The *C. elegans* RUNX transcription factor RNT-1/MAB-2 is required for asymmetrical cell division of the T blast cell

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Abstract

The RUNX genes encode conserved transcription factors, which play vital roles in the development of various animals and human diseases. *Drosophila runt* is a secondary pair-rule gene, which regulates embryo segmentation. Human RUNX1, previously known as AML1, is essential for hematopoiesis. *C. elegans rnt-1* is co-orthologous to the human RUNX genes. We found that RNT-1:GFP is expressed in the H0-2, V1-6, and T blast cells in the embryo, and predominantly in the seam cells during larval to adult stages. *rnt-1* mutants exhibit a loss of polarity in the asymmetrical T cell division in hermaphrodites and abnormal ray morphology in the male tail. Genetic and molecular analysis revealed that *rnt-1* is allelic to *mab-2*. Mutant analysis suggested that *rnt-1/mab-2* is involved in regulating T blast cell polarity in cooperation with the Wnt signaling pathway. Expression studies of GFP:POP-1 and TLP-1:GFP reporters in *rnt-1/mab-2* mutants indicated that this gene functions upstream of *tlp-1* and downstream, or in parallel to, *pop-1* in the genetic cascade that controls asymmetry of the T cell division. All our data suggest that RNT-1/MAB-2 functions with POP-1 to control the asymmetry of the T cell division.

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Introduction

The RUNX family of transcription factors is known to be intimately involved in controlling both proliferation and differentiation during the development of metazoans (Coffman, 2003). The RUNX genes share a region, termed the Runt domain (Kagoshima et al., 1993; Ogawa et al., 1993), which is responsible for specific DNA binding and heterodimerization with its partner subunit, PEBP2β/CBFβ/brother (Kagoshima et al., 1993; Kamachi et al., 1990; Li and Gergen, 1999; Ogawa et al., 1993; Wang et al., 1993). The founding gene of the RUNX family is *Drosophila runt*, which was initially identified as a primary pair-rule gene involved in establishing the pattern of embryo segmentation (Gergen and Butler, 1988). This gene also plays an important role in sex determination and neural development (Duffy and Gergen, 1991; Duffy et al., 1991). lozenge is the second RUNX gene in *Drosophila*, controlling eye development and hematopoiesis (Canon and Banerjee, 2000; Daga et al., 1996). In mammals, there are 3 types of RUNX genes: RUNX1 (AML1/PEBP2αA/CBFαA) (Bae et al., 1994), RUNX2 (AML3/PEBP2αB/CBFαB) (Miyoshi et al., 1993; Ogawa et al., 1993; Wang et al., 1993), and RUNX3 (AML2/PEBP2αC/CBFαC) (Baehner et al., 1995). RUNX1 is known as the most frequent target of chromosomal translocations associated with leukemia (Miyoshi et al., 1993). Analysis of RUNX1 knock-out mice revealed that RUNX1 is essential for the establishment of definitive hematopoiesis (Okuda et al., 1996; Takakura et al., 2000; Wang et al., 1996). RUNX2 has been shown to be a critical transcription factor for skeletal ossification (Komori et al., 1997); and RUNX3 controls axonal
projection in the dorsal root ganglion and differentiation of the gastric epithelial cell (Inoue et al., 2002; Li et al., 2002). The RUNX family proteins show versatile functions in different situations: (1) their expression is observed in a variety of organs, which appear functionally unrelated to each other (i.e. blood, bone, epithelia, and neuron); (2) their mutation affects various biological processes (i.e. segmentation, sex determination, organ development, and maintenance); (3) depending upon the context of cell-type, developmental stage, gene organization, and physiological or experimental conditions, the RUNX proteins exhibit both transcriptional activation and repression activities. As a result of their dual function, the RUNX proteins are known as both proto-oncogenes and tumor suppressors in mammals (Ito and Miyazono, 2003). RUNX protein activity is modified by at least two signaling pathways. RUNX2 regulates bone development by interacting with SMAD transcription factors, which mediate TGFβ/BMP signaling (Carey, 1998; Hanai et al., 1999; Ji et al., 2004). RUNX1 is an important interacting partner for the TCF/LEF-1 transcription factor to transduce the Wnt signaling that controls blood cell proliferation and differentiation (Carey, 1998; Giese et al., 1995; Osato et al., 1999).

In C. elegans, Wnt signaling controls the polarity of cell division in early embryo and certain blast cells. In particular, asymmetrical cell division of the T blast cells is regulated by LIN-44/Wnt (Herman et al., 1995), LIN-17/Frizzled (Sawa et al., 1996), and POP-1/TCF/LEF-1 (Herman, 2001). In normal development, the anterior daughters of the T cells (T.a) produce primarily hypodermal cells, while the posterior daughters (T.p) generate neural cells. However, mutations of Wnt signaling genes disrupt asymmetrical cell division of the T cells. In the lin-44 mutants, the polarity of the T cell division is reversed; T.a produces neural cells and T.p produces hypodermal cells (Herman and Horvitz, 1994). In the lin-17 mutants and pop-1 RNA interference (pop-1(RNAi)) animals, the T cells undergo symmetrical cell division; both T.a and T.p produce hypodermal cells (Herman, 2001; Sternberg and Horvitz, 1988).

