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DEVELOPMENT AND DISEASE

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

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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

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 (Schwanzel-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 (Schwanzel-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 (Schwankhaus 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 of cerebellar neurons in vitro (Soussi-Yanicostas et al., 1998). It has been suggested that KAL is involved in terminal steps of olfactory axon guidance to the bulb and that olfactory bulb hypoplasia/aplasia in KS is secondary to lack of innervation. Similarly, GnRH migration impairment has been

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 (Blelloch 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 as described (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 *evIs82a* [*unc-129* ^{ns}*::GFP; dpy-20(+)*], which was a kind gift from J. G. Culotti (Toronto, Canada). The following mutant strains were used: CB2769 *eDf3 / eDf24 (*I); MT8189 *lin-15(n765)* X; DR466 *him-5*(*e1490*) V; and CB1489 *him-8*(e1489) IV. In this work, a mutant strains was also generated: NA 654 *kal-1(gb503)* I.

Identification of *C. elegans kal-1* and sequence alignment

Standard molecular biology methods were used (Sambrook et al., 1989), unless otherwise noted. The genomic sequence of *kal-1* spans the sequences of cosmids K03D10 (Z81561) and C37A5 (Z92828) and includes the predicted gene K03D10.1. A nematode cDNA clone was identified by database searching (yk172f9). This clone, kindly provided by Y. Kohara, was sequenced using an ABI automatic sequencer and found to contain an almost full-length cDNA. Eleven nucleotides at the 5′ end of coding sequence were deduced from the corresponding genomic region. Sequence analysis and prediction of domains was performed using the SMART algorithm (Schultz et al., 1998). Sequence alignment was performed using the ClustalW program. GenBank *kal-1* cDNA sequence Accession Number is AF342986.

Isolation of a *kal-1* deletion mutant

The *kal-1* mutant *gb503* was isolated after Trimethyl Psoralen (TMP) + UV light mutagenesis (Yandell et al., 1994) and PCR screening. The procedure combines steps from several established protocols for gene knockout with chemical mutagens listed at http://cobweb.dartmouth.edu/cgi-bin/cgiwrap/~ambros/protocol.cgi?id=24. Briefly, 200 cultures were set up, each founded by about 1250 synchronized F1 derived from mutagenized mothers. When the F2 was produced, DNA was prepared from one-third of each culture and tested by PCR to identify cultures in which a deletion in the *kal-1* locus had occurred. Primers and conditions were designed to amplify preferentially bands deriving from a deleted locus. After a positive culture was identified, four rounds of screening and sibling selection were necessary before cultures could be started with single worms. Eventually a homozygous culture of the deletion carrying strain was established. Mutants were backcrossed to *N2* four times before phenotypic analysis. After genetic crosses wild-type and mutant *kal-1* alleles were detected by single worm PCR. The backcrossed strain will be deposited at the *C. elegans* Genetic Center. Sequences of diagnostic primers are available upon 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 *Sph*I and *Bam*HI 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 HuKAL 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); p*lin-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 Axiophot 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 p*lin-15*(+) construct. *gbEx17* is an extrachromosomal array containing plasmid HuKAL and, as selectable markers, plasmid pJM67 (*elt-2*::GFP) and the p*lin-15*(+) construct. 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 (40× 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 *eDf3*, 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 ingression 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

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10 20 30 40 50 60 70
KAL      M V P G V P G A V L T L C L W L A A S S G C L A A G P G A A A R R L D E S L S A G S V Q R A P C A S R C L S L Q I T R I S - - A F F Q H F Q N N G S L V W
KALc     M V R R A P G A S L A L L L W V T A V S G S - P A G P G A A T A R R Q D E A F S T - - - - - A R C T S R C L S L Q I T R I S - - A F F K H F Q N N G S L A W
zKAL1.1        M R D G L T G L C I G I I L L L N G - - - - G L V V M R R Q D S S S A L G S I F R T R C A A R C L S L H S T R I A - - L S P R H F Q S N G S L G W
zKAL1.2        M N L L L R N L C C C L L C C V F E - - - - - - V S V A R R T E E A E S L E K M E S A R C V S R C L T L H I T Q I T - - A A F R H L Q N D R V L G W
CeKAL                      M I L L L L F I L G - - - - - - - S V S P - - D E L I S T - - - - - - R C Q A Q C L H L M D Q R L Q N N M E L R K T L K H H I I Q L

