the cleft. At this stage the epidermis consists of two dorsal, two lateral (future seam cells) and two ventral rows of cells aligned longitudinally on the dorsal side of the embryo. In the next major morphogenetic process, ventral enclosure of the embryo, the epidermal cells spread, from their dorsal position and over the ventral neuroblasts, to surround the embryo and join with contralateral epidermal cells at the ventral midline. Ventral enclosure is followed by elongation, which transforms the embryo from an ovoidal mass into an elongated, worm-shaped
**A**

Fig. 1. Evolutionary conservation of KAL proteins. (A) Amino acid sequence alignment of known KAL homologs. KAL, human KAL protein; KALc, chicken KAL protein; zKAL1.1 and zKAL1.2, zebrafish KAL proteins (Ardouin et al., 2000); and CeKAL, *C. elegans* KAL protein. Stars highlight conserved cysteines. The WAP domain is underlined. In the most conserved part of the protein, containing the cysteine-rich region, the WAP domain and the first FNIII repeat, identity and similarity of residues between KAL and CeKAL are approximately 30% and 50%, respectively. (B) The domain topology of KAL proteins. The order and the relative distance of all domains are conserved. Abbreviations: SP, signal peptide; C-rich, cysteine-rich domain; WAP, WAP domain; FNIII, fibronectin type III repeat; GPl, glycosyl-phosphatidylinositol anchoring site.

**B**
organism (Priess and Hirsh, 1986; Williams-Masson et al., 1997).

Affected kal-1 mutant embryos are defective in ventral enclosure and rupture ventrally with cells protruding out of the embryonic mass (Fig. 3B-D). These embryos neither complete development nor hatch, contributing to the embryonic lethality of kal-1 mutants. As it occurs in other C. elegans mutants that are defective in ventral enclosure (Chin-Sang and Chisholm, 2000; Roy et al., 2000; Simske and Hardin, 2001), some embryos seem to present later or milder enclosure defects that affect especially the extremities, head and tail (not shown). Some of these embryos complete development and give rise to abnormal larvae (Fig. 3J-L).

Ventral enclosure defects are due to abnormal contacts of epithelial cells

To describe in more detail the cellular basis for the phenotypes of kal-1 mutants, we visualized the boundaries of epithelial cells during ventral enclosure by rhodaminated phalloidin staining (Priess and Hirsh, 1986) and, at later stages, by staining adherens junctions using the anti JAM-1 monoclonal antibody MH27 (Francis and Waterston, 1991). In wild-type embryos, the ventral epidermal cells joining at the midline are positioned according to a stereotyped symmetric pattern (Fig. 3E). In mutant kal-1 embryos, abnormal positions and contacts between epithelial cells can already be observed during enclosure (Fig. 3F). At later stages, in the wild type, the lateral epidermal cells have a regular geometric shape and are arranged in an ordered pattern, with each cell contacting only one anterior and one posterior partner (Fig. 3G). In mutants, these cells have irregular shapes and abnormal/ectopic contacts and form clusters; as a consequence MH27 staining assumes a star like shape (Fig. 3H). Visualization of JAM-1 in newly hatched abnormal larvae again indicates that the shape and arrangement of the epithelial cells are altered (Fig. 3M,N). In some cases (Fig. 3N), a group of the lateral epithelial cells becomes detached from the line of lateral seam cells and appears to underlie the external bulges seen with visible light (Fig. 3K,L). Thus visualization of epithelial cell boundaries indicates that in both loss-of-function and overexpressing kal-1 mutants epithelial cells are born, differentiate correctly and migrate to surround the embryo, but their migration, reciprocal contacts and shape are abnormal.