Here, we report that rnt-1 (runt-related), the only C. elegans ortholog of the RUNX genes, is involved in regulating asymmetrical cell division of the T cells. Expression studies using RNT-1:GFP fusion constructs in transgenic C. elegans demonstrate that rnt-1 gene is predominantly expressed in the H0 cells and in the postembryonic blast cells, the H1, H2, V1-6, and T cells. In the rnt-1 mutant, the T cells lose their polarity and divide symmetrically in hermaphrodites, and in males, the tail structure is disrupted. Genetic and molecular analyses revealed that rnt-1 is identical to mab-2, which was previously isolated from an abnormal male phenotype. Phenotype analysis of rnt-1 and the Wnt signaling mutants demonstrates that rnt-1 has a genetic interaction with this signaling pathway. Expression studies of the T.a and T.p marker genes, pop-1 and tlp-1, indicate that rnt-1 functions upstream of tlp-1 and downstream, or in parallel to, pop-1 in the genetic cascade controlling asymmetrical cell division of the T cell. Based on these results, we will discuss about the molecular function of RNT-1/MAB-2 in the asymmetric T cell division.

Materials and methods

Isolation of rnt-1/mab-2 strains

mab-2/rnt-1 alleles were isolated from trimethylpsoralen/ultraviolet (TMP/UV) mutagenesis screens (Gengyo-Ando and Mitani, 2000) and Ethyl Methanesulfonate (EMS) mutagenesis screens (Sawa et al., 1996). The sequences of mutant alleles were determined from PCR-amplified genomic fragments.

Reporter gene constructs and transgenic animals

To generate phK192, a 12.9-kb fragment of cosmid B0414 (3933–16797 nt) was amplified with primers, rnt-3 (ectGATCCTcggccgggttctttcct) and rnt-8 (ctaacGATCCTcggccgggttctttcct), and cloned into the Kpn1–BamH1 fragment of GFP reporter vector pDP9579. All the exon sequences of the rnt-1 in phK192 were confirmed by DNA sequencing. Germline transformation was performed by coinjecting reporter DNA at a concentration of 20–50 ng/µg and marker DNA at a concentration of 20 ng/µg into the gonad of animals (Mello et al., 1991). We used the following constructs for transgenic markers: pRF4; containing rol-6(su1006) for selecting a rolling phenotype (Mello et al., 1991), pMH86; containing dpw-20(+); for rescuing dpw-20(e2017) (Han and Sternberg, 1991), myo-2::GFP; containing the myo-2 promoter fused to GFP for selecting worms with GFP in the pharynx (T. Ishihara personal communication). Multiple, independent lines were established for each construct.

To examine the expression pattern of rnt-1, a transgenic strain carrying phK192 and pRF4 was irradiated with ultraviolet light for the chromosomal integration of the extrachromosomal array. The integrated strains, YK114: msls114[pKHK192, pRF4] and YK115: msls115[pKHK192, pRF4], were backcrossed twice with wild-type strain N2. For the expression analysis of rnt-1 reporter under the Wnt signaling mutation background, YK114 and YK115 were crossed with lin-17(n3091)I and lin-44(n1792), resulting in strains YK116: msls114; lin-17(n3091)I, YK117: msls115; lin-17(n3091)I, YK118: msls114; lin-44(n1792) and YK119: msls115; lin-44(n1792). All the expression analyses were performed with these integrated strains. The animals were mounted on a 2% agarose pad containing 10 mM sodium azide as an anesthetic and examined by using a Zeiss LSM510 confocal microscope.

Worm strains


Dfy and Psa phenotype analysis

The Dfy (dynein-filling defect) phenotype analyses were performed in the same way as the FITC staining previously described (Hedgecock et al., 1985). Live animals on NGM plates were washed out with water and transferred to a test tube. The animals were incubated for 30 min at 20°C in water containing 10 µg/ml 1,1'-diodooctadecyl-3,3',3'-tetramethlyindocarbocyanine perchlorate (DiI). After staining, the animals were transferred to NGM plates without dye for more than 60 min for destaining of the gut. Stained worms were viewed by fluorescence microscopy using a rhodamine filter. We counted L2 to young adult animals with stained amphid neurons, which served as an appropriate control for the staining procedure.
The \textit{Psa} (plasmid socket \textit{absent}) phenotype was determined by observing the morphology of the nuclei in the cell on each side just anterior to \textit{hyp}8 and \textit{hyp}11 (Sawa et al., 2000). In wild-type animals, this cell shall be a plasmid socket cell, which has a neural morphology (a small nucleolus with granular nucleoplasm). In animals that show the \textit{Psa} phenotype, this cell has a hypodermal morphology (a large nucleolus with smooth nucleoplasm).