80 90 100 110 120 130 140 150
KAL      C Q N H K Q C S K C L E P C K E S G D L R K H Q C Q S F C E P L F P K K S Y E C L T S C E F L K Y I L L V K Q G D C P A P E K A S G F A A A C V E S C E V D
KALc     C Q N H K Q C S K C L E P C K E S W R L K K N H C Q S F C E P L F P K K N Y E C L T S C E F L K Y I L S V K Q G D C P A P E K A S G F A A A C V E S C E A D
zKAL1.1  C Q S H K Q C A K C L E P C K D S W E L K D G P C R D L C E H A F P K R H G E C V T S C E F L R S V M V V K Q G D C P P P F R A S G F A A A C V E G C E E D
zKAL1.2  C E N H R R C S Q C L Q P C K E L W E T R R A L S P K T C E - - - - - - H H E C V T S A E F L R S L R V Q E K Q G D C P P A Q R A S G F A A A C V E S C A Q D
CeKAL    C K E D A T C S A C S A P C R E Q F D - D I K A C K K S C V P - - R D D R E T C E D S C H Y L Q R I Y Q E K P G A C P S V S N Q S - - N Y E C S A L C Q M D

160 170 180 190 200 210 220 230
KAL      N E C S G V K K C C S N G C G H T C Q V P K T - L Y K G V P L K P R K E L R F T E L Q S G Q L E V K W S S K F - - N I S I E P - - V I Y V V Q R R W N Y G I
KALc     S E C S G V K K C C S N G C G H T C Q V P K N - L Y K G V P L K P R K E L K F I E L Q S G D L E V K W S S K F - - N I S I E P - - V I Y V V Q R R W N Q G I
zKAL1.1  G E C S G Q K K C C P N G C G H T C Q S P K N - L Y F G A P L K P R K F I V F F F I E S G V L E V K W S S K F - - N V S A E P - - V L N V L Q R R W N Y G I
zKAL1.2  R E C S G V K K C C S N G C G H T C Q S P A N - L F K G V P L K F R R D I S F S E D H Q G H V K V T W M S K F - - N V S V E P - - V L Y V L Q R R W N H G I
CeKAL    G E C P E T Q K C C S S G C S R Q C L K P R S S D I K L L P I P R N I S V Q E R K R K R S - I I I R W A T G R L S K A Q Q N E N A N L F L V Q W R W - - G L

240 250 260 270 280 290 300 310
KAL      H P S E D D A T - - H W Q T V A Q T T D E R V - - - - - - - Q L T D I R P S R W Y Q F R V A A V N V H G T R G F T A P S K H F R S S K D P S A P P A P A N L
KALc     H P S E D D A T - - N W Q T V A Q T T D E R V - - - - - - - Q L S D I R A S R W Y Q F R V A A V N V H G T R G F T A P S K H F R S S K D P S A P P A P S N I
zKAL1.1  H P S E D G A T - - E W Q V V A R T S G E R V - - - - - - - W L T D I R P G R W Y Q F R V A A V N V H G T R G Y T T P S R H F R S S K D P P P P P A P S D L
zKAL1.2  H P S E D E A T - - A W Q T V L M T M E D R S - - - - - - - V L K D I R P H R W Y Q F R V S A V N S Q G T R G F T T P S K H F F S V R C P L P P E T P Q N A
CeKAL    H A D E S A M T - - E W Q T V T V R N K P Y A I - - - - - - L K H L L S P G R F Y E F R T A A V S Q E G S L G F S S P S K P F K I S K E A K A P P P P K D L

320 330 340 350 360 370 380 390
KAL      R L A N S T V N S D G S V T V T I V W D L P E E P D I P V H H Y K V F W S W M V S S K S - L V P T K K K R R K T T D G F Q N - - - S V I L E K - - - - - - L
KALc     R I A N I S A N N D G T V N V M I T W D L P E E P D I P V H H Y K V F W S W T Y S - K Y - V I P A K K K R R K I T D G P Q N - - - Y V V L E G - - - - - - L
zKAL1.1  H V S D M T F G A D H S A S V R L S W S I S A D L D I P V N H Y K L S W S S S D H - - - - N L P P K L K K R E T V N G D S T - - - Y A E L D D - - - - - - L
zKAL1.2  R T A N Q T L H P D G T V S V L V L W E T P R E S D L V V H H Y K V T W S P R G T P - - - - S S Q S R A G R V T D G D V T - - - Q M F I Q G - - - - - - L
CeKAL    S L G A S K V V A S G L W N Q M V H W N P P P - S D L P I K N Y Q I S W A S S T K S E A D A F E E Q M R K K S T L E F A A H E K R S L Q S D D D E E F I G I