### Table 1. Embryonic lethality

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Lethality</th>
<th>n</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>kal-1(+); him-8(e1489)</td>
<td>13.8%</td>
<td>745</td>
<td>Control</td>
</tr>
<tr>
<td>kal-1(gb503); him-8(e1489)</td>
<td>25.7%*</td>
<td>288</td>
<td>Loss of function</td>
</tr>
<tr>
<td>kal-1(+)</td>
<td>0.5%</td>
<td>204</td>
<td>Control</td>
</tr>
<tr>
<td>kal-1(+)</td>
<td>56.1%*</td>
<td>228</td>
<td>Overexpression of C. elegans kal-1 (cDNA)</td>
</tr>
<tr>
<td>kal-1(+)</td>
<td>72.4%*</td>
<td>232</td>
<td></td>
</tr>
<tr>
<td>kal-1(+)</td>
<td>45.9%*</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>kal-1(+)</td>
<td>1.1%</td>
<td>609</td>
<td>Control</td>
</tr>
<tr>
<td>kal-1(+)</td>
<td>9.8%*</td>
<td>286</td>
<td>Overexpression of human KAL-1 (cDNA)</td>
</tr>
<tr>
<td>kal-1(+)</td>
<td>20.2%*</td>
<td>724</td>
<td></td>
</tr>
<tr>
<td>kal-1(+)</td>
<td>7.5%*</td>
<td>509</td>
<td></td>
</tr>
</tbody>
</table>

kal-1(gb503) is the deletion allele described in this paper; gbEx13 and gbEx17 are extrachromosomal arrays containing plasmids CeKAL and HuKAL, respectively. In each case, results from three independent lines, a-c, are reported. Control worms for the overexpression experiments carried extrachromosomal arrays containing the same selectable markers but no test plasmid. n is the total number of transformed embryos scored for each genotype.

*Significantly different from control (P<0.001).
Male tail morphogenesis is defective in kal-1 mutants

The tail of *C. elegans* males is a symmetric structure required for mating and composed of a heart-shaped cuticular fan and nine sensory rays on each side (Fig. 4A). Although they mate with reasonable efficiency, the majority of *gb503* males show various tail abnormalities (Table 2). Overexpression in wild-type worms of *C. elegans* kal-1 from an array carrying the CeKAL plasmid also produces male tail defects (Table 2). In general, the whole structure appears irregular and distorted. In addition, the presence, position, shape, and size of the nine rays are variably altered (Fig. 4B-F). The most common defects are reduction of sensory rays, which often take a ball-like shape (rays 1 to 3), presence of an extra ray in the region between E. I. Rugarli and others

![Fig. 3. kal-1 mutants show embryonic lethality and L1 larvae morphological abnormalities. (A-D) DIC photomicrographs of embryos.](image)

(A) Control embryo at one and a half-fold stage; (B-D) mutant embryos in which ventral enclosure has failed; cells protrude ventrally outside of the embryonic mass (white arrows). These embryos will not hatch and they will eventually die. Confocal images of embryos stained with rhodaminated phalloidin (E, F) or AbMH27 (G, H).

(E) Wild-type comma stage embryo showing the ordered pattern of cell boundaries. (F) This pattern is disrupted in a mutant, where cells establish ectopic contacts and are abnormally oriented (white arrow), clumping together and generating star-like shapes (white arrowheads).

(G) In later wild-type embryos (threefold stage), each epithelial cell contacts only one anterior and one posterior partner. In mutant embryos of comparable stage (H), epithelial cells clump together (white arrow) and the normal pattern cannot be recognized. (I-L) DIC photomicrographs of L1 larvae. (I) Control L1 larva. (J-L) Mutant L1 larvae with abnormal body shape consisting in enlargements and bulges (white arrows), most often present in the head and tail regions. (M, N) Confocal images of larvae stained with AbMH27. In M, the line of lateral epithelial cells in a newly hatched larva is disorganized and the shape of the individual cells is altered. The white arrow points to a three partners boundary. In N, a group of epithelial cells has detached from the main lateral line and has organized a separate islet of epithelial cells. The outline of the animals (broken white lines) is drawn from parallel visible light micrographs (G, M and N).

