**mab-2 encodes RNT-1, a C. elegans Runx homologue essential for controlling cell proliferation in a stem cell-like developmental lineage**

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**Summary**

In this report, we demonstrate that *C. elegans* mab-2 mutants have defects in the development of a male-specific sense organ because of a failure in the proliferation of the stem cell-like lateral hypodermal (seam) cells. We show, by positional cloning, that mab-2 encodes RNT-1, the only *C. elegans* member of the Runx family of transcriptional regulators, which are postulated to act both as oncogenes and tumour suppressors in mammalian cells. Importantly, we find that *rnt-1* is a rate-limiting regulator of seam cell proliferation in *C. elegans*, as overexpression of *rnt-1* at particular developmental stages is capable of driving extra cell divisions, leading to seam cell hyperplasia. Loss of *rnt-1* is correlated with upregulation of *cki-1*, a CDK inhibitor. Deregulated expression of Runx genes in humans is associated with various cancers, particularly leukaemias, suggesting a conserved role for Runx genes in controlling cell proliferation during development, especially in stem cell lineages. *C. elegans* is therefore an important model system for studying the biology, and oncogenic potential, of Runx genes.

Key words: Caenorhabditis elegans, Runx genes, Cell proliferation, Male tail development

**Introduction**

During development, it is essential for cell division to be properly regulated in time and space so that the correct number of cells are produced in the appropriate location at the required time. These controls ensure that the developmental process culminates in an organism of the appropriate size and complexity. Failure of such controls over cell proliferation can result in tumourigenesis in many metazoan organisms.

Coordination of cell division with growth and differentiation is achieved, at least in part, through the integration of extracellular signals during the G1 phase of the cell cycle (Zhu and Skoultchi, 2001). Cells respond to such signals by either advancing into, or withdrawing from, the next division cycle. Ultimately, signals impinge on the cell-cycle machinery intrinsic to each cell, composed of cyclin-dependent kinases (CDKs) and CDK inhibitors (CDKIs) among other factors.

During *C. elegans* development, cell divisions are almost completely invariant and well characterised (Sulston and Horvitz, 1977; Sulston et al., 1983). The effects of mutations on cell proliferation during development can thus be analysed at high resolution. Normal development gives rise to 959 somatic nuclei in the wild-type hermaphrodite and 1031 in the male. As an organism develops, individual cells must either continue to divide (proliferate) or differentiate and adopt a specialised function. Many cell lineage mutants have been identified that do not produce the correct number of cells, and these may be subdivided in various ways. For example, some mutants are defective in cell division itself, others have defects in the timing of particular cell divisions and a third group have defects in the polarity, or asymmetry, of divisions, leading to fate changes that may influence the choice between subsequent proliferation and differentiation.

The *C. elegans* male tail is an excellent system for genetic studies because this sensory organ is dispensable for viability. The male tail includes the proctodeum, which houses two sclerotized spicules and associated musculature used for the transfer of sperm, and a cuticular fan containing sensory rays used to locate the hermaphrodite vulva during mating (Sulston et al., 1980). The male tail forms from postembryonic divisions of male-specific blast cells and extra divisions of posterior lateral hypodermal (seam) cells and is particularly sensitive to alterations in cell number, which result in recognisable developmental defects. A well-defined set of mutants, the Mab (male abnormal) mutants, have a range of defects in male tail development (Hodgkin, 1983). *mab-2* mutants have variable defects in sensory ray formation (Hodgkin, 1983).

In this report, we show that the *mab-2* phenotype is caused by reduced proliferation of seam cells in both sexes, resulting in the loss of V and T derived rays in males. We show that *mab-2* encodes a Runx domain transcription factor, RNT-1, which is similar to Runx proteins from other species. In humans, mis-expression of Runx genes is associated with
leukaemias and other cancers (reviewed by Coffman, 2003). Most importantly, we find that overexpression of mab-2/rnt-1 in *C. elegans* causes seam cell hyperplasia, suggesting that mab-2/rnt-1 is a conserved factor required to control the extent of cell proliferation during the development of metazoan organisms.

**Materials and methods**

**Strains and *C. elegans* maintenance**

All *C. elegans* strains were derived from the wild-type Bristol strain N2. Routine maintenance of worms and genetic manipulations were performed as described previously (Sulston and Hodgkin, 1988). The *him-5(e1490)* and *him-8(e1489)* alleles were used where necessary to generate a high proportion of males. Phenotypic analyses were carried out using a Zeiss Axiopt microscope fitted with DIC and fluorescence optics as appropriate. Worms were anaesthetized on 2% agarose pads as previously described (Sulston and Hodgkin, 1988). Statistical analysis of quantitative data was performed using the unpaired two-tailed heteroscedastic *t*-test function of Microsoft Excel.

**Growth curves**

Well fed young adult hermaphrodites were picked to a fresh plate and allowed to lay eggs for 1 hour at 20°C. Hatched larvae were then measured after 1, 2, 3 and 4 days growth at 20°C by mounting on 2% agarose gel.