400 410 420 430 440 450 460
KAL      Q P - D - C D Y V V K L Q A I T Y W G Q T R L K S A K V S L H F T S T H A T - N N K E Q L V K T R - - K G G I Q T Q L P F Q R R R P T R P L E V G A P F Y Q
KALc     Q P - N - S N Y N V E L Q A V T R W G Q I R L K S A K V S L H F S T A Q D N R N N N E Q T S A G K P P K G L V D P Y P T F Q R R K P T R F L K I G T P F Y Q
zKAL1.1  R E - N - G S Y T V E L Q A V S Y W G Q V P L K S S K A I L Q F T T S Q T Q - S D L E P T V S - - - - - - - P V F I K P - - - - - Q S D L L D V G T P F Y Q
zKAL1.2  Q P - N - T P Y T A Q I Q A I S Y W G Q N R L K S S R A H V S F T T T S Q N - S A T S S N G E - - - - - - F S N E L P V S H S S P S V L R L E A A A P H Y H
CeKAL    Q E R D R Q S V V V P S H A T S S E I T G L F P N S V Y L V E I H A S V D S S D G E L H G E K G V V F I R T A D S L S A V P E D R E P Y T G P K A R I A H G

470 480 490 500 510 520 530 540
KAL      D G Q L Q V K V Y W K K T E D P T - - - - - V N R Y H V R W F P E A C A H N R T T G S E A S S G M T H E N Y I I L Q D L S - - F S C K Y - K V T V Q P I R P
KALc     D N Q L Q V K V Y W K K T D I N - - - - - - M N Q F Q V H S L L E S C V H N D T K G L E K V T E L T Y E N Y M I L K D L S - - F S C K Y - K V T V L P A K S
zKAL1.1  D G Q L Q V R V Y W K K K D P T - - - - - - V N R Y R V Q W A P E F C S H N G S R - - T Q G K L I T Q E N F A S L P G L Q - - F S C K Y - K V I I Q P V G L
zKAL1.2  D N Q L Q V K V F W K S R V Q E S Q - - K D S S S Y I L R W Y P E V C S H N V T K - - D E K T A T V Q G T H F V I T G L L - - F A C K Y - R V A V K P V A E
CeKAL    S E E L K I E A S L K D L K T S S - - - H A P N R P N S P Q P A H L E I Q T P F F D T E L Q T Q L N W L N A A H C T P A K R Q F S V K V R K T L C R E Y Q P

550 560 570 580 590 600 610 620
KAL      K S H S K A E A V F F T T P P C S A L K G K S - - - - - - H K P I G C L G E R G - H V L S K V L A K P E N L S A S F I V Q D - V N I T G H F S W K M A K A N
KALc     K S R F K A E S I F F V T P S C S A F K E K T - - - - - - H K H I N C A A E E V - P V L P K V L A K P E N L S A S F I V Q E - G N I T G H F S W K I S K A V
zKAL1.1  K G R T Q V E S I T F Y T P S C A T I Q S K S - - - - - - P K P I P C S S V P - - V V Q P K V L V K A E N L T A A F S V Y L - G N V T G L F S W V V A M P Q
zKAL1.2  Q K - - S E V T T S V T T P L C L S L M G R R - - - - - - K K P A A C A R E E R H P Q L K K A L R R P E K L A V L F Q P V N - G S L Q A L F S W Q L S P E S
CeKAL    N H H S D T R W H D L R V T D C S A L L K G L S F D C D Y K V E V T D T L T G D F V V D G V F S T D T C D Q T S S L T P I D C S S V T T P I Q C Q V T S E S

630 640 650 660 670 680 690 700
KAL      L Y Q - P M T G F Q V T W A E V T T E S R Q N S L P N S I I S Q S Q I L P S D H Y V L T V P N L R P S T L Y R L E V Q V L T P G G E G P A T I K T F R T P E
KALc     L H Q - P M T G F Q V T W A E V T T E S R Q N S L P N S I I S Q S Q I L P A D H Y V L T V P N L R P S M L Y R L E V Q V L T T G G E G P A T I K L F R T P D
zKAL1.1  Q P Q - Q V T G Y Q V T W A E V I T E S R R N N L P N S L I S Q S Q I L P P E R N I L V V S G L Q L A S L Y R L F V Q V I T A A G Q G P A T S R T F Q T P T
zKAL1.2  S G Q T A S S G F Q F S W V K V S S G S - - S N - - G S L I S Q T H I L P P D Q L S L T V D A L Q P S S V Y R V Q V Q V L S E A G N G P S V A K T L H T P A
CeKAL    S A H C H W T R H H D A M Q T V I G Y R I L L S S P I N H D T N T T I N Q P Q L R E V R Y E N L A P G Y V Y T V E V Q S I T N K G L G R T V S T Q F V T H P

