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RUNX Regulates Stem Cell Proliferation and Differentiation: Insights From Studies of *C. elegans*

Hiroshi Kagoshima,[1]* Katsuya Shigesada,[2] and Yuji Kohara[1]

[1]Genome Biology Laboratory, National Institute of Genetics, Mishima, Japan
[2]Faculty of Engineering, Doshisha University, Kyoto, Japan

Abstract The RUNX genes encode conserved transcription factors that play vital roles in the development of various animals and human diseases. Recent studies by a few groups including ours have demonstrated that this gene family, as represented by a single ortholog designeated *rnt-1*, also occurs and plays intriguing roles in the simple model organism, *Caenorhabditis elegans.* Our genetic and molecular analyses revealed that *rnt-1* is allelic to *mab-2*, which had previously been known to cause an abnormal development of the male tail. *rnt-1* was further shown to be predominantly expressed in the stem cell-like lateral seam hypodermal cells. These cells are characterized by their abilities to undergo stem cell-like asymmetric divisions giving rise to self-renewing seam cells and various differentiated descendants of hypodermal and neuronal fates. We found that *rnt-1* mutants exhibit an impaired asymmetry in the division of T cells, the posterior-most member of the seam cells. Mutant analysis indicated that *rnt-1* is involved in regulating T blast cell polarity in cooperation with the Wnt signaling pathway. On the other hand, Nimmo et al. independently discovered that *rnt-1* acts as a rate limiting regulator of cell proliferation in the seam cells, V1-6. In this review, we will outline these new findings and discuss their general implications in the mechanism of coordination between proliferation and differentiation of stem cells. J. Cell. Biochem. 100: 1119–1130, 2007.

Key words: *Caenorhabditis elegans*; RUNX; runt domain; asymmetrical cell division; stem cell; proliferation; CBFβ/brother

The balance between two cellular processes, proliferation and differentiation, is a perpetual theme in the development of multicellular organisms. These processes are often thought of as mutually exclusive. However, stem cells provide a fundamental mechanism(s) that coordinates both processes. The most definitive property of stem cells is their capacity to undergo asymmetric divisions, giving rise at each time to one daughter remaining a stem cell and another daughter committed to differentiation (self-renewal maintenance) [Horvitz and Herskowitz, 1992]. Sometimes, they can also divide symmetrically into twins, which retain the stem-cell character (self-renewal expansion), thereby providing a means either to increase their number or to compensate their occasional loss. Stem cells are the essential source of highly differentiated but short-lived cells in any developing or regenerating organs including blood, bone, epithelia, and so on. Thus, the mechanisms regulating when and whether stem cells divide either symmetrically or asymmetrically are of prime importance in determining the size, shape, and functional integrity of multicellular organisms.

The RUNX family of transcription factors has been attracting growing attention as a key player in controlling both proliferation and differentiation in metazoan development [Coffman, 2003]. The RUNX family genes are distributed in all the animal phyla ranging from sponge to mammals but not in protozoa, fungi,

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*Correspondence to: Hiroshi Kagoshima, Genome Biology Laboratory, National Institute of Genetics, Yata 1111, Mishima, Shizuoka 411-8540, Japan.
E-mail: hkagoshi@lab.nig.ac.jp

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and plants as far as examined to date (Fig. 1). The RUNX genes share a highly conserved 128 amino acid domain, termed the Runt domain [Kagoshima et al., 1993; Ogawa et al., 1993], which is responsible for both DNA binding (consensus recognition motif: RACCRCA) and heterodimerization with its cofactor protein, CBFβ/PEBP2β [Kagoshima et al., 1993; Ogawa et al., 1993; Li and Gergen, 1999]. One of the founding genes 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 roles 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 [Daga et al., 1996; Canon and Banerjee, 2000]. *Drosophila* genome project revealed that the fly has two additional *runt*-related genes (CG15455 and CG1379), though their functions remain to be studied. In mammals, there are three types of RUNX genes: RUNX1 (AML1/PEBP2αB/CBFA2), RUNX2 (AML3/PEBP2αA/CBFA1), and RUNX3 (AML2/PEBP2αC/CBFA3) [van Wijnen et al., 2004]. RUNX1 knock-out mice revealed that RUNX1 is essential for the establishment of definitive hematopoiesis [Wang et al., 1996; Takakura et al., 2000]. RUNX2 has been shown to be a critical regulator of skeletal development [Komori et al., 1997]; and RUNX3 controls neuronal development or axonal projection in the dorsal root ganglion [Inoue et al., 2002] and differentiation of gastric epithelial cells [Li et al., 2002]. It is suggestive that vertebrate RUNX genes function in the organs like blood,