Table 2. Male tail phenotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Wild-type sides</th>
<th>Ray 1-2</th>
<th>Ray 3-4</th>
<th>Ray 5-6</th>
<th>n</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>kal-1(+); him-8(e1489)</td>
<td>96.9%</td>
<td>5r</td>
<td>1r</td>
<td></td>
<td>192</td>
<td>Control</td>
</tr>
<tr>
<td>kal-1(gb503)/+; him-8(e1489)</td>
<td>96.3%</td>
<td>4r; 1e</td>
<td>1r</td>
<td>2i</td>
<td>136</td>
<td>Heterozygote</td>
</tr>
<tr>
<td>kal-1(gb503); him-8(e1489)</td>
<td>18.1%*</td>
<td>20r; 6e; 3l</td>
<td>24r; 1l; 4f</td>
<td>10r; 63; 15f</td>
<td>315</td>
<td>Loss of function</td>
</tr>
<tr>
<td>kal-1(gb503); him-8(e1489); gbEx10</td>
<td>68.2%*†</td>
<td>22r; 2l</td>
<td>2r; 1f</td>
<td>1i; 3f</td>
<td>88</td>
<td>Rescue with kal-1 (genomic)</td>
</tr>
<tr>
<td>kal-1(gb503); him-8(e1489); gbEx13b</td>
<td>59.0%*†</td>
<td>39r; 1l</td>
<td>9r; 5f</td>
<td>1r; 5i; 2f</td>
<td>101</td>
<td>Rescue with <em>C. elegans</em> kal-1 (cDNA)</td>
</tr>
<tr>
<td>kal-1(gb503); him-8(e1489); gbEx17a</td>
<td>48.9%*†</td>
<td>24r; 3l</td>
<td>2r; 6f</td>
<td>3r; 16i; 2f</td>
<td>88</td>
<td>Rescue with human KAL-1 (cDNA)</td>
</tr>
<tr>
<td>kal-1(+); him-5(e1490)</td>
<td>92.9%</td>
<td>5r</td>
<td></td>
<td></td>
<td>70</td>
<td>Control</td>
</tr>
<tr>
<td>kal-1(+); him-5(e1490); gbEx13b</td>
<td>65.4%*</td>
<td>13r; 7e; 10l; 2f</td>
<td>1r; 2f</td>
<td>1r; 4i</td>
<td>107</td>
<td>Overexpression of <em>C. elegans</em> kal-1 (cDNA)</td>
</tr>
<tr>
<td>kal-1(+); him-5(e1490); gbEx17a</td>
<td>79.0%*†</td>
<td>29r; 6e; 1l</td>
<td>4r; 1l</td>
<td>1r; 1f</td>
<td>195</td>
<td>Overexpression of human KAL-1 (cDNA)</td>
</tr>
</tbody>
</table>

kal-1(gb503) is the deletion allele described in this paper; gbEx10 is an extrachromosomal array containing cosmID10, which includes the complete sequence of kal-1; and gbEx13b and gbEx17a are extrachromosomal arrays containing plasmids CeKAL and HuKAL, respectively. Defects are grouped on the basis of the rays involved. n is the total number of sides observed for each genotype. For each group of rays, the number of observed tails showing a particular defect is indicated and is followed by a letter code indicating the type of defect: r, reduction; e, extra-ray; l, large; i, inversion; f, fusion.

*Significantly different from control (P<0.001).
†Significantly different from mutant (P<0.001).
Because the male tail defects of the loss-of-function mutant \textit{gb503} are recessive and represent the most penetrant phenotype of the mutation, we selected this phenotype for the following rescue experiments. The male tail defects are largely rescued in \textit{gb503} worms carrying cosmId K03D10 (which contains the complete sequence of \textit{kal-1}) as a transgene on an extra-chromosomal array (Table 2). Rescue of the male tail defects could also be obtained by transformation with the construct CeKAL (Table 2) in which expression of the \textit{C. elegans} \textit{kal-1} cDNA is driven by 4.3 kb of sequences upstream of the start codon (Fig. 2B).