710 720 730 740 750 760 770 780
KAL      L P - P S S A H R S H L K H R H P H H Y K P S P E R Y
KALc     L P - P F L P H R P H L K Q H H P H H Y K P P P E K Y
zKAL1.1  H S K P V L H Y K P R I K K - - - H H L R S V I E R H
zKAL1.2  L N - - S I I H
CeKAL    D L D S N A I Q R F P G G E I I E L P L E S S G K L S I F S I F S S F F L V L L L R L L
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B

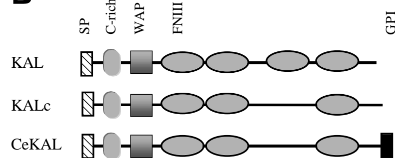

Fig. 1. Evolutionary conservation of KAL proteins. (A) Amino acid sequence alignment of known KAL homologs. KAL, human KAL protein; KALc, chicken KAL protein; zKAL1.1 and zKAL1.2, zebrafish KAL proteins (Ardouin et al., 2000); and CeKAL, *C. elegans* KAL protein. Stars highlight conserved cysteines. 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; GPI, glycosyl-phosphatidyl-inositol anchoring site.

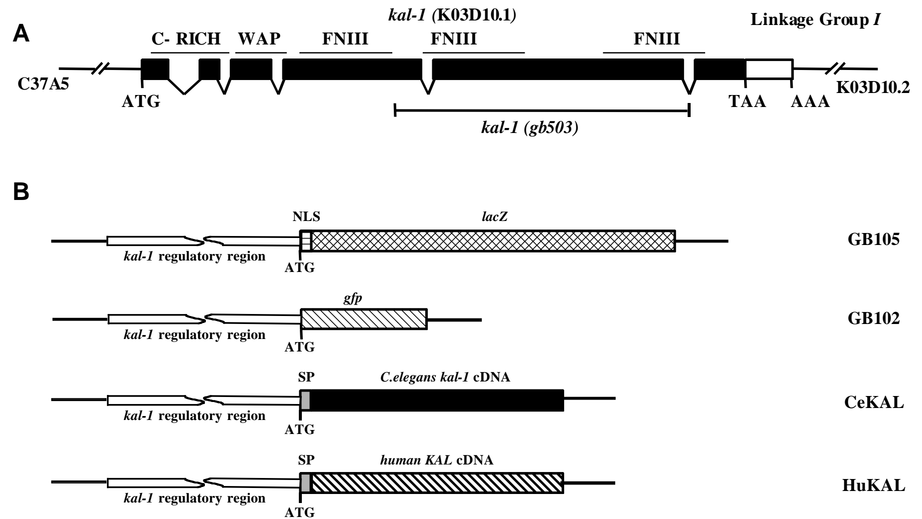

Fig. 2. Structure of the *kal-1* locus and of the constructs used in this study. (A) Structure of the *kal-1* locus on cosmid K03D10. Introns 1, 2, 3, 4 and 5 (not drawn to scale) are 7025, 1968, 949, 471, 888 nucleotides long, respectively. There is a putative transplicing site 3 bp upstream of the ATG. The 3′ untranslated region is 211 nucleotides long. *kal-1* is at one extremity of linkage group 1, near the gene *unc-54* (map coordinates 22,88). The regions corresponding to the conserved protein domains are indicated. The approximate position of the *gb503* deletion is indicated. (B) Reporter expression constructs and KAL proteins overexpression constructs used in this work. The *kal-1* regulatory region, present in all constructs, corresponds to 4.3 kb of genomic sequences upstream and including the ATG of *C. elegans kal-1*. It drives expression of an *E. coli lacZ* gene (GB105), a *gfp* gene (GB102), the *kal-1* cDNA, including the signal peptide (CeKAL), or the human *KAL* cDNA (HuKAL, see Materials and Methods). Abbreviations: SP, signal peptide; C-rich, cysteine-rich domain; WAP, WAP domain; FNIII, fibronectin type III repeat; NLS, nuclear localization signal.

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 epidermal cells are born, differentiate correctly and migrate to surround the embryo, but their migration, reciprocal contacts and shape are abnormal.