\textbf{Mating assay}

Two transgenic lines, \textit{msEx359} and \textit{msEx360}, were obtained by injecting pHK192 and \textit{myo-2}:GFP into YK102, \textit{him-8(e1489); rnt-1(tm388)}. Mating assays were performed with five males and two hermaphrodites of CB3775, \textit{dpy-20(e2017)}, in five 3.5-cm NGM plates for each transgenic line (total ten mating plates) (Hodgkin, 1980). As a negative control, we also tested animals that had lost the transgene (without GFP in pharynx) from the \textit{msEx359} and \textit{msEx360} strains. As a positive control, \textit{him-8} animals were assayed on 15 mating plates. We counted the plates with non-Dpy hermaphrodites as successful mating experiments.

\textbf{Results}

\textit{rnt-1} is identical to \textit{mab-2}, whose mutations result in male tail abnormality

The \textit{rnt-1} (runt-related) gene was previously identified as the only \textit{C. elegans} homolog of the RUNX family genes (accession #: AB027412, (Bae and Lee, 2000)). The members of the RUNX family share a 128 amino acid domain, termed the Runt domain, which is an evolutionarily conserved DNA binding and protein–protein interaction domain (Fig. 1) (Kagoshima et al., 1993, 1996; Ogawa et al., 1993). To understand the role of \textit{rnt-1} in \textit{C. elegans} development, we isolated a deletion strain \textit{rnt-1(tm388)} by using a trimethylpsoralen/ultraviolet (TMP/UV) mutagenesis screen (Gengyo-Ando and Mitani, 2000). \textit{rnt-1(tm388)} has a deletion spanning from intron 3 to intron 4, resulting in a frame shift and a premature stop codon in the Runt domain (Fig. 1). Molecular data indicate \textit{rnt-1(tm388)} is a null mutation. The \textit{tm388} transcript lacks the C-terminal half of the RNT-1 coding sequences, including residues critical for DNA recognition in the Runt domain. \textit{rnt-1(tm388)} homozygous animals were viable and showed no gross morphological defects in hermaphrodites. However, the \textit{rnt-1(tm388)} males showed striking phenotypes in which many copulatory rays in the tail were lost and the overall structure of the male tail was often destroyed (Fig. 2). \textit{rnt-1(tm388)} males failed to mate with hermaphrodites (Table 1). According to physical and genetic maps, the \textit{rnt-1} locus was located close to \textit{rnt-1} (male abnormal), which showed an abnormal male phenotype (Hodgkin, 1983). The \textit{rnt-1(tm388)} mutation failed to complement the male abnormality of \textit{mab-2(e1241)}, suggesting that \textit{rnt-1} could be identical to \textit{mab-2}.

To identify the molecular lesions in four other existing \textit{rnt-1/mab-2} alleles, we amplified genomic DNA from mutant animals by PCR and determined the sequences of the coding regions. We found sequence alterations in the \textit{rnt-1} loci of all four alleles (Fig. 1B). The reference allele, \textit{mab-2(e1241)}, is a missense mutation that changes an amino acid from Ile to Lys within the Runt domain (Fig. 1B). \textit{mab-2(os58)} is a nonsense mutation that changes the TGG (Trp) codon to a TAG (amber) codon. \textit{rnt-1(tm491)} deletes a region from intron 3 to intron 4, which produces the same transcript to that of the \textit{rnt-1(tm388)} mutation. \textit{mab-2(os11)} is a large deletion allele, which lacks not only the \textit{rnt-1} locus, but also a neighboring locus, \textit{cpb-3} (cytoplasmic polyadenylation element binding protein). These data demonstrated that \textit{rnt-1} is identical to \textit{mab-2}. Thus we hereafter refer to \textit{mab-2} as \textit{rnt-1}. Because all of the mutants, except for the \textit{e1241} allele, invariably harbor functionally destructive alterations in the Runt domain, they may well be regarded as null alleles. These null \textit{rnt-1/mab-2} alleles were viable in our analyses. Although it was previously claimed that a deletion mutant lacking the entire \textit{rnt-1} locus was lethal (Lee et al., 2004) and \textit{rnt-1(RNAi)} caused embryonic lethality (~5%) (Nam et al., 2002), the viability of the \textit{rnt-1(oc351)} strain was shown by the same group. (Ji et al., 2004). The viability of the \textit{rnt-1} mutations is also supported by the \textit{rnt-1(RNAi)} analyses by six independent groups, including our own (Fraser et al., 2000; Maeda et al., 2001; Rual et al., 2004; Sonnichsen et al., 2005 and R. Nimm, A. Woollard, personal communication).