**Mapping**

*mab-2* was originally mapped to the region between *dpy-5* (0.00, cloned map position) and *unc-13* (2.06, cloned map position) on LGI (Jonathan Hodgkin, personal communication). Further mapping revealed *mab-2* to be to the right (or very close to the left) of *unc-40* (0.32, cloned map position) and to the left (or very close to the right) of *air-2* (0.49, cloned map position). The order of the nine cosmid in this region (left to right) is as follows: T19B4 (contains *unc-40*), C04F1–F56H1–T22E7–B0414–C32F10–F33D11–C34G6–B202 7 (contains *air-2*). Confirmed single nucleotide polymorphism (SNP) markers assayed by restriction enzyme analysis, which differ between the Bristol N2 strain and the Hawaiin strain CB4856, are available for cosmids C04F1 (C04F1:3815) and C34G6 (C34G6:15466). Recombinants were picked from the heterozygous *dpy-5* mab-2 *unc-13* CB4856. Ten out of 10 Dpy Mab non-Unc recombinants were found to be wild type for SNP C04F1:3815, and nine out of nine Unc non-Mab non-Dpy recombinants were found to be Hawaiian for SNP C34G6:15385. These data indicate that *mab-2* is to the right of C04F1:3815 (or very close to the left). Four out of four Unc Mab non-Dpy recombinants were found to be wild type for SNP C34G6:15466, and seven out of nine Unc non-Mab non-Dpy recombinants were found to be Hawaiian for SNP C34G6:15466. These data indicate that *mab-2* lies to the left of C34G6:15466. The five cosmids between C04F1 and C34G6 were tested for rescue of *mab-2(e1241)*, and cosmid B0414 was found to rescue the mutant phenotype completely. Oligos used for SNP C04F1:3815 were RN3 (5′GGATTTATCAGGATGATCAG) and RN4 (5′CATTGCCA-GAGGGATGGAAC), yielding a 784 bp PCR product, cut by *Tsp*51I in N2 to give bands of 490 bp and 294 bp. For SNP, C34G6:15466 oligos were RN5 (5′GGATTTATCAGGATGATCAG) and RN6 (5′CAGAGGTGCAGGATGGAAC), yielding a 992 bp PCR product, cut by *Taql* in N2 to give bands of 42 bp, 225 bp, 276 bp and 449 bp, and in CB4856 to give bands of 25 bp, 42 bp, 225 bp, and 251 bp and 449 bp. These differences could be easily resolved on a 2% agarose gel.

**Lineage analysis**

Worms were mounted on 2% agarose pads (Sulston and Hodgkin, 1988) in 2 μl of M9 to which a smear of OP50 bacteria had been added and mixed. A 12×12 mm coverslip was gently lowered onto the worm and the divisions of the seam cells were observed using Nomarski DIC optics and a 63× oil immersion objective (Zeiss) over a period of 18-20 hours.

**Transgenic worms**

Plasmids were injected into the syncytial gonad of young adult hermaphrodite worms at a concentration of 30 ng/μl as described (Mello and Fire, 1995), along with the *rol-6* transformation marker (at 50-100 ng/μl). Rol progeny were picked and stable lines selected for analysis. Several lines were generated for each construct.

**Transformation rescue of rnt-1**

Cosmid B0414 was found to rescue the *rnt-1(e1241)* mutant phenotype. Subclones were generated to test for single gene rescuing activity and a fragment containing the single gene B0414.2 was found to rescue this subclone was generated by PCR amplification from cosmid B0414-1 TA cloned into TOPOXL (Invitrogen) in two halves using oligos RN64 and RN83 (5′ half) and RN68 and RN82 (3′ half). The 3′ half (7902 bp) was then subcloned into the 3′ end of the 5′ half (8428 bp) using *XbaI* and *BglIII* to generate the full-length B0414.2 genomic clone pAW258. The rescued strain referred to in this report carrying the whole cosm B0414 is AW44 (rnt-1(e1241); *him-5(e1490); ouEx15[B0414 + rol-6]*), and the rescued strain referred to in this report carrying the single gene B0414.2 (rnt-1) is AW68 (rnt-1(e1241); *him-5(e1490); ouEx17[B0414.2 + rol-6]*)

**Sequencing of rnt-1 alleles**

Primers were used to amplify rnt-1 coding sequences from appropriate mutant worms for sequencing using the high-fidelity Expand DNA polymerase (Roche). PCR from worms was performed as described previously (Williams, 1995). Sequences (both strands) of at least two independent PCR products were analysed using Pairwise Alignment in BioEdit.

**GFP reporter constructs**

A rnt-1 translational GFP fusion construct was made using pPD vectors kindly supplied by the Fire Lab (Stanford University). All constructs were verified by sequencing. To make rnt-1::GFP, GFP from pPD19.16 was excised using Smal and inserted into the StuI site at the 3′ end of the 3′-rnt-1 clone. Then, this GFP-tagged 3′ clone was inserted into the 3′ end of the 5′ clone using *XbaI* and *BglIII* as above, to generate a full-length genomic GFP-tagged rescuing construct, pAW260. The rescued strain referred to in this report carrying this rnt-1::GFP translational fusion is AW109 (rnt-1(e1241); *him-5(e1490); ouEx26[rnt-1::GFP + rol-6]*). rnt-1(ok351) *him-5(e1490) was crossed into the *scm::GFP* cell line rescuing strain (JR667) to generate the strain AW58, into a *myo-3::GFP* cell muscle cell reporter (strain PD4251) to generate the strain AW106 and into a *cki-1::GFP* reporter (strain VT825) to generate the strain AW148.

**Heat-shock constructs**

A full-length rnt-1 cDNA construct was generated by PCR from a *C. elegans* cDNA library (gift from R. Barstead, Oklahoma University) and used to generate two *hspl-16* driven constructs (F primer 5′GCTAGCCAGTCTGGAGGATTTCCGG, RN87; R primer 5′GAGCTCTTGAAGAGGGTTTACGTT, RN88; product 994bp, pAW261 consists of the rnt-1 cDNA cloned into pPD49.78 (*hspl-16* driven) as a *NheI*-SaciI fragment. pAW262 consists of rnt-1 cDNA cloned into pPD49.83 (*hspl-16* driven) as a *NheI*-SaciI fragment. The transgenic line generated for pAW261 described in this study is AW87 (him-5(e1490); ouEx19[hspl-16::rnt-1 + rol-6*]). AW87 was crossed into the *scm::GFP* cell line reporter strain to generate the strain AW102 and into rnt-1(e1241); *him-5(e1490)* to generate the strain AW103. A transgenic line was also constructed carrying pAW262 as well as an *elt-2::GFP* intestinal reporter (gift from Joel Rothman, University of California, Santa Barbara) by co-
injecting both reporters in addition to the rol-6 transformation marker, to generate the strain AW100 (him-5(e1490); ouEx23[hsp16-41::rtt-1 + elt-2::GFP + rol-6]).