Table 1. Embryonic lethality

Genotype	Lethality	*n*	Notes
kal-1(+); *him-8*(*e1489*)	13.8%	745	Control
kal-1(gb503); *him-8*(*e1489*)	25.7%*	288	Loss of function
kal-1(+); *gbEx12*	0.5%	204	Control
kal-1(+); *gbEx13a*	56.1%*	228	Overexpression of *C. elegans kal-1* (cDNA)
kal-1(+); *gbEx13b*	72.4%*	232	
kal-1(+); *gbEx13c*	45.9%*	98	
kal-1(+); *lin-15(n765); gbEx16*	1.1%	609	Control
kal-1(+); *lin-15(n765); gbEx17a*	9.8%*	286	Overexpression of human *KAL-1* (cDNA)
kal-1(+); *lin-15(n765); gbEx17b*	20.2%*	724	
kal-1(+); *lin-15(n765); gbEx17c*	7.5%*	509	

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 ($P<0.001$).

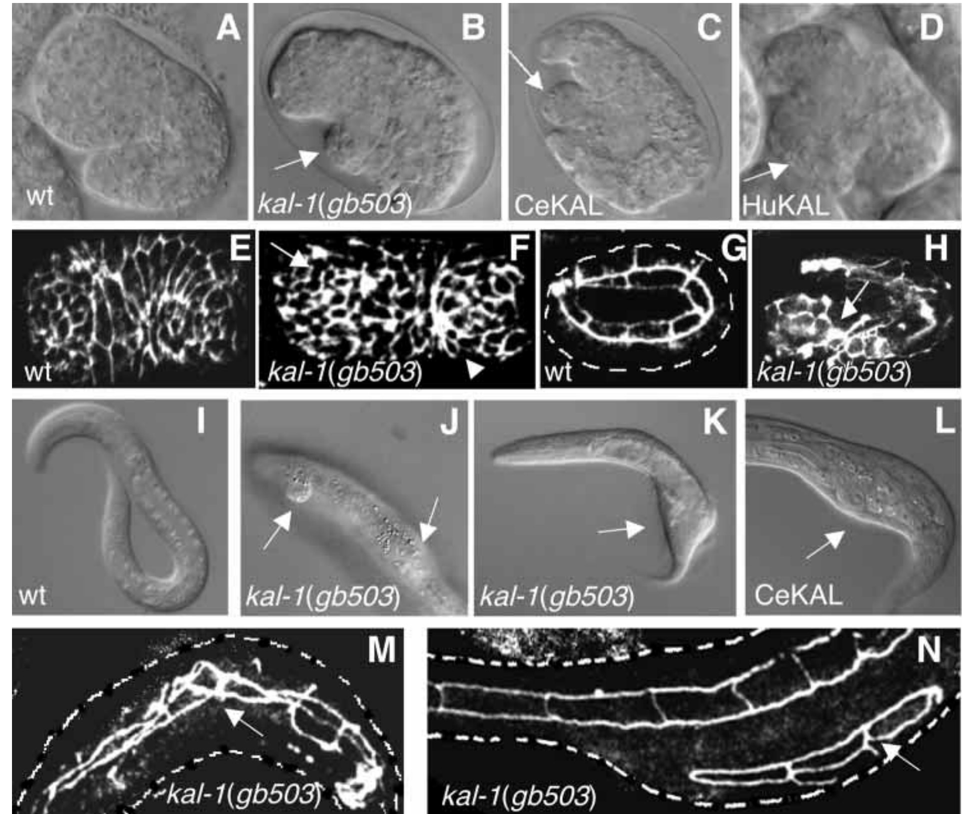

Fig. 3. *kal-1* mutants show embryonic lethality and L1 larvae morphological abnormalities. (A-D) DIC photomicrographs of embryos. (A) Control embryo at one and a half-fold stage; (B-D) mutant embryos in which ventral enclosure has failed; cells protrude ventrally outside of the embryonic mass (white 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 arrowhead). (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).