In accordance with the earlier observation made on the original \textit{mab-2} mutant (Hodgkin, 1983), all the newly isolated \textit{rnt-1/mab-2} mutants showed no gross phenotypic defects in hermaphrodites. However, it was also noted that \textit{mab-2(e1241)} showed abnormal hypodermal divisions of the V and T cells in hermaphrodites as well, and hence should be taken to represent a lineage mutant, rather than a male-specific one (Hodgkin, 1983). We subsequently directed our efforts to explore the potential function of \textit{rnt-1} as a regulator of the T cell division using hermaphrodites, which is easier to handle experimentally than males.

\textbf{RNT-1::GFP is predominantly expressed in the seam cells from embryo to adult stages}

To investigate the expression pattern of \textit{rnt-1}, we generated a reporter construct pHK192 in which the entire \textit{rnt-1} locus was fused to the Green Fluorescent Protein (GFP) gene at the carboxyl terminal of its coding sequences (Fig. 1A) (Chalfie et al., 1994). This construct was able to rescue all the \textit{rnt-1} mutant phenotypes, demonstrating that this full length \textit{RNT-1::GFP} fusion protein is functional (see below). At the pre-comma stage, at about 260 min of embryonic development, \textit{RNT-1::GFP} expression began in the nuclei of the seam cells (H0-2, V1-6 and T cells in Fig. 3A) (Sulston and Horvitz, 1977). At the comma stage, at about 400 min, expression also appeared in the body wall muscle cells (Fig. 3B). At the L1 larval stage, \textit{RNT-1::GFP} expression was observed in the seam cells, the body wall muscle cells, and occasionally in the intestinal cells (Fig. 3C). The H1, H2, V1-6, and T cells are post-embryonic blast cells, which divide with a typical stem cell-like division pattern. They undergo several rounds of asymmetrical cell division, producing a self-renewing cell and a differentiating cell (Fig. 4A). Interestingly, in the H, V, and T cell lineages, \textit{RNT-1::GFP} expression was always observed in the seam.
(self-renewing) cells, but not in the hypodermal syncytial (differentiating) cells, from L1 to adult stages (Fig. 3C). In addition, we observed RNT-1 expression in the descendants of the M blast cell from late L1 to adult stages. The expression began after two rounds of M cell division, and continued in the body wall muscle descendants (data not shown). We have not characterized the expression in the intestinal cells and the body wall muscle cells in detail, since we did not observe any defects in these tissues in rnt-1 mutants.
We examined RNT-1::GFP expression in the T cell descendants in further detail. RNT-1::GFP was strongly expressed in the T cells until just before the initiation of mitosis (Fig. 3D). The expression was diminished in prophase and not visible from metaphase to telophase of the T cell division (Fig. 3E). The expression was not detectable in either daughter of the T cells, T.a and T.p (Fig. 3F), but reappeared in the posterior daughters of the T.a cells, T.ap, several hours after the T.a cell division in late L1 stage. After this stage, RNT-1::GFP expression was exclusively observed in T.app and T.appa, but not in either of the other T.ap descendants or any T.p descendants (Fig. 3G). We tried to observe RNT-1::GFP expression in T.a and T.p by anti-GFP antibody staining to enhance the GFP signal intensity, and by cultivating worms at 15°C for accumulation and maturation of GFP in T.a and T.p. However, we were not able to detect the RNT-1::GFP expression in T.a and T.p. We thus conclude RNT-1::GFP is absent in the T.a and T.p cells. Overall, these results revealed that RNT-1::GFP is specifically expressed in the seam cell lineage of the T cell descendants (Fig. 4A).

Fig. 2. Male tail in rnt-1 mutants. (A) In wild-type males, the nine pairs of sensory rays are observed. (B, C) In rnt-1(tm388) males, several to all pairs of lay neurons are lost in the tail, and often overall structure of the male tail is destroyed.

We examined RNT-1::GFP expression in the T cell descendants in further detail. RNT-1::GFP was strongly expressed in the T cells until just before the initiation of mitosis (Fig. 3D). The expression was diminished in prophase and not visible from metaphase to telophase of the T cell division (Fig. 3E). The expression was not detectable in either daughter of the T cells, T.a and T.p (Fig. 3F), but reappeared in the posterior daughters of the T.a cells, T.ap, several hours after the T.a cell division in late L1 stage. After this stage, RNT-1::GFP expression was exclusively observed in T.app and T.appa, but not in either of the other T.ap descendants or any T.p descendants (Fig. 3G). We tried to observe RNT-1::GFP expression in T.a and T.p by anti-GFP antibody staining to enhance the GFP signal intensity, and by cultivating worms at 15°C for accumulation and maturation of GFP in T.a and T.p. However, we were not able to detect the RNT-1::GFP expression in T.a and T.p. We thus conclude RNT-1::GFP is absent in the T.a and T.p cells. Overall, these results revealed that RNT-1::GFP is specifically expressed in the seam cell lineage of the T cell descendants (Fig. 4A).