Heat-shock experiments

Worms were staged by bleaching gravid adult hermaphrodites to produce a pure egg population that was then allowed to hatch in the absence of food to produce a synchronous population of arrested L1 stage animals (Sulston and Hodgkin, 1988). These animals were then re-fed on OP50 seeded NGM plates and heatshocked at 33°C for 1 hour at different larval stages.

RNAi

PCR primers, including T7 or T3 RNA polymerase promoter sites were designed to be specific for rnt-1, and to amplify 504 bp of mostly exonic sequence (F primer 5’-ATTACCTCAAAAGGTTCAG-GTTGATGGAC, RN84; R primer 5’-AATACGACTCTATAGCGAGAAGAAGCTATTCG, RN85). Primers specific for cki-1 amplified 602 bp (F primer 5’-ATTACCCCTCAAAAGGTTCAG-TGCTCGTGGGCC, RN90; R primer 5’-AATACGACTCTATAGGAGAGCATGAGATCGAGTCTG, RN91). dsRNA was synthesised directly from gel-purified PCR product as previously described (Fire et al., 1998) and injected into young adult hermaphrodites at a concentration of ~1 mg/mL. Injected worms were transferred to fresh plates 6 hours following injection and thereafter every 24 hours for 3 days.

Results

mab-2 mutants have missing V and T lineage derived rays

mab-2(e1241) mutant males have a variable missing ray phenotype, typically lacking 5 rays from each side of the tail (Fig. 1, Fig. 2D). The phenotype is identical in two other alleles of mab-2, os11 and os58 [kind gifts from Hitoshi Sawa (Fig. 2 and data not shown)]. Both V and T derived rays are variably missing. We also noticed a low (5-10%) penetrance ray fusion phenotype in mab-2 males (Fig. 1E). Ray fusions may simply be a consequence of missing intervening rays. However, it is also possible that mab-2 has some downstream function in specifying ray identity, separate from its function in specifying ray number.

To examine the cellular basis of the missing ray phenotype, we examined tail development using the ajm-1::GFP reporter (Strain SU93), which marks adherens junctions and shows the outlines of male tail precursor cells derived from the posterior seam. We found fewer ray precursor cells in mab-2 mutants compared with wild type (Fig. 1), indicating either a failure of cell proliferation or a cell fate transformation in V5, V6 and T lineages.

mab-2 encodes RNT-1, a member of the Runx group of transcription factors

We mapped mab-2 to the centre of chromosome 1, between unc-40 and air-2, using three-factor crosses (see Materials and methods). SNP mapping was then used to further map mab-2 to a region spanned by seven cosmids (see Materials and methods) and these cosmids were tested for rescuing activity. The cosmid B0414 was found to completely rescue the phenotype of mab-2 mutant worms, and a subclone including the full ORF of only one gene, B0414.2, was subsequently found to provide all of this rescuing activity (Fig. 2A). Examination of the sequence of B0414.2 revealed it to encode a transcription factor of the Runx family, members of which include human RUNX1, RUNX2, RUNX3 and Drosophila runt and lozenge (Fig. 2C). B0414.2 has been given the name RNT-1 in Wormbase.
and is the only Runx orthologue present in the worm genome.

A deletion allele of \textit{rnt-1} is available from the \textit{C. elegans} Knockout Consortium (Oklahoma, USA, allele \textit{ok351}). We found that this allele fails to complement the male tail phenotype of \textit{mab-2(e1241)} (Fig. 2A), demonstrating that \textit{mab-2} and \textit{rnt-1} are allelic. For simplicity we will henceforward refer to \textit{mab-2} as \textit{rnt-1}. The male tail phenotype of the \textit{rnt-1} deletion allele, \textit{ok351}, is indistinguishable from \textit{e1241}, with the same number of
Development

The molecular nature of the lesions associated with these alleles are shown in Fig. 2. The mutation in e1241 results in a single amino acid substitution, 1112K, in a conserved part of the DNA-binding domain, changing a hydrophobic residue into a hydrophilic one. os58 contains a premature stop codon near the N terminus of the protein (H. Kagoshima, personal communication). This allele is therefore a likely null allele and has the same male tail phenotype as e1241 and ok351 (Fig. 2D). The trans-heterozygote os58/e1241 is indistinguishable from a e1241 or os58 homozygote (Fig. 2A; data not shown). The deletion in ok351 removes 10 amino acids from the Runt domain plus an additional 29 amino acids downstream, but leaves the reading frame intact.

Silencing rnt-1 using RNA interference (RNAi) also gave rise to males with missing rays, similar to the loss of function alleles described (Fig. 2D). There was a small but significant amount of embryonic lethality associated with rnt-1 RNAi (18% embryonic lethality at 25°C in him-8(e1489); rnt-1(RNAi) animals compared with 3.6% embryonic lethality in him-8(e1489) animals kept at 25°C but not subjected to RNAi.