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 shows 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 also 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

Table 2. Male tail phenotype

Genotype	Wild-type sides	Ray 1-2	Ray 3-4	Ray 5-6	*n*	Notes
kal-1(+); him-8(e1489)	96.9%	5r	1r		192	Control
kal-1(gb503)/+; him-8(e1489)	96.3%	4r; 1e	1r	2i	136	Heterozygote
kal-1(gb503); him-8(e1489)	18.1%*	207r; 6e; 3l	24r; 1l; 4f	10r; 63i; 15f	315	Loss of function
kal-1(gb503); him-8(e1489); gbEx10	68.2%†	22r; 2l	2r; 1f	1i; 3f	88	Rescue with *kal-1* (genomic)
kal-1(gb503); him-8(e1489); gbEx13b	59.0%†	39r; 1l	9r; 5f	1r; 5i; 2f	101	Rescue with *C. elegans kal-1* (cDNA)
kal-1(gb503); him-8(e1489); gbEx17a	48.9%†	24r; 3l	2r; 6f	3r; 16i; 2f;	88	Rescue with human *KAL-1* (cDNA)
kal-1(+); *him-5(e1490)*	92.9%	5r			70	Control
kal-1(+); *him-5(e1490); gbEx13b*	65.4%*	13r; 7e; 10l; 2f	1r; 2f	1r; 4i	107	Overexpression of *C.elegans kal-1* (cDNA)
kal-1(+); *him-5(e1490); gbEx17a*	79.0%*	29r; 6e; 1l	4r; 1l	1r; 1l; 1f	195	Overexpression of human *KAL-1* (cDNA)

kal-1(gb503) is the deletion allele described in this paper; *gbEx10* is an extrachromosomal array containing cosmid K03D10, 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).

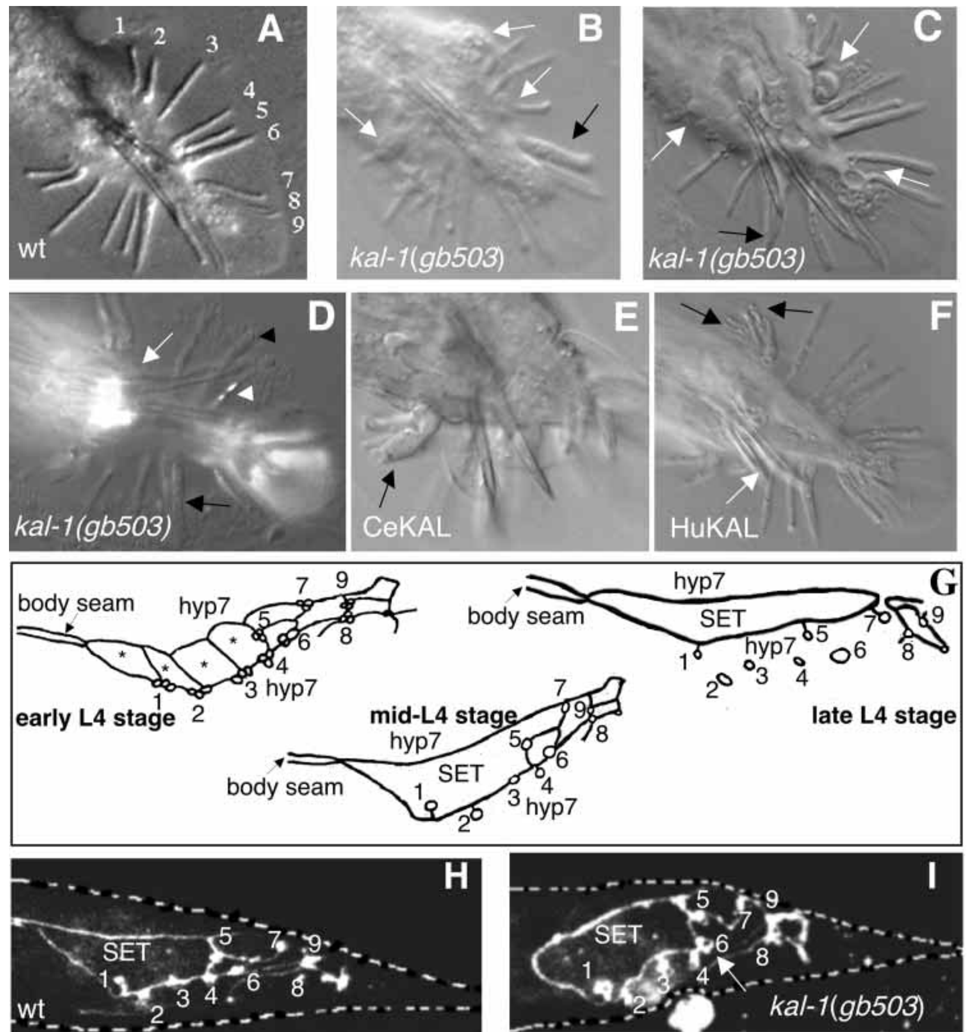

Fig. 4. *kal-1* mutants show altered male tails. (A-F) DIC photomicrographs; ventral views. Control tail (A): the nine rays on each side are indicated. (B-F) *kal-1* mutants. White arrows indicate loss or strong reduction of rays; black arrows indicate abnormal shape, extra rays or fusion of rays. In D, combination of DIC and epifluorescence image of a *kal-1(gb503)* mutant worm, which is also transgenic for *evIs82a* [*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; *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.

ray 1 and 2, and inversion of the position or fusion of two rays (especially ray 5 and 6) (Table 2).