rnt-1 mutants are defective in the T cell lineage pattern

We next investigated whether and how rnt-1 mutations would affect the proliferation and differentiation of the T cells. In wild-type hermaphrodites, the T cells divide asymmetrically during the L1 stage. The anterior daughter of each T cell (T.a) produces four hypodermal cells and one neuron, whereas each posterior daughter (T.p) generates five neural cells, including the phasmid socket cells (Fig. 4). In most cases, defects in asymmetrical T cell division causes abnormal differentiation of the T.p sublineage, which is easily monitored by the Psa (phasmid socket absent) phenotype analysis (Sawa et al., 2000). The abnormality can be also detected by the Dyf (dye-filling defect) phenotype of the phasmid neurons (Hedgecock et al., 1985; Perkins et al., 1986; Sawa et al., 2000). We first examined the Psa phenotype in the T cells of hermaphrodites of all the rnt-1 alleles. A large proportion (51%–77%) of the mutants exhibited the Psa phenotype (Table 2). We next performed phasmid dye-filling analysis on hermaphrodites from all the alleles. Again, a large proportion (46%–73%) of the mutants displayed the Dyf phenotype (Table 2). All the defects observed in the rnt-1(tm388) and mab-2(os58) mutants were rescued by the pHK192 construct (Tables 1, 2), confirming that rnt-1 is allelic to mab-2. The defect of rnt-1 mutants in asymmetrical division was also ascertained by a direct cell lineage analysis. In eight out of the eleven T lineages examined in the rnt-1(tm388) animals, the T cell divided symmetrically, giving rise solely to hypodermal cells from both T.a and T.p (Figs. 4B, C). All these data demonstrated that mutations in rnt-1 result in the loss of asymmetry in the T cells and T.p sublineage.

In rnt-1 mutants, although the T cells fail to divide asymmetrically, their seam-lineage descendants appear to keep features of the seam cells; (1) they express the seam cell specific marker scm::GFP (data not shown); (2) they fuse with the anterior seam cells to form the seam syncytium at the L4 stage like in wild-type; (3) they retain an eye-shaped morphology typical to the seam cells until seam syncytium formation as seen in Fig. 5. These data indicated that RNT-1 is required for some of the T cell functions, i.e. the asymmetrical (stem-like) cell division, but not for the seam cell differentiation.

Genetic interactions between rnt-1 and the Wnt signaling pathway

Previous genetic studies have indicated that the asymmetrical division of the T cells in C. elegans is regulated by Wnt signaling...
pathway genes including \textit{lin-44}, \textit{lin-17}, and \textit{pop-1} (Herman, 2001; Herman et al., 1995; Sawa et al., 1996). \textit{LIN-44} is a member of the Wnt signaling family, and \textit{LIN-17} is a member of the Frizzled receptor family. \textit{LIN-17} is thought to act as a receptor for \textit{LIN-44} that specifies the polarity of cell division. \textit{POP-1} is a homolog of the vertebrate TCF/LEF-1 proteins that belong to the high mobility group (HMG) transcription factor family and function as the primary transcriptional regulator transducing the Wnt signal. In contrast to wild-type, the polarity of the T cell division is reversed in \textit{lin-44} mutants: T.a and T.p generate neural cells and hypodermal cells, respectively, rather than vice versa (Herman and Horvitz, 1994; Herman et al., 1995). In \textit{lin-17} mutants and \textit{pop-1(RNAi)} animals, the polarity of the T cell division is lost and the T cells divided symmetrically, producing two daughters that generate hypodermal cells (Herman, 2001; Sawa et al., 1996; Sternberg and Horvitz, 1988).

Symmetrical T cell division observed in the \textit{rnt-1} mutants resembles the phenotype of \textit{lin-17} mutants and \textit{pop-1(RNAi)} animals, suggesting that \textit{rnt-1} might be involved in the Wnt signaling pathway. We therefore analyzed the phenotypes of the double mutants, \textit{rnt-1 lin-44} and \textit{rnt-1 lin-17}, by counting the number of hypodermal cells produced by the T.a and T.p cells at the late L1 stage, as described in (Sawa et al., 2000). The percentage of T cells showing symmetrical cell division was dramatically increased in \textit{rnt-1 lin-44} double mutant animals (96%) compared to the respective single mutants (\textit{rnt-1}, 51%; \textit{lin-44}, 20\%) (Table 3). Notably, there was no case of polarity reversal. With respect to polarity reversal, the \textit{rnt-1 lin-44} double mutant is more similar to the single \textit{rnt-1} mutant than the single \textit{lin-44} mutant. Thus, \textit{rnt-1} appears to be epistatic to \textit{lin-44}. Similarly, the \textit{rnt-1} and \textit{lin-17} double mutations also caused a marked increase in the frequency of symmetrical cell division (88\% against 51\% in \textit{rnt-1} and 72\% in \textit{lin-17}) (Table 3). Furthermore, while both \textit{rnt-1} or \textit{lin-17} single mutants still permitted normal asymmetrical cell division to occur in a certain sizable proportion (\textit{rnt-1}, 28\%; \textit{lin-17}, 22\%), the \textit{rnt-1} and \textit{lin-17} double mutant no longer showed this residual phenotype except for various irregular types of cell division in a small proportion (12\%). Since the mutational effects of \textit{rnt-1} and \textit{lin-17} are similar in pattern and synergistic in action to each other, the two genes may directly or indirectly cooperate to reinforce the proper polarity of asymmetrical cell division.