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**Fig. 2.** mab-2 encodes RNT-1, a Runx transcription factor. (A) (i) him-5(e1490) male tail. (ii) mab-2(e1241); him-5(e1490) male tail. (iii) mab-2(e1241); him-5(e1490); osEx15[B0414 + rol-6] male tail. Wild-type appearance is restored using this cosmid. (iv) mab-2(e1241); him-5(e1490); osEx17[B0414 + rol-6] male tail. Wild-type appearance is restored using the single gene B0414, encoding the Runx transcription factor rnt-1. (v) Male tail from a e1241(ok351) trans-heterozygote, showing that these two alleles fail to complement each other. (vi) Male tail from a e1241/os58 trans-heterozygote, again showing non-complementation between the two alleles. Scale bar: 10 μm. (B) Schematic representation showing the genomic structure of the rnt-1 gene, the nature and position of the mutations in e1241 and os58 alleles, and the deletion in ok351. The genomic region shown corresponds to the region cloned in the rescuing construct pAW258. The position of the GFP insertion in pAW260 is also indicated. (C) Alignment of Runx genes from various species showing the conserved DNA-binding domain. Identical amino acids are shown in black; similar amino acids are in grey. The Runt domain of rnt-1 (Accession Number OA1834), 51% similar to Drosophila Runt (Accession Number CA9398), 50% similar to human RUNX1 (Accession Number NP_001545), and 50% similar to mouse Runx1 (Accession Number Q03347). Sequence alignments were performed using ClustalW and processed using BioEdit software. The nature of the mutations in e1241, os58 and ok351 animals are indicated with asterisks. The ok351 deletion removes 10 amino acids of the Runt domain plus 29 amino acids downstream in an in-frame deletion. (D) Bar chart showing a quantitative analysis of ray number in wild-type (n=33), rnt-1(os58) (n=37), rnt-1(e1241) (n=31), rnt-1(ok351) (n=35), rnt-1(RNAi) (n=28) and rnt-1(e1241); osEx26[rnt-1::GFP+rol-6] (n=35) males. Ray number in all three rnt-1 alleles, as well as in rnt-1(RNAi) males is significantly different from wild type (P<0.0001). Ray number in rnt-1(e1241); Ex[rnt-1::GFP] males is not significantly different from wild type (P=0.05), indicating rescue. Error bars represent the standard error of the mean. (E) Graph showing length growth curves of wild-type and rnt-1(ok351) animals. There is no significant reduction in the length of ok351 animals compared with wild-type animals until the worms have reached adulthood, 4 days after eggs were laid. A ~5% decrease in ok351 body length is seen at this time point (n=33 for wild type, 30 for ok351, P<0.0001). Error bars represent the s.e.m. 14.4% lethality is thus attributable to the effects of rnt-1 RNAi). A similar amount of embryonic lethality is seen in rnt-1(os58) animals (50% embryonic lethality in rnt-1(os58); him-5(e1490) animals kept at 25°C compared with 38% embryonic lethality in him-5(e1490) animals alone kept at 25°C; 12% lethality is thus associated with the os58 allele). None of the other rnt-1 alleles displays any significant embryonic lethality, so it is possible that those alleles, including the deletion allele ok351, are non-null. rnt-1(os11) was found to be largely inviable at 25°C, with much reduced fertility, but the lesion in os11 animals appears to be a large deletion also affecting a neighbouring gene (R.N. and A.W., unpublished), which known from genome-wide RNAi screens to be essential (Kamath et al., 2003).

rnt-1 hermaphrodites have no defects at the gross morphological level, except a slight (5%) reduction in body length at adulthood (Fig. 2E). The slight reduction in body length is similar in all alleles tested (data not shown). We noticed that the reduction in body length was more pronounced in rnt-1 worms recovering from starvation, and these worms were found to be very sick (data not shown), suggesting that rnt-1 may have some function in stimulating growth following starvation. No other defects were observed in any of the rnt-1 mutant strains tested, or in rnt-1(RNAi) animals.

**Seam cell number is reduced in rnt-1 animals**

In wild-type males, the rays are generated by seam cells. Seam cell divisions in both sexes provide hypodermal nuclei and a postdeirid neuroblast (Sluson and Horvitz, 1977). In males, extra divisions in the posterior seam cells V5, V6 and T and subsequent specification of ray neuroblasts result in generation of the 18 rays.

We analysed the number of seam cells in rnt-1 mutants using the seam cell marker scm::GFP. Wild-type adult hermaphrodites usually contain 16 seam cells on each side of the animal at the end of development, derived from the 10 embryonically derived blast cells H0, H1, H2, V1-V6 and T (Fig. 3). rnt-1 mutant hermaphrodites contain fewer, typically 13 (Fig. 3). Male rnt-1 mutant worms also contain fewer seam cells, typically 11, compared with 18 in wild type (Fig. 3). This indicates that rnt-1 activity is required in both males and hermaphrodites for either seam cell proliferation or differentiation, and that this is the basis of the male tail phenotype, as sensory rays are generated from posterior seam cells. Cells in rnt-1 animals (albeit reduced in number) fuse normally in L4 (visualised with ajm-1::GFP, data not shown), indicating that they maintain the correct fate. In hermaphrodites, cells are responsible for the formation of alae, cuticular ridges on the surface of the worm (Sluson and Horvitz, 1977). Despite the lack of seam cells in rnt-1 mutants, alae do not appear to be defective (data not shown), again indicating that correct seam cell fate is maintained in the remaining seam cells.

**rnt-1 is expressed in seam cells**

The expression pattern of a rescuing translational rnt-1::GFP fusion construct is shown in Fig. 4. rnt-1::GFP is visibly expressed in the nuclei of seam cells in embryos from around 260 minutes post fertilisation, just after the time at which seam cell are formed. Seam cell expression in both males and hermaphrodites is visible during all developmental stages, but
is particularly strong until late L2. In males, *rnt-1::GFP* is expressed additionally in seam cell derived ray precursor cells and ray sublineages. *rnt-1::GFP* is also expressed transiently in body wall muscle nuclei from late embryogenesis until the end of L2 (Fig. 4). We could find no obvious phenotype associated with expression of *rnt-1* in body wall muscle. Both the number and arrangement of body wall muscle nuclei (assayed using a *myo-3::GFP* reporter) in *rnt-1* mutants appears normal (data not shown). We did not observe *rnt-1::GFP* expression in any other cell types. Expression of *rnt-1* has been previously reported in intestinal cells (Nam et al., 2002), but we did not find any intestinal expression using our rescuing GFP reporter construct, nor did we observe any intestinal defects in *rnt-1* mutant alleles or *rnt-1(RNAi)* animals. Our rescuing GFP reporter contains the endogenous *rnt-1* 3’UTR that was absent from the reporter described by Nam et al. It is possible that this difference in reporters accounts for the slight difference in the *rnt-1* expression pattern observed.