As for ventral enclosure, also in the case of male tail formation the defects presented by \textit{kal-1} mutants appear to be due to changes in the position and the shape of the rays rather than in the specification of their identity. For example, in the frequently observed inversion/fusion of rays 5 and 6, ray 5 is found posterior or fused to ray 6 but maintains its identity (Fig. 4D). Visualization of epidermal cell boundaries during tail morphogenesis confirms that in \textit{kal-1} mutants epithelial cells of the tail have abnormal contacts, shapes and positions. At the L4 stage, in males, the nine clusters of cells that will give rise to the sensory rays have already been generated, on each side. Tail morphogenesis continues with cell movements and changes of shape and contacts that result in the correct positioning and separation of the precursors of the rays (Fig. 4G). These processes are impaired in mutants and result in defects in the arrangement of the ray precursors clusters and in abnormal shape of some of the cells involved (Fig. 4I).

\textbf{Fig. 4.} \textit{kal-1} mutants show altered male tails. (A-F) DIC photomicrographs; ventral views. Control tail (A): the nine rays on each side are indicated. (B-F) \textit{kal-1} mutants. White arrows indicate loss or strong reduction of rays; black arrows indicate abnormal shape, extra rays or fusion of rays. In D, combination of DIC and epifluorescence image of a \textit{kal-1}(gb503) mutant worm, which is also transgenic for evIs82a \textit{unc-129 ns::GFP; dpy-20(+)} (Colavita and Culotti, 1998). This transgene specifically expresses GFP in one of ray 5 sensory neurons (white arrowhead), allowing to establish that ray 5 maintains its identity, but is posterior to ray 6 (black arrowhead). (G) Schematic representation of the outline of epithelial cells at three different times during tail formation in wild-type L4 males [modified from Baird et al. (Baird et al., 1991)]. During early L4, the tail seam cells (indicated by stars), which are next to the clusters of ray precursors (numbered 1 to 9), are still separated from each other. At mid L4, tail seam cells have partially fused together to form the SET (seam tail) cell, which maintains its connection with the most posterior body seam cell and with the ray clusters. At late L4, the fusion of tail seam is complete and the flanking hyp7 cell has engulfed the ray clusters. The SET cell maintains its contact with body seam throughout the process. (H,I) Confocal images of developing male tails at the L4 stage stained with AbMH27: wild type in H; \textit{kal-1}(gb503) in I. Numbers from 1 to 9 indicate the clusters of precursors of sensory rays. The typical triangular arrangement of precursors to rays 4, 5 and 6 of the control tail (H) is changed to an almost straight line in the mutant tail (white arrow in I). This arrangement of epithelial cells in L4 will result, in the adult tail, in the inversion between the position of ray 5 and 6 (D) or in their fusion. In I, the shape of the SET cell, which does not contact the posterior body seam cell, is also abnormal.

\textbf{kal-1} mutants show neurite outgrowth defects

Some of the most important symptoms of Kallmann syndrome appear to be derived from neuronal and axonal migration
neurons, these defects demonstrate for the first time that a KAL protein can affect neurite outgrowth in vivo and suggest that it might function in this process as a regulator of neurite branching.

**Functional conservation of the human KAL gene in C. elegans**

In order to test experimentally the extent of functional conservation of the KAL gene between man and worm, we transformed nematodes with a construct in which expression of human KAL cDNA is driven by the 5’ regulatory sequences of the C. elegans kal-1 gene (HuKAL, Fig. 2B). Expression of human KAL cDNA in the loss-of-function mutant kal-1 (gb503) rescues its male tail defects (Table 2). In addition, overexpression of human KAL cDNA in wild-type worms results in embryonic lethality (Table 1 and Fig. 3D) and male tail defects (Table 2 and Fig. 4F) that are indistinguishable, although less penetrant, than those caused by overexpression of C. elegans kal-1. These results indicate that conservation of the KAL protein between man and worm is not limited to structure, but is also functional, and give further support to the hypothesis that kal-1 is the ortholog of the human gene for X-linked Kallmann syndrome. They also define the experimental setting for future studies aimed at analyzing the structure-function relationship of KAL proteins in vivo.