Because the male tail defects of the loss-of-function mutant *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 *gb503* worms carrying cosmid K03D10 (which contains the complete sequence of *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 *C. elegans 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 *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 *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). For example, in mutants the tail seam cells fuse to form the SET cell as in the wild type, but this cell often loses contact with the most posterior body seam-cell and acquires a rounded shape (Fig. 4I).

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

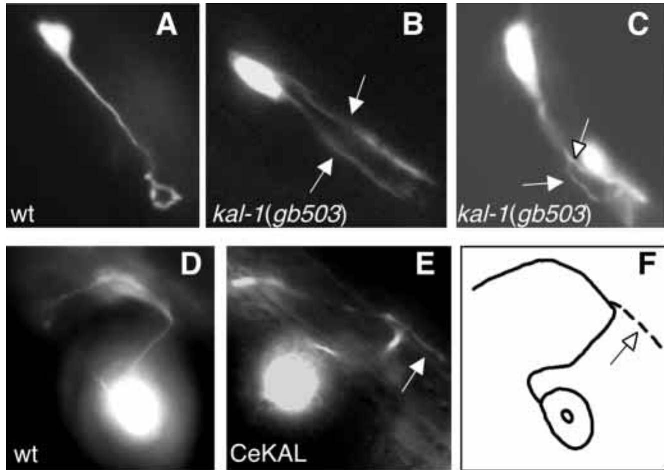

Fig. 5. *kal-1* mutants show neuronal growth defects. Epifluorescence images of neurons of adult worms, harboring the reporter plasmid GB102 on an extrachromosomal transgenic array. In all panels, anterior is towards the left. (A,D) control; (B,C,E) *kal-1* mutants. (A-C) The EF3 male-specific neuron is visible in these worms because of GFP expression from the transgene. In mutant males, the EF3 dendrite presents an extra-branching with the formation of a second dendrite running in the same direction and parallel to the normal one. (D-F) A neuron of the head, RIC, is visible in these worms because of GFP expression from the transgene. In mutant worms overexpressing CeKAL the axon presents an extra-branching with the formation of a spike running parallel but in the opposite direction to the normal path. In F, the trace of the neuron is drawn from micrograph D, and the broken line represents the extrabranching visible in E. Arrows indicate extra-branching in the mutants.

defects. Analysis of movement and of some sensory-based behaviors of *kal-1* mutants did not show significant differences from wild type (not shown). However, expression of GFP under neuron-specific promoters allowed us to visualize directly neuronal processes and detect neurite growth defects in *kal-1* mutants. EF3 is a male-specific neuron whose cell body is placed ventrally in the pre-anal ganglion (Sulston et al., 1980) (Fig. 5A). The EF3 dendrite runs posteriorly along the midline and makes connection with the projections of several of the sensory ray neurons of the male tail. EF3 is one of the neurons expressing GFP under the *kal-1* regulatory sequences (see below) and thus it is easily visible in transgenic strains carrying the expression construct GB102 (Fig. 2B). In 15% of *kal-1(gb503)* male worms, which are transgenic for GB102, the EF3 neuron has an extra-branching of the dendrite that usually runs in the same direction and parallel to the normal one, but terminates prematurely and thus apparently does not connect to a target (Fig. 5B,C).

More penetrant neurite branching and growth defects are visible in *kal-1* overexpressing mutants, especially in neurons of the head ganglia that express GFP under the *kal-1* regulatory sequences. One of these, tentatively identified as RIC, frequently shows an extra branching of the axon, usually running in the opposite direction to the normal path (Fig. 5D-F).