*rnt-1* is specifically required for seam cell proliferation, not cell fate determination

One possible interpretation of our data could be that *rnt-1* is required for cell fate determination, rather than cell proliferation per se. For example, controls acting on the polarity of seam cell divisions can influence cell proliferation. The seam cells normally undergo polarized divisions during larval development, such that the posterior daughters (Vn.p) adopt the seam cell (proliferative) fate and the anterior daughters (Vn.a) adopt the non-proliferative, syncytial fate, fusing with the hypodermal syncytium hyp7 (Sulston and Horvitz, 1977). It is therefore possible that *rnt-1* mutants could fail to maintain seam cell number if, at particular seam cell divisions, the posterior daughter adopted the hypodermal fate like its anterior neighbour. Alternatively, divisions could fail as a direct result of a failure to enter or progress through the cell cycle. In order to distinguish between these two possibilities, we performed a detailed lineage analysis of seam cell divisions in *rnt-1* mutants. A lineage trace of seam cell divisions in a *rnt-1(ok351)* hermaphrodite from late L1 up to mid L3 is shown in Fig. 5B. Various seam division failures are evident in this animal, as discussed in the figure legend. A *rnt-1(ok351)* him-8(e1489) male seam cell lineage trace is shown in Fig. 5D.

The most common lineage defect in *rnt-1* animals involves failures in L2 and L3 seam cell divisions. L1 divisions were...
found to be normal in all animals analysed. Thus, the main role of \( rnt-1 \) is to stimulate divisions of seam cells from L2 onwards. The lineage traces shown illustrate the variable nature of the division failures in different seam lineages. Moreover, different animals displayed different seam lineage failures (data not shown). This explains why \( rnt-1 \) males end up with a variable number of missing rays. L4 divisions and male ray sublineages were not extensively lineaged. The lineage defects observed do not appear to be heterochronic alterations, as lineages are only partially affected (usually the posterior branch). Two examples of potential cell fate transformations (in the anterior branch of V1 in the hermaphrodite and in the posterior branch of T in the male) were observed, but this type of defect was found to be rare.

**Ectopic expression of \( rnt-1 \) causes seam cell hyperplasia**

Deregulated expression (both loss and gain of function) of Runx genes in humans is associated with various cancers (reviewed by Cameron and Neil, 2004). In particular, amplification of \( Runx1 \) has been associated with leukaemia (Roumier et al., 2003). To test the effects of overexpressing \( rnt-1 \) in \( C. elegans \), we constructed transgenic worms carrying a full-length \( rnt-1 \) cDNA driven by the heat shock promoter \( hsp16-2 \), which drives high level expression in seam, hypodermal and neuronal cells (see Materials and methods).

We found that heat shock of transgenic worms prior to L2 or after L3 had no effect on adult seam cell number (as assayed by \( scm::GFP \) expression), but heat shock during L2 and L3 caused a significant increase in the number of seam cells present in adult hermaphrodites (Fig. 6), indicating that \( rnt-1 \) is both necessary and sufficient for seam cell proliferation. Moreover, in males, these extra seam cells are capable of differentiating into extra rays (Fig. 6D).

Overexpression of \( rnt-1 \) does not appear to cause hyperplasia in other cell types. We looked at the number of intestinal nuclei (using an \( elt-2::GFP \) intestinal reporter) in heat shocked worms carrying an \( hsp16-41::rnt-1 \) construct (which would be expected to drive high level expression in the intestine) and found it to be normal (data not shown). We likewise found no increase in the number of body wall muscle cells, assayed using a \( myo-3::GFP \) reporter strain in heat shocked worms carrying an \( hsp16-2::rnt-1 \) construct (data not shown).

**Suppression of \( rnt-1 \) seam cell division failures by \( cki-1 \) RNAi**

\( cki-1 \) is a member of the KIP/CIP family of CDK inhibitors and is most similar to p27kip1, which functions to link developmental programmes to cell cycle progression (Boxem and van den Heuvel, 2001; Fukuyama et al., 2003; Hong et al., 1998). KIP/CIP CDK inhibitors are thought to act by inhibiting the activity of the cyclin E/CDK2 complex during G1 (reviewed by Sherr, 2000). \( cki-1 \) in \( C. elegans \) has been reported to be required for developmental cell cycle arrest in several lineages and is known to be expressed in seam cells (Fukuyama et al., 2003; Hong et al., 1998). The temporal pattern of \( cki-1 \) expression in seam cells partially overlaps with that of \( rnt-1 \). Both genes are expressed strongly during embryogenesis and until mid-L1 (this report) (Fukuyama et al., 2003). High level expression of \( rnt-1 \) persists during larval
**Fig. 5.** *rnt-1* is necessary for seam cell proliferation, not fate determination. Lineage traces are shown up to mid L3. The L1 asymmetric division is omitted for simplicity. Seam cells are indicated by circles, hyp7 nuclei by squares, and glial and neuronal cells by diamonds. Broken lines indicate incomplete lineages. The data shown are lineage traces for single animals. Five animals were lineaged and found to give similar results. (A) Wild-type hermaphrodite V1-V6 and T lineages. (B) *rnt-1(ok351)* hermaphrodite lineage trace of V1-V6 and T divisions. V2 and V3 divisions display a similar pattern in the lineage trace shown; the anterior branch divides normally, but there is a failure of the L2 asymmetric division in the posterior branch, leading to a reduction in hypodermal nuclei. V4 and V6 display a different defect; this time the L2 proliferative division fails, causing a reduction in seam and hypodermal nuclei. V5 divisions are normal in this lineage trace, leading to the correct formation of the post-deirid neuroblast. Normal post-deirids appeared to be present in all *rnt-1* animals analysed (data not shown).