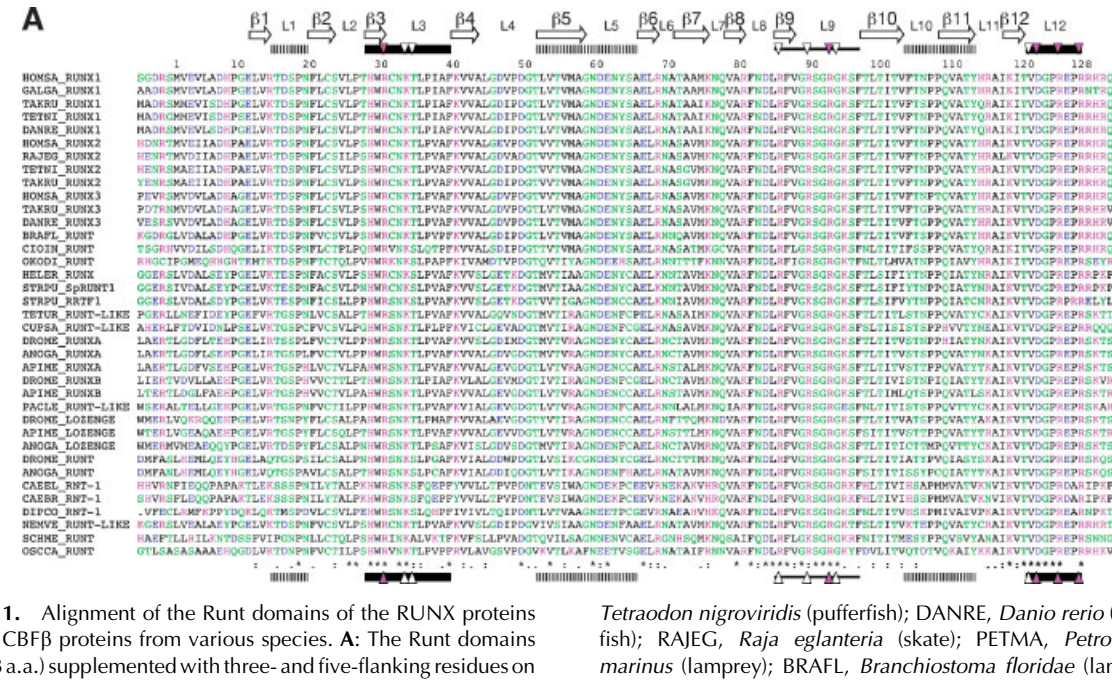

Fig. 1. Alignment of the Runt domains of the RUNX proteins and CBFβ proteins from various species. **A**: The Runt domains (128 a.a.) supplemented with three- and five-flanking residues on the N-terminal and C-terminal ends, respectively. **B**: The CBFβ proteins. The secondary structure profiles determined for mouse RUNX1 and CBFβ [Tahirov et al., 2001] are shown on the top of each corresponding alignment. The α helices and the β sheets are indicated by gray bars and open arrows, respectively. The regions for major and minor groove contacts, and for protein–protein interaction between RUNX1 and CBFβ are shown by thick, thin, and dotted lines, respectively. The amino acids involved in base recognition and phosphate backbone interaction are shown by magenta and white triangles. Note: OIKDI_RRTF1 and OSCCA_RUNT contain extra residues (PI and DI) at the position between 50–51 and 45–46, respectively. Abbreviations: HOMSA, *Homo sapiens* (human); MUSMU, *Mus musculus* (mouse); GALGA, *Gallus gallus* (chicken); XENLA: *Xenopus laevis* (frog); TAKRU, *Takifugu rubripes* (pufferfish); TETNI, *Tetraodon nigroviridis* (pufferfish); DANRE, *Danio rerio* (zebrafish); RAJEG, *Raja eglanteria* (skate); PETMA, *Petromyzon marinus* (lamprey); BRAFL, *Branchiostoma floridae* (lancelet); CIOIN, *Ciona intestialis* (tunicate); OIKDI, *Oikopleura dioica* (tunicate); HELER, *Heliocidaris erythrogramma* (urchin); STRPU, *Strongylocentrotus purpuratus* (urchin); BIOGL, *Biomphalaria glabrata* (mollusk); TETUR, *Tetranychus urticae* (mite); CUPSA, *Cupiennius salei* (spider); DROME, *Drosophila melanogaster* (fluit fly); ANOGA, *Anopheles gambiae* (mosquito); APIME, *Apis mellifera* (honey bee); BOMMO, *Bombyx mori* (silk worm); TOXCI, *Toxoptera citricida* (aphid); PACLE, *Pacifastacus leniusculus* (crayfish); CAEEL, *C. elegans* (nematode); CAEBR; *C. briggsae* (nematode); DIPCO, *Diploscapter coronatus* (nematode); SCHME, *Schmidtea mediterranea* (planaria); NEMVE, *Nematostella vectensis* (sea anemone); HYDMA, *Hydra magnipapillata* (hydra); OSCCA, *Oscarella carmela* (sponge). The sources of these sequences are given in supplemental tables.