\textbf{RNT-1 function is required for asymmetrical expression of TLP-1, but not of POP-1}

We examined the effects of the \textit{rnt-1(tm388)} mutation on the expression pattern of two transcription factors, \textit{pop-1} and \textit{tlp-1}, which are necessary for the asymmetry of the T cell division (Herman, 2001; Zhao et al., 2002). In particular, \textit{POP-1} has been identified as functioning downstream of the Wnt signaling pathway, and its asymmetrical distribution in the T cell daughters plays a pivotal role in controlling the polarity of
Fig. 4. T cell lineages in wild-type and rnt-1 hermaphrodites. (A) Wild-type T cell lineage in hermaphrodite. The directions of the cell divisions are shown with anterior to the left and posterior to the right. The hyp7 cells join the hypodermal syncytium. PHso1 and PHso2 are socket cells that support phasmid sensory neurons, PVN, PHC, PWV, and PLN are neurons. Those cells have neural-type nuclear morphologies. The seam cell (se) is a specialized hypodermal cell. x indicates programmed cell death. Green lines indicate the RNT-1 expressing cells. (B, C) T cell lineages in rnt-1(tm388) mutant hermaphrodites at the L1 stage. Eight out of eleven T cell lineages exhibited abnormal (symmetrical) cell division pattern (B), and the remaining three T cell lineages exhibited normal (asymmetrical) cell division pattern (C). We did not necessarily follow the subsequent divisions after cells showed an apparent neural morphology, but we confirmed that they did not produce any hypodermal cells. (D) Simplified cell lineage pattern of the T cells in wild-type and mutants at early L2 stage, which is shown in panel A. In wild-type, each T.a cell generates two hypodermal cells and each T.p cell generates two neural cells. In the rnt-1 mutant, polarity of the T cell division is lost and both T.a and T.p generate two hypodermal cells in the lin-44 mutant, the polarity of the division is reversed, and T.a generates two neural cells and T.p generates two hypodermal cells. In the rnt-1 lin-44 double mutant, the division pattern is similar to that of rnt-1 (also see in Table 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Penetrance of mab-2 defects

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<th>Phasmid dye-filling</th>
<th>Psa phenotype</th>
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Phasmid dye-filling was used as an indicator of normal T-cell polarity. Psa phenotype (see Materials and methods) was scored during the L2 stage.

* For rescue analysis, two and three independent lines, carrying functional fusion-construct pHK192, were tested for mab-2(tm388) and mab-2(os58), respectively.

the T cell divisions. Previous immunofluorescent measurements demonstrated that in wild-type animals, the nuclear POP-1 level is higher in T.a than in T.p (T.a > T.p, Fig. 5F) (Herman, 2001; Zhao et al., 2002). With lesions of the Wnt pathway, this POP-1 polarity is either lost in lin-17 mutants (T.a = T.p), or reversed in lin-44 mutants (T.a < T.p). In the present analysis, we used a GFP:POP-1 reporter to monitor the distribution of POP-1 (Kidd et al., 2005; Siegfried et al., 2004). Since asymmetrical distribution of POP-1 is critical for timing immediately after the T cell division, we examined the worms whose T cells had just divided. In wild-type, the level of GFP:POP-1 was higher in T.a (T.a > T.p) in 100% of the wild-type T cell lineages examined (Figs. 5A, E). In rnt-1, most of the T cell lineages (91%) exhibited a higher level of GFP:POP-1 in T.a (T.a > T.p), and the rest of the lineages (9%) showed the same level of expression in T.a and T.p (T.a = T.p) (Figs. 5B, E). These results indicate that rnt-1 exhibits almost no effect on the asymmetrical distribution of POP-1 immediately after T cell division. Nevertheless, in rnt-1 mutants, we also observed that POP-1 asymmetry became obscured near the next round of cell division (data not shown), suggesting that rnt-1 might be involved in the maintenance of POP-1 asymmetry in T.a and T.p cells. Since the POP-1 asymmetry in the rnt-1 mutant was normal in newborn T.a and T.p, rnt-1 defects may not be primarily attributed to an abnormal distribution of POP-1.