V1 displays a similar defect to V2 and V3 in the posterior branch, a failure of the L2 asymmetric division, but there is an additional defect in the anterior branch. Both daughter nuclei from the L2 ‘asymmetric’ division appear to fuse with the hypodermal syncytium, rather than the posterior daughter undergoing the proliferative fate. In the T lineage, the anterior branch is normal, but there is a division failure in the posterior branch, yielding just one seam cell, rather than a seam cell plus a glial cell. (C) Wild-type male V1-V4, V5, V6 and T lineages. A description of these divisions is given in the legend to Fig. 3. (D) *rnt-1(ok351); him-8(e1489)* male seam cell lineage trace. In V1 the L2 proliferative division occurs normally but there are no further divisions. Both daughters resemble seam cells. In V2, the posterior daughter of the L2 proliferative division does not divide further in L2 or L3 (it remains as a seam cell), while the anterior daughter undergoes one further asymmetric division in L2 to produce a hypodermal daughter and a seam daughter that fails to divide further in L3. In V3, the L2 proliferative division occurs normally and the anterior branch undergoes the normal asymmetric divisions in L2 and L3, while the posterior branch undergoes one asymmetric division in L2, after which the posterior seam daughter fails to divide further. In V4, the L2 proliferative division occurs normally and the anterior branch displays a similar division pattern to the anterior branch of V2, while the posterior branch undergoes the normal L2 and L3 asymmetric divisions. In V5, the anterior branch is normal but the posterior branch fails after the first L3 proliferative division, with both daughters failing to divide. In V6, L2 divisions are normal but there are failures in L3 divisions. The wild-type male V6 lineage normally undergoes two rounds of division in early L3, whereas in this *rnt-1* male, only one round of division occurs in each branch. In the T lineage, the anterior branch was normal but there was, unusually, an extra proliferative division in the posterior branch during L2.

development, whereas *cki-1* expression declines sharply at mid-L1, to be restored during resting phases between larval moults and at L4, coincident with seam cell terminal differentiation (Fukuyama et al., 2003).

To test for a genetic interaction between *rnt-1* and *cki-1*, we removed *cki-1* expression by RNAi in a *rnt-1* mutant background and assayed seam cell number. *cki-1* RNAi in wild-type worms causes an increase in seam cell number in treated animals which survive to adulthood (Fig. 7A), indicating that *cki-1* normally acts to limit seam cell proliferation. This is in agreement with a previous report showing seam cell hyperplasia induced by *cki-1* RNAi in embryos (Fukuyama et al., 2003). When *rnt-1(ok351)* animals (which normally have reduced seam cell proliferation) are subjected to *cki-1* RNAi, adult seam cell number is restored to almost wild-type levels (Fig. 7A). This suggests that *rnt-1* and *cki-1* may act at a similar point in the cell cycle to control seam cell proliferation in opposing ways.