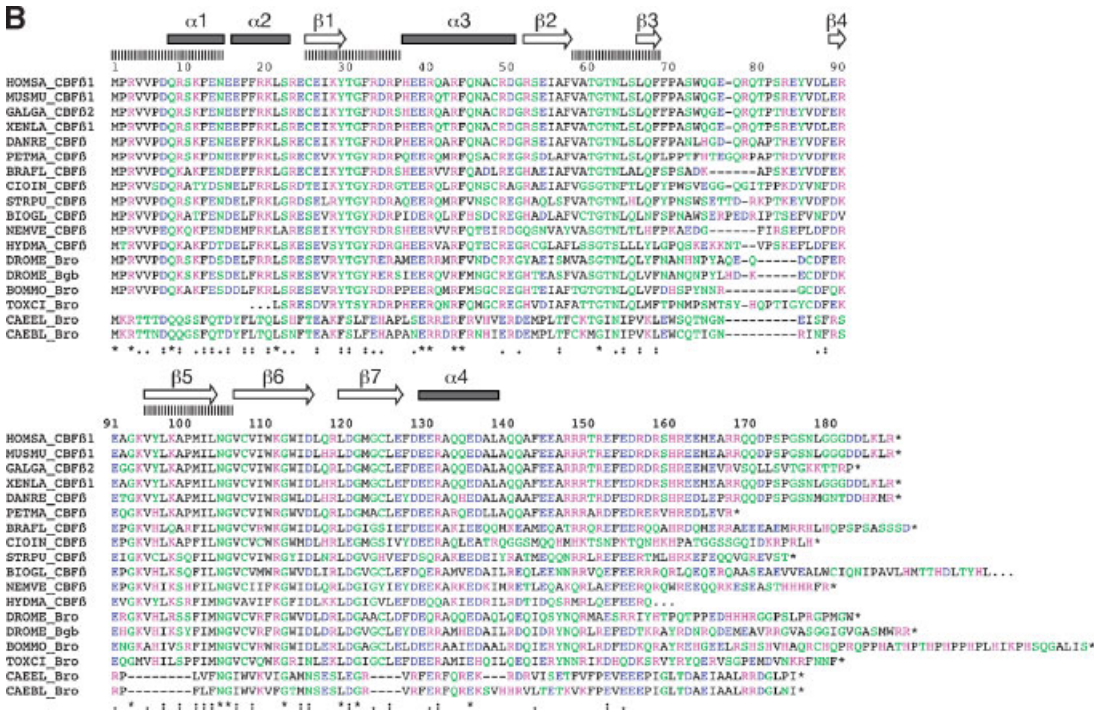

Fig. 1. (*Continued*)

bone, and gastric epithelia, where a continual and active replenishment of differentiated cells from stem cells is particularly important. A RUNX homolog has also been identified and thoroughly characterized in sea urchin [Coffman, 2003].

In mammals, the partner protein CBFβ is encoded by a single gene [Ogawa et al., 1993], whereas *Drosophila* has two homologous genes, *Brother* (*Bro*) and *Big-brother* (*Bgb*) [Golling et al., 1996]. CBFβ itself does not bind to DNA, but forms heterodimer with RUNX proteins to increase their DNA binding activity [Kagoshima et al., 1993; Ogawa et al., 1993]. The phenotypes of CBFβ null mice are nearly identical to those observed with RUNX1 null mice [Okuda et al., 1996; Wang et al., 1996]. Similar to RUNX1, CBFβ is frequently involved in acute myeloid leukemia through its chromosomal translocations to form various chimeric gene products [Ito, 2004]. Recent transgenic studies have demonstrated that CBFβ also required for bone formation in a manner cooperating with RUNX2 [Kundu et al., 2002; Miller et al., 2002; Yoshida et al., 2002]. *Drosophila Bro* and *Bgb* have also been reported to be important for the biological activities of *runt* and *lozenge* [Li and Gergen, 1999; Kaminker et al., 2001]. All these observations support the notion that RUNX proteins and CBFβ/*Bro* function together as evolutionarily conserved, obligatory partners.