*tlp-1* encodes a C2H2 zinc-finger protein that is a member of the Sp family of transcription factors. Its mutation causes defects in the specification of asymmetrical cell fates in T blast cell descendants, resulting in the loss of neural cells (Herman, 2001). *tlp-1* is supposed to function downstream of *pop-1*, because POP-1 polarity is not affected by *tlp-1* mutations (Zhao et al., 2003). TLP-1:GFP is expressed predominantly in T.p (T.a < T.p, Figs. 5C, F). The expression pattern of TLP-1:GFP is reversed in lin-44 (T.a > T.p) and becomes symmetrical in lin-17 (T.a = T.p) (Herman, 2001; Zhao et al., 2002). In the present study, we also confirmed that the level of TLP-1:GFP was higher in T.p (T.a < T.p) in 76% of wild-type animals. In rnt-1(tm388), however, the expression level of TLP-1:GFP is significantly reduced and the proportion showing a normal, asymmetrical expression of TLP-1:GFP
(T.a < T.p) was decreased to 20% (Figs. 5D, E). Taken together, these results indicate that *rnt-1* functions upstream of *tlp-1* and downstream of, or in parallel to, *pop-1* in the genetic cascade that controls asymmetrical T cell division.

**RNT-1::GFP expression in the Wnt signaling mutants**

We used the RNT-1::GFP reporter to see whether the expression of *rnt-1* might in turn be affected by the Wnt signaling mutations, *lin-44(n1792)* and *lin-17(n3091)* (Herman et al., 1995; Sawa et al., 1996). Since RNT-1::GFP expression disappears in both T.a and T.p, we observed the later expression that appears in the four T granddaughter cells at the late L1 stage, starting when RNT-1::GFP expression is resumed in T.ap. We first confirmed that the expression of RNT-1::GFP has resumed in the T.ap cells in most (90%) of the 60 wild-type animals examined (Fig. 6C). In both *lin-17* and *lin-44* mutants, the expression pattern of RNT-1::GFP was indistinguishable from that of wild-type until the middle L1 stage. *rnt-1* appeared to be unresponsive to the Wnt signaling up to this stage. Subsequently, however, *lin-17* and *lin-44* mutants began to show irregular expressions of RNT-1::GFP among the granddaughter cells. In the analysis of the *lin-17* mutation, we examined 50 animals in which the T cell underwent symmetrical cell division giving rise to four hypodermal descendants (Figs. 6 A, C). Only 14 animals (28%) showed a normal pattern of RNT-1::GFP expression (detectable in T.ap alone), whereas the great majority (72% in total) exhibited either aberrant expression in various cell combinations such as T.ap and T.pa or T.aa and T.pa, etc. (38%), or no expression at all in any of the four granddaughters (34%). In no case was RNT-1::GFP detected in T.aa and T.ap that were altered to take neural cell fates.

Notably, the T granddaughter cells expressing RNT-1::GFP in the Wnt signaling mutants had a seam cell (T.ap)-like appearance. The putative linkage between the *rnt-1* expression and the seam cell lineage persisted even when the T cell polarity was lost by *lin-17* or reversed by *lin-44*. These results indicate that Wnt signaling affects *rnt-1* expression after the

---

**Table 3**

<table>
<thead>
<tr>
<th>Cell division patterns of the T cell</th>
<th>WT (%)</th>
<th>Sym (%)</th>
<th>Rev (%)</th>
<th>Others (%)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>&gt;100</td>
</tr>
<tr>
<td><em>mab-2(tm388)</em></td>
<td>28</td>
<td>51</td>
<td>0</td>
<td>21</td>
<td>150</td>
</tr>
<tr>
<td><em>lin-44(n1792)</em></td>
<td>3</td>
<td>20</td>
<td>60</td>
<td>17</td>
<td>98</td>
</tr>
<tr>
<td><em>lin-17(n3091)</em></td>
<td>22</td>
<td>72</td>
<td>2</td>
<td>4</td>
<td>82</td>
</tr>
<tr>
<td><em>mab-2(tm388)</em></td>
<td>1</td>
<td>96</td>
<td>0</td>
<td>3</td>
<td>112</td>
</tr>
<tr>
<td><em>lin-44(n1792)</em></td>
<td>22</td>
<td>72</td>
<td>2</td>
<td>4</td>
<td>82</td>
</tr>
<tr>
<td><em>lin-17(n3091)</em></td>
<td>1</td>
<td>96</td>
<td>0</td>
<td>3</td>
<td>112</td>
</tr>
</tbody>
</table>

The cell division patterns of the T cell were categorized into four classes, (WT) Wild-type, (Sym) Symmetrical, (Rev) Reversed, and Others, by the number of hypodermal cells produced from the T.a and T.p cells at late L1 stage. In each class, the number of hypodermal cells produced from the T.a and T.p cells were indicated by two numbers in the brackets: WT: [2:0], Sym: [2:2], Rev: [0:2], and Others [1:2] (mainly seen in *mab-2*), [2:1] (mainly seen in *lin-44*), [1:1] or [1:0].
first T cell division. However, the expression pattern of RNT-1::GFP was very different from that of POP-1, the primary target of Wnt signaling, suggesting that the regulatory influence of the Wnt signaling pathway on rnt-1 expression must be indirect. The exact mechanism that keeps RNT-1::GFP specifically expressed in the seam cell lineage among T cell descendents, in an on/off cycling manner during larval development, remains an intriguing question.