One model for the opposing roles of *rnt-1* and *cki-1* in controlling seam cell proliferation would be that RNT-1 negatively regulates *cki-1* expression in seam cells. We tested this possibility by examining *cki-1::GFP* expression in a *rnt-1* mutant. The best situation in which *cki-1* expression could be robustly analysed in seam cells during larval development was found to be during L1 (we found *cki-1::GFP* reporter expression to be too faint to analyse reliably after L1). Although, as discussed above, loss of *rnt-1* does not normally affect the L1 stem cell division, we found that this division fails in *rnt-1* animals that have been arrested in L1 diapause by hatching in the absence of food, before being allowed to recommence larval development by introducing food (Fig. 7B). By contrast, wild-type animals undergo this division normally.
Development division failure is correlated with increased animals whose seam cells divide normally. In other words, seam cells fail to divide in L1 is higher than in wild-type cki-1::GFP we found that somehow downregulating rnt-1 promotes seam cell proliferation Discussion sensitis the L1 division to loss of cki-1 rnt-1 precursor cells and execute the correct ray sublineages, hence T lineage divisions is to produce the correct number of ray hermaphrodites. In males, the cumulative effect of V5, V6 and asymmetric divisions of seam cells that occur in males and hermaphrodites. In males, the cumulative effect of V5, V6 and T lineage-derived rays. The cellular basis of the ray loss phenotype is a reduction abnormalities owing to the loss of V and T lineage-derived components of the Sma/Mab TGF signalling pathway (Ji et al., 2004). Although we do observe a small (5%) decrease in length in rnt-1 adult hermaphrodites, this is much less severe than in Sma animals. In the case of rnt-1, we suggest that this slight reduction in size is caused by a reduction in the number of nuclei in the hypodermal syncytium, which is caused by seam cell division failures. The relationship between hypodermal ploidy and body size in C. elegans has been previously discussed (Flemming et al., 2000). Moreover, we can see no phenotypic similarities between rnt-1 and Sma male tails. The Sma phenotype is typified by fused rays (Hodgkin, 1983; Morita et al., 1999; Savage-Dunn et al., 2003), whereas the rnt-1 phenotype is typified by missing rays caused by failures in seam cell proliferation, as indicated by our lineage analysis. It is possible that rnt-1 has some minor downstream role in ray morphogenesis, as evidenced by the very low penetrance ray fusion phenotype we observe in rnt-1 animals, but it is certain that the major focus of rnt-1 action is in the regulation of seam cell proliferation. Given that missing rays are not observed in Sma mutants, we consider it unlikely that this major function of rnt-1 involves an interaction with the Sma/Mab TGFß pathway. Other genes known to influence ray development tend to affect specific ray sublineages. For example, mab-5, a posterior Hox gene, is required for proliferation and ray production in V5 and V6 sublineages but does not affect T lineage-derived rays (Kenyon, 1986; Salser and Kenyon, 1996). By contrast, ray loss in mab-19 mutants is restricted to when subjected to the same treatment (Fig. 7B), i.e. starvation sensitises the L1 division to loss of rnt-1. As shown in Fig. 7B, we found that cki-1::GFP expression in rnt-1 mutants whose seam cells fail to divide in L1 is higher than in wild-type animals whose seam cells divide normally. In other words, division failure is correlated with increased cki-1::GFP expression. This is good evidence that rnt-1 normally acts during G1 of the cell cycle to promote cell division by somehow downregulating cki-1 expression. It is not clear at present whether this interaction is direct or indirect. Discussion rnt-1 promotes seam cell proliferation All alleles of rnt-1 examined display similar male tail abnormalities owing to the loss of V and T lineage-derived rays. The cellular basis of the ray loss phenotype is a reduction in seam cell proliferation during larval development. Our data suggest that rnt-1 is required for both the proliferative and asymmetric divisions of seam cells that occur in males and hermaphrodites. In males, the cumulative effect of V5, V6 and T lineage divisions is to produce the correct number of ray precursor cells and execute the correct ray sublineages, hence rnt-1 mutants have missing rays. We found that silencing of cki-1, a G1 inhibitor normally active in seam cells, suppresses the seam cell proliferation defect, suggesting that rnt-1 and cki-1 act on the cell cycle to regulate cell proliferation in opposing ways. Intriguingly, we found that cki-1::GFP seam cell expression is upregulated in rnt-1 mutants, suggesting that RNT-1 acts in G1 to promote seam cell divisions via the downregulation of cki-1. Our data support the view that the loss of seam cells is mainly the result of a defect in cell proliferation per se, rather than a change in cell fate. The rare cell fate, as opposed to cell proliferation, defects we did observe in rnt-1 animals may be explained by signalling defects. It is known that signalling among seam cells (acting in conjunction with lineage cues) is important for cell fate determination. This has been demonstrated most clearly in ablation studies, where extensive ablation of seam cells was found to cause various cell fate changes in the remaining seam cells (Sulston and White, 1980). Thus, cells with inappropriate neighbours may be expected to take on the wrong fate under certain circumstances. In this context, it becomes somewhat artificial to postulate a clear distinction between cell proliferation and cell fate determination. A recent report suggests that rnt-1 functions to regulate body size and ray morphogenesis in C. elegans by interacting with components of the Sma/Mab TGFß signalling pathway (Ji et al., 2004). Although we do observe a small (5%) decrease in length in rnt-1 adult hermaphrodites, this is much less severe than in Sma animals. In the case of rnt-1, we suggest that this slight reduction in size is caused by a reduction in the number of nuclei in the hypodermal syncytium, which is caused by seam cell division failures. The relationship between hypodermal ploidy and body size in C. elegans has been previously discussed (Flemming et al., 2000). Moreover, we can see no phenotypic similarities between rnt-1 and Sma male tails. The Sma phenotype is typified by fused rays (Hodgkin, 1983; Morita et al., 1999; Savage-Dunn et al., 2003), whereas the rnt-1 phenotype is typified by missing rays caused by failures in seam cell proliferation, as indicated by our lineage analysis. It is possible that rnt-1 has some minor downstream role in ray morphogenesis, as evidenced by the very low penetrance ray fusion phenotype we observe in rnt-1 animals, but it is certain that the major focus of rnt-1 action is in the regulation of seam cell proliferation. Given that missing rays are not observed in Sma mutants, we consider it unlikely that this major function of rnt-1 involves an interaction with the Sma/Mab TGFß pathway. Other genes known to influence ray development tend to affect specific ray sublineages. For example, mab-5, a posterior Hox gene, is required for proliferation and ray production in V5 and V6 sublineages but does not affect T lineage-derived rays (Kenyon, 1986; Salser and Kenyon, 1996). By contrast, ray loss in mab-19 mutants is restricted to
Defective male tail formation is the only highly penetrant gross morphological phenotype associated with loss of rnt-1 function, and consistent with this we found seam cells, and ray sublineage cells in the male, to be the major focus of rnt-1 expression. However, we did notice a low level of embryonic lethality associated with rnt-1 RNAi and in the presumed null allele os58. The reason for this low level of embryonic lethality is not clear. Perhaps rnt-1 acts partially redundantly with some other factor in embryos. We also found mt-1 to be transiently expressed in body wall muscle nuclei from late embryogenesis until the end of L2, but this expression does not appear to be associated with any obvious muscle function. Using a rescuing GFP reporter construct, we did not see rnt-1 expression in any other cell type. In particular, we did not see expression in hyp7 nuclei, suggesting that rnt-1 expression is switched off in cells derived from the seam, whose fate is to fuse with hyp7, and therefore to stop dividing.

**rnt-1 overexpression drives ectopic cell divisions**

Loss of rnt-1 function is associated with a failure of seam cell proliferation, whereas overexpression of rnt-1 drives seam cell hyperplasia, indicating that rnt-1 can function as a rate-limiting regulator of cell proliferation. It is noteworthy that seam cell hyperplasia occurs only if rnt-1 is overexpressed in L2 or L3. This is consistent with the normal expression pattern of rnt-1 in seam cells becoming fainter from L2 onwards, suggesting that seam cell lineages may not be able to cope with high level rnt-1 expression after this stage. High level expression during L2 and L3 is therefore sufficient to drive inappropriate cell divisions, leading to seam cell hyperplasia. Perhaps during L4 there are other constraints on cell proliferation, rendering seam cells recalcitrant to high-level rnt-1 expression during late larval development.