**Expression of kal-1 reporters is restricted to subsets of neuronal cells and is consistent with the phenotypes of kal-1 mutants**

To study the expression of kal-1 in *C. elegans* and to try to correlate it with the phenotypes observed in mutants, we analyzed worms transgenic for constructs in which expression of reporters (GFP or β-galactosidase) was driven by sequences at the 5’ end of the kal-1 gene. A variety of constructs were prepared using 5’ regulatory sequences of kal-1 spanning from 1000 to 4300 bp upstream of the ATG. In all cases expression was restricted to the same subsets of neuronal cells. Expression was highest with a 4300 bp fragment (plasmids GB102 and GB105 of Fig. 2B). This regulatory region was also used to drive the expression of KAL cDNAs in the rescue and overexpression experiments (constructs CeKAL and HuKAL).

During embryogenesis kal-1 expression is first detectable at about the 160- to 200-cell stage in a group of eight to ten neuroblast descendants of the AB blastomere (Fig. 6A,B). These neuroblasts are located on the ventral surface of the embryo where they first surround and later close the gap left by the gastrulation process. kal-1-expressing neuroblasts are then covered by epithelial cells, which migrate around the embryo to join at the ventral midline for ventral enclosure. Thus, kal-1 is expressed by the cells that act as a substrate for the epithelial migration during enclosure. This expression underlies the function of kal-1 during embryogenesis and can explain the defects of embryonic development present in kal-1 mutants. At no stage during embryogenesis were kal-1 driven reporters detectably expressed by epidermal cells.

As it is covered by the ventral epidermal cells, the group of kal-1-expressing neuroblasts splits in an anterior and a posterior group (Fig. 6C). After elongation of the embryo is completed, the expressing cells have roughly reached the positions they will occupy in larval stages (Fig. 6D). During
As during ventral enclosure, also during male tail formation, Fig. 5A) and one of the neurons of sensory ray 5 (not shown). We identified two male-specific neurons, the interneuron EF3 (see Fig. 6I) and another interneuron, possibly PVW (Fig. 6J). We also identified two male-specific neurons, the interneuron EF3 (see Fig. 5A) and one of the neurons of sensory ray 5 (not shown). We provide the first in vivo evidence that KAL is involved in epithelial morphogenesis and in neurite branching. Morphogenesis, the development of the spatial organization of tissues and organs, involves dramatic changes in cell shapes, cell-cell interactions and cell movements. This requires a tight control of cell adhesion to reinforce appropriate contacts and prevent ectopic ones (McNeill, 2000). The formation of adhesive bonds is a selective process mediated through specific cell-to-cell and cell-to-substrate interactions, and leading to the activation of cytoplasmic signaling cascades (Song and Poo, 2001). Modulation of adhesion is crucial also to neurite outgrowth, branching and guidance. These processes are now regarded as specialized form of cell movements, as emphasized by recent findings that the same repertoire of molecules control movements of cells during morphogenesis, and of neurons and axons during brain development (Song and Poo, 2001; Wilkinson, 2001). We now show that CeKAL-1 is one of such molecules.

**The role of** kal-1 **in epidermal morphogenesis**

This study shows that kal-1 is involved in at least two distinct morphogenetic events in the worm: ventral enclosure and male tail formation. Both processes involve a regulated series of dynamic epithelial and neuronal cell contacts and of cell-shape changes, and have been shown to depend on the action of partially redundant molecular cues (Chin-Sang et al., 1999; George et al., 1998; Roy et al., 2000; Wang et al., 1999b). In kal-1 mutants, epithelial cell contacts and shapes are variably disrupted during morphogenesis, suggesting that kal-1 might modulate the formation or stabilization of contacts between cells. These inappropriate contacts can be due either to the failure to establish stable adhesive bonds in the right position or to the stabilization of an ectopic contact that would otherwise collapse. The identification of kal-1-interacting...
proteins and of downstream signaling pathways will shed new insights into these two possibilities.