Discussion

During C. elegans development, asymmetrical divisions take place in many cells, providing a fundamental mechanism for generating diverse cell fates. The postembryonic division and subsequent differentiation of the T blast cells represents one remarkable example. In wild-type worms, the T cells divide asymmetrically, giving rise to a hypodermal seam cell, T.a, and a neural precursor cell, T.p. In rnt-1 mutants, the T cells frequently show symmetrical cell divisions that produce two T.a-like daughter cells, both of which adopt the hypodermal cell fate. The failures in the asymmetrical division of the T cells, and probably of V cells as well, would underlie the tail ray loss phenotype in rnt-1 males, which was originally observed in rnt-1 mutants. All of our studies revealed that rnt-1 is required to ensure that asymmetrical T cell division occurs correctly and efficiently. In the following discussion, we address the potential roles of RNT-1 in regulating asymmetrical stem cell-like divisions, as well as the general biological implications of our results for the RUNX family of genes.

RNT-1 regulates the asymmetry of the T cell division

We have demonstrated that rnt-1 genetically interacts with the Wnt signaling genes, lin-44 and lin-17, which are known to instruct the polarity of the T cell division. Expression studies using GFP::POP-1 and TLP-1::GFP reporters in rnt-1 mutants further suggested that rnt-1 functions upstream of tlp-1 and downstream of, or in parallel to, pop-1 in the Wnt signaling cascade that controls asymmetry of the T cell division. The penetrance of the T cell defects in double mutants of the rnt-1 and Wnt signaling genes was significantly higher than in either of the single mutants, suggesting that rnt-1 functions in parallel with the Wnt signaling pathway (Fig. 7). The failure of asymmetrical T cell division in rnt-1 may suggest that rnt-1 is participating in regulating POP-1 asymmetry in the T daughter cells. However, the asymmetry of GFP::POP-1 expression in T.a and T.p was not affected in the rnt-1 mutants, indicating that rnt-1 is not involved in asymmetric expression of pop-1. Thus, RNT-1 may regulate POP-1 activity asymmetrically in the T daughter cells. However, rnt-1 functions before the T cell division, because RNT-1::GFP expression was diminished at prophase. How can rnt-1 affect POP-1 activity in cells lacking RNT-1?

A simple model is to suppose that RNT-1 might induce another molecule (factor X in Fig. 7), which is required for the T daughter cells (Fig. 7). Factor X might be merely transmitted to the daughter cells. Alternatively, RNT-1 initiates the expression of factor X, which maintains its own expression level by auto-regulation thereafter. Another model is that rnt-1 might mediate a cellular memory mechanism that marks the
target genes that express asymmetrically in the T daughter cells. This memory could be a chromatin modification, which is directly or indirectly induced by the action of RNT-1 and enables the subsequent asymmetrical gene expression. It has been reported that PSA-1 and PSA-4, homologs of the components of the SWI/SNF chromatin remodeling complex, are required for proper T cell division at telophase (Sawa et al., 2000). Alternatively, the memory could be RNT-1 itself. Two mammalian homologs of RNT-1, RUNX1 and RUNX2, have been shown to localize at punctuate subnuclear foci in the interphase nucleus (Zaidi et al., 2004). These Runx foci persist in part through mitosis and undergo a complete restoration in interphase nucleus (Zaidi et al., 2004). These observations suggest that RUNX genes may also play a role in regulating stem cell division in vertebrates similar to the one we demonstrated here for the T cell lineage in C. elegans. In accordance with this possibility, several reports demonstrated that functional Wnt signaling proteins (LIN-44 homologs: Wnt3A and Wnt5A; POP-1 homolog: TCF/LEF-1) are required for normal proliferation and differentiation of hematopoietic stem cells or downstream progenitors (Giese et al., 1995; Reya et al., 2003; van de Wetering et al., 2002). In a more specific context, both RUNX1 and RUNX3 were shown to be essential in their own ways for CD4 silencing in thymocyte differentiation: Runx1 is required for active repression in CD4+CD8− thymocytes, whereas Runx3 is required for establishing epigenetic silencing in cytotoxic CD4+CD8+ lineage thymocytes (Taniuchi et al., 2002, 2004). Furthermore, chromatin remodeling has been implicated in hematopoietic gene regulation (Chi et al., 2002; Kitabayashi et al., 1998). Nevertheless, little is known about the RUNX and Wnt interaction in vertebrate stem cell division. This is because direct analysis of stem cell division is difficult in vertebrate organs. Hence, studies of C. elegans rnt-1 and vertebrate RUNX genes can complement each other and provide new insights into the molecular function of the RUNX genes, both at the level of asymmetrical cell division of stem cells and the cause of RUNX-related diseases.

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References