Although we cannot distinguish if normal outgrowth of EF3 or RIC requires the CeKAL-1 protein secreted by the affected neurons themselves, or by nearby *kal-1*-expressing 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

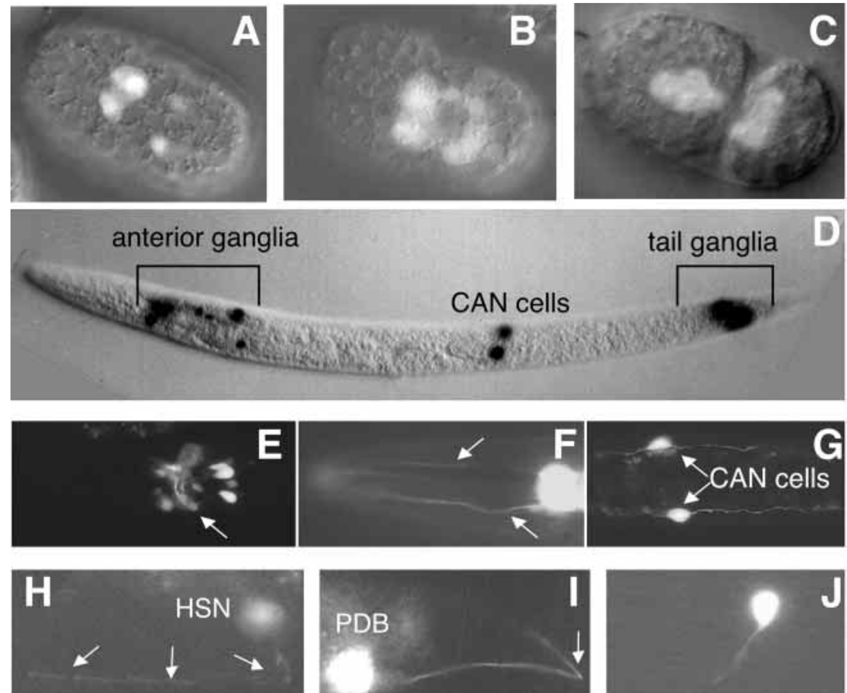

Fig. 6. Expression of *kal-1* reporter constructs in transgenic worms (anterior is towards the left). In all cases, except in D, worms harboring plasmid GB102 (no nuclear localization signal) are shown and GFP expression was detected by epifluorescence; in D, the plasmid was GB105, (containing a NLS) and β-galactosidase expression was detected histochemically. (A,B) Embryonic expression begins in a group of ventral neuroblasts in 120- to 200-cell stage embryos, ventral view, superficial focal planes; (C) ventral view, intermediate focal plane of an embryo between 310 and 360 minutes after fertilization; the expressing neuroblasts have split in an anterior and a posterior group and have been covered by the epithelial cells that have joined at the ventral midline. (D) β-gal staining of an L3 larva showing three groups of neurons expressing the transgene after hatching, see text. (E) Neurons of the anterior ganglia express GFP; arrow points to their axonal projections in the nerve ring. (F) Arrows point to the dendrites of sensory neurons, in the head of an adult male. (G) Ventral view of the mid section of an L3 larva; the canal associated neurons, CAN cells, express the construct. (H) Lateral view of the mid section of an L4 hermaphrodite; the cell body of an HSN neuron is visible, partially out of focus; arrows indicate its anteriorly directed process. (I,J) Tail region of L4 hermaphrodites. In I, the PDB cell body is partially out of focus and the arrow points to the characteristic process reaching the tail tip before turning anteriorly. In J, a tail interneuron, possibly PVW can be seen.

post-embryonic development, the expressing neurons remain largely the same, except for the recruitment of some sexually dimorphic neurons. Three groups of neurons express the *kal-1* constructs post-embryonically (Fig. 6D). One group is located in the anterior ganglia. Here, about 15 neurons express the gene. They include some interneurons and some sensory neurons (Fig. 6E,F). The processes of some of these neurons are affected in *kal-1* mutants (Fig. 5D,E). A second group is located at mid body and is composed of the two canal associated neurons (CANs) (Fig. 6G) and, in adult hermaphrodites, of the hermaphrodite specific neurons (HSN) (Fig. 6H). The neurites of CAN cells run along the length of the animal underneath the lateral seam cells. It is possible that seam cells use *kal-1* expressed from CAN cells for their spatial organization, such that in *kal-1* mutants their reciprocal contacts are sometime altered (Fig. 3M,N). The third group is located in the tail region, where three to six neurons express the construct (Fig. 6D). Among these are the PDB neuron (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). As during ventral enclosure, also during male tail formation, *kal-1* is not expressed by epithelial cells but by neurons. We do not know which of the *kal-1*-expressing neurons are responsible for the effects on tail morphogenesis, but plausible candidates are the neurons in the tail and the CAN cells, the neurites of which run along the length of the animal underneath the lateral seam cells.

DISCUSSION

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/ephrins 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-branching 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.,

1975). 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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