Our expression data strongly suggest that CeKAL-1 is acting in a non-cell-autonomous way, being secreted by neurons and influencing epithelial cells. Although these studies have been performed using reporters driven by kal-1 regulatory sequences, we believe that they reproduce reasonably well the endogenous kal-1 expression. This is strongly supported by the ability of KAL cDNAs, whose expression is driven by the same regulatory sequences, to rescue the most penetrant phenotype of the kal-1 loss-of-function mutant.

We observe similar phenotypes in loss-of-function and overexpressing kal-1 mutants. A strict dose control of the secreted molecules involved in contact guidance during morphogenesis and axonal growth may underlie this phenomenon. Indeed, similar findings have been reported for mutants of other extracellular molecules involved in adhesion and axon guidance (Ackley et al., 2001; Powell et al., 2001; Roy et al., 2000). Tight regulation of CeKAL-1 dose might explain an apparent paradox of previous results obtained in cell culture systems, where KAL was found to stimulate cerebellar axon outgrowth, when added in a uniform concentration, and to induce cessation of growth when provided as a discontinuous substrate in high local concentrations (Soussi-Yanicostas et al., 1998). This notwithstanding, we observe that, while embryonic ventral enclosure is more sensitive to an increase than to a reduction of kal-1 function, the opposite appears to be true for male tail formation. A more sophisticated phenotypic analysis might reveal opposite effects of hypomorph and hypermorph kal-1 mutations.

The association of ventral enclosure defects and malformation of the male tail has been previously described in C. elegans mutants of the Eph receptors/ephrin pathways (vab-1, vab-2, efn-2 and efn-3) (Chin-Sang et al., 1999; George et al., 1998; Wang et al., 1999b; Zallen et al., 1999) and semaphorin-2a (mab-20) (Roy et al., 2000). Similarly to kal-1, mab-20/Ce-sema-2a seems to regulate proper contacts between hypodermal cells (Chin-Sang and Chisholm, 2000; Roy et al., 2000). As mab-20 is ubiquitously expressed, it has been suggested that the ability of a cell to respond to semaphorin signaling must be controlled by the expression of a receptor, a co-factor, or downstream signaling components. The neuroblast-restricted expression pattern of kal-1 makes it a candidate to be a regulated element of this pathway.

A crucial role of neuronal cells in epidermal cell movements during ventral enclosure has been inferred from the analysis of ventral enclosure defects in vab-1 and vab-2 mutants (Chin-Sang et al., 1999; George et al., 1998; Wang et al., 1999b). VAB-2 signaling to VAB-1 occurs before and during enclosure between neuronal precursors, regulating their adhesion or movement. Some vab-1 or vab-2 mutant embryos die owing to failure of the neuroblasts to close the ventral gastrulation cleft. However, in other embryos, enclosure fails even though the gastrulation cleft is sealed, suggesting that neuroblasts may provide a permissive substrate or a guidance molecule to epidermal cells (Chin-Sang et al., 1999; George et al., 1998). It is plausible that kal-1, expressed by the ventral neuroblasts during enclosure, is one of such cues. Although CeKAL-1 harbors a GPI anchor, we have preliminary data indicating that, at least in cell culture conditions, it is partially diffusible. One possible scenario is that CeKAL-1, either associated with the surface of the expressing neurons or released into the ECM, signals to a yet unidentified receptor present on epithelial cells. An alternative model is that CeKAL-1 specifically modulates other negative and positive guidance cues, for example, through its anti-protease domain. In fact, there is emerging evidence that metalloproteases and their inhibitors are important regulators of contact-mediated attraction or repulsion, by controlling the number of functional extracellular receptors (Galko and Tessier-Lavigne, 2000; Hattori and Flanagan, 2000).