Overexpression of rnt-1 was not found to be associated with hyperplasia in other cell types. The tissue and stage specificity of RNT-1-induced hyperplasia suggests that the ability of RNT-1 to drive cell proliferation may be limited by the expression of some co-factor or of some other proliferation 'licensing factor'. Runx genes have been shown to associate with a binding partner, CBFβ, in other species (reviewed by Coffman, 2003). An orthologue of CBFβ, bro-1, exists in *C. elegans*, but the function and expression pattern of this subunit has not yet been reported. Perhaps if rnt-1 and bro-1 were co-expressed, more widespread hyperplasia would result.

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**Fig. 7.** Suppression of rnt-1 cell division failures by silencing of cki-1. (A) Graph showing the number of seam cells present in adult hermaphrodites (assayed by scm::GFP expression) in wild-type (n=30), rnt-1(ok351) (n=32), cki-1(RNAi) (n=36) and rnt-1(ok351); cki-1(RNAi) (n=31) animals. All strains were in a him-5(e1490) background. cki-1 RNAi causes an increase in seam cell number relative to wild type (P<0.0001). The number of seam cells in rnt-1(ok351); cki-1(RNAi) animals is restored to near wild-type levels (no significant difference compared with wild type, P=0.05). Error bars represent the standard error of the mean. (B) cki-1::GFP expression in the seam cells of L1 larvae. The left hand panel shows the L1 stem cell division pattern of V1-V6 in wild-type and rnt-1(ok351) animals. Seam cells are identified by circles, hyp7 nuclei by squares. Crosses in the lineage diagrams indicate where divisions failed. (i,ii) Worms hatched in the absence of food, kept 24 hours at 20°C then re-fed and the division pattern examined by observing hypodermal and seam nuclei present in late L1 or mid L2 stages. In this situation, the L1 stem cell division failed in rnt-1 mutants 52% of the time (n=84). In wild type, the division pattern was always normal (n=66). (iii,iv) Worms were examined in late L1 after hatching on food, having never been subjected to starvation. Wild-type animals always underwent the normal L1 division (n=60) and rnt-1(ok351) animals displayed the normal division pattern 98% of the time (n=60). The right-hand panel shows cki-1::GFP expression under these conditions, prior to the time of the expected L1 stem-cell division. Individual seam cells are labelled. The increased cki-1::GFP expression observed in rnt-1 L1 larvae hatched in the absence of food, compared with wild-type animals subjected to the same treatment, was consistently observed (n=30). Scale bars: 20 μm.
Conservation of Runx function

Runx genes have previously been characterised from a variety of metazoan organisms (reviewed by Coffman, 2003). In mammals there are three members of the family and they appear to have lineage-specific functions. Runx1 (also known as AMLI, PEBP2aB and CBFA2) is required for definitive haematopoiesis (reviewed by Okuda et al., 2001). Recently, it has also been shown that Runx1 is required for the proliferation of early developing thyocytes (Sato et al., 2003). Clinically, Runx1 is strongly associated with human leukaemia. Indeed, Runx1 is one of the genes most frequently deregulated in leukaemia through different mechanisms involving translocation, mutation and amplification (reviewed by Roumier et al., 2003). Runx1 has been shown to actively drive cultured mammalian cells from G1 into S phase (Strom et al., 2000), and overexpression of Runx1 in NIH3T3 cells has been reported to lead to neoplastic transformation (Kurokawa et al., 1996). Runx1 has been regarded in the literature both as a tumour suppressor and as a proto-oncogene (Cameron and Neil, 2004; Coffman, 2003).

An intriguing link between the function of rnt-1 in C. elegans seam cells and the function of Runx1 in haematopoiesis is that both of these developmental lineages have stem cell-like properties: relatively undifferentiated cells continue dividing (self renewal), throwing off daughter cells that can undergo terminal differentiation into particular cell types. Perhaps a particular kind of signal, involving Runx genes, must be generated in stem cell-like lineages to promote proliferation. Runx2 and Runx3 also appear to function in developmental lineages with stem cell-like properties. Runx2 mice fail to undergo osteogenesis, dying shortly after birth (Komori et al., 1997; Otto et al., 1997). In humans, Runx2 (also known as Cbfa1) is commonly associated with the congenital bone malformation cleidocranial dysplasia (Otto et al., 2002). Runx3 is required in mice for neurogenesis of the dorsal root ganglia (Inoue et al., 2002; Levanon et al., 2002). Runx3 has also been reported to be involved in controlling growth of the gastric epithelium in mice, and has been associated with human gastric cancers where it appears to act as a tumour suppressor gene (Li et al., 2002).

Overall, it has been suggested that Runx genes are required to maintain the balance between cell proliferation and differentiation in a variety of developmental contexts (reviewed in Coffman, 2003). One of the most important controls concerns the regulation of cell number. Too few cells in a given lineage and formation of a particular structure will not be possible, too many cells and an aberrant structure or a tumour may form. rnt-1 in C. elegans is part of the control that ensures developmental outputs are composed of the appropriate number of cells. In other systems, deregulated cell proliferation is at the heart of carcinogenesis.

It is noteworthy that the C. elegans genome contains only one Runx gene, whereas most other genomes so far examined contain multiple Runx orthologues. This makes C. elegans an organism of choice for the further study of this important family of transcription factors. Perhaps rnt-1 in C. elegans represents the primitive Runx function, promoting cell proliferation in developmental lineages where choices must be continually made between proliferative and differentiative division patterns.

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