**kal-1 is involved in neurite branching**

The analogies among vab-1, mab-20 and kal-1 mutants are not restricted to their role in morphogenesis. vab-1 and mab-20 mutants in fact also display aberrant axon growth, such as guidance and fasciculation defects (George et al., 1998; Roy et al., 2000; Zallen et al., 1999). In vertebrates, these molecules have been mostly studied for their function in axon guidance, and only recently have been involved in morphogenetic events (Flanagan and Vanderhaeghen, 1998; Holder and Klein, 1999; Ito et al., 2000; Raper, 2000; Tessier-Lavigne and Goodman, 1996).

Abnormal neurite growth of the EF3 and RIC neurons was observed in kal-1 mutants. The abnormal neurite is characterized by the appearance of an extra-branch, suggesting that CeKAL-1 may act as a modulator of branch formation. In all these mutants, neurite defects may, in principle, result from defective morphogenesis leading to the aberrant positioning of other cues. However, the role of Eph receptors, ephrins, and semaphorins in axon guidance in vertebrates and the phenotype of KS make a strong case in favor of a direct effect. Extra-branckding can be interpreted as a reinforcement of an ectopic contact between an axon collateral and the environment, underlying the same mechanism of CeKAL-1 action that we have postulated during morphogenesis.

Although a role of KAL in some aspects of axon guidance has been always suggested, based on the human KS phenotype, this is the first time a specific function on neurite growth is documented. So far the only factor for which a role as positive regulator of axonal elongation and branching has been demonstrated is the mammalian Slit2 (Wang et al., 1999a). Slit2 is a bifunctional molecule, implicated both in repelling migrating cells and axons, and in stimulating axonal branching, further supporting the idea that there are general mechanisms controlling cell migration, axon pathfinding and axon branching (Brose and Tessier-Lavigne, 2000).

**Relevance to Kallmann syndrome pathogenesis**

We have clearly shown significant functional conservation of the human KAL protein in the nematode. Therefore, we think that our findings in C. elegans are relevant to the function of the human KAL gene and justify a re-examination of the mechanisms that underlie the pathogenesis of KS. KAL proteins may be involved in morphogenesis during development, both in the brain and in other tissues, by regulating cell adhesion and preventing the formation of ectopic cell contacts. Defective morphogenesis and perturbation of cell migration may explain some symptoms observed in individuals with KS, such as cleft lip and palate, and unilateral renal hypoplasia/agenesis (Colquhoun-Kerr et al., 1999; Hermanussen and Sippell, 1985; Wegenke et al., 2000).
KAL involvement in brain morphogenesis has been so far underestimated, but our results strongly induce to reconsider the possibility that olfactory bulb hypoplasia in KS may be due to defective bulb formation, rather than to bulb involution caused by lack of innervation.

Our data also suggest that KAL may directly affect specific axonal populations, by regulating neurite branching and therefore the formation of axon collaterals and the establishment of target connections. KAL may specifically affect olfactory axon outgrowth by regulating branching and by stabilizing contacts of growth cones with the matrix and neurons of the bulb. Specific axonal defects might underlie the occurrence of mirror movements and other neurological symptoms in individuals with KS.

Finally, KAL appears to be involved in highly redundant pathways in both human and worm. All the kal-1 mutant phenotypes described in this paper are observed with incomplete penetrance. As no other C. elegans protein is found with the same domain composition as CeKAL-1, we can rule out the presence of a homologous gene with a similar role, and conclude that redundancy of CeKAL-1 function is due to other molecules and pathways active in the same developmental processes. For many symptoms described in individuals with KS, even as dramatic as unilateral renal aplasia, incomplete penetrance has been reported. Furthermore, KS is a genetically heterogeneous disease and mutations in KAL have been found to be responsible in only a limited percentage of cases (Georgopoulos et al., 1997).

In conclusion, we have established the nematode as a system to study the function of the KS gene in vivo. The advantage of using C. elegans as a model lies in the possibility of performing protein structural-functional studies in vivo and in the power of epistatic studies. We predict that these studies will aid in the identification of domains relevant for KAL function, and of other components of the same pathway.

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