Over a decade since the identification of the RUNX family, extensive studies have been done to delineate the functional roles and molecular mechanisms of RUNX in the regulation and pathogenic alterations of development in mammals, *Drosophila* and sea urchin. The results of those studies have been amply documented in several recent reviews [Coffman, 2003; Ito, 2004]. On the other hand, an intriguing problem remains to be explored as to what roles the RUNX family play in lower metazoan species, where its occurrence has been suggested in increasing cases by recent expansions of genome sequencing projects. Since such animals appear to have only a single RUNX gene in most cases, they may provide opportunities to unravel fundamental (ancestral) roles of the RUNX factor in the development and evolution of metazoans. Toward this goal, a few groups including ours have recently started to investigate *rnt-1*, the only ortholog of the RUNX in the simple model organism, *Caenorhabditis elegans* [Ji et al., 2004; Lee et al., 2004; Kagoshima et al., 2005; Nimmo et al., 2005]. These studies have collectively revealed that *rnt-1* has dual roles in coordinating proliferation and differentiation of

lateral hypodermal cells designated the seam cells, which show stem-like division patterns during post-embryonic larval development. First, *rnt-1* facilitates asymmetrical cell division of the tail seam (T) cell during the L1 stage in a manner cooperating with the Wnt signaling pathway [Kagoshima et al., 2005]. Second, it promotes both symmetric and asymmetric divisions of anterior seam cells, V1-6, during the later larval stages so that these processes could occur at the normal timing and frequencies [Nimmo et al., 2005]. In this review, we will mainly focus on these novel findings and discuss their general implications in the mechanism of coordination between proliferation and differentiation of stem cells.

PHYLOGENETIC COMPARISON OF RUNX AND CBFβ PROTEINS

Figure 1A presents alignments of the Runt domain sequences from the RUNX homologs identified or predicted thus far in various animal species. Among them, the *r*u*nt*-related (*rnt-1*) gene was first identified as the only *C. elegans* homolog of the RUNX family by in silico searches against the *C. elegans* genome sequence and subsequently reconfirmed by isolation of its full-length cDNA clones [Kagoshima and Bürglin, 1999; Bae and Lee, 2000]. The Runt domain of the *C. elegans* RNT-1 protein has a high sequence similarity to human RUNX1 (50% identity, 63% similarity) and *Drosophila runt* (53% identity, 65% similarity). The conservation is particularly high amongst residues in certain β sheet (β) and adjacent loop (L) structures, which are required for DNA recognition (β3-L3, β9-L9, and β12-L12) and protein–protein interactions (β5-L5, L10-β11) [Tahirov et al., 2001]. Of note, the residues determined to make direct contacts with DNA are perfectly conserved in *rnt-1* as in all the other RUNX homologs sequenced to date. Thus, RNT-1 is predicted to recognize basically the same consensus DNA motif, RACCRCA, as do well-characterized vertebrate and fly homologs.

The alignments also include RUNX homologs recently found in various animals such as amphioxus, ascidians, planarian, cnidarians, and porifera. Shown in Figure 1B are alignments of CBFβ homologs from phylogenically diverse species similar to those seen in the alignments for the Runt domain. Taken together, these alignments indicate that the RUNX and CBFβ proteins are distributed together in virtually all phyla of metazoans. This suggests that these protein partners would have been intimately associated with, and has some important relevance to, the metazoan evolution from its earliest stage.

rnt-1 IS IDENTICAL TO *mab-2*, WHOSE MUTATIONS RESULT IN MALE TAIL ABNORMALITY

To understand the role of *rnt-1* in *C. elegans*, we first performed genetic analysis using a deletion mutant of *rnt-1(tm388)* as generated by an ultraviolet-trimethylpsoralen (UV-TMP) mutagenesis [Kagoshima et al., 2005]. *rnt-1(tm388)* is predicted to form a functionally inactive protein lacking the C-terminal half of the RNT-1 coding sequences, including residues critical for DNA recognition in the Runt domain. *rnt-1(tm388)* homozygous animals were viable and showed no gross morphological defects in hermaphrodite. However, the *rnt-1(tm388)* males showed striking phenotypes that many copulatory rays in the tail were lost and the overall structure of the male tail was often destroyed (Fig. 2). As a consequence, *rnt-1(tm388)* males failed to mate with hermaphrodites. In this phenotype, *rnt-1(tm388)* is very similar to a known mutant, *m*ale *ab*normal (*mab-2*) [Hodgkin, 1983]. Indeed, *rnt-1(tm388)* failed to complement the male abnormality of *mab-2(e1241)*, indicating that *rnt-1* is identical to *mab-2*. This conclusion was directly confirmed by the demonstration that known alleles of *mab-2* have missense or deletion mutations within the *rnt-1* coding sequence. Similar results were also reported from two other groups [Ji et al., 2004; Nimmo et al., 2005].

Notable here is the previous intriguing report by Nam et al. [2002] that RNAi targeting *rnt-1* caused either embryonic death (5%) or larval death associated with malformation of intestine (20%–30%). However, no or only low embryonic lethality nor any intestinal malformation was observed in similar *rnt-1(RNAi)* analyses done by several other independent groups [Fraser et al., 2000; Nimmo et al., 2005] as well as in mutational analysis with various homozygous *rnt-1* mutants including even a complete null allele (*os11*) [Kagoshima et al., 2005; Nimmo et al., 2005]. The reason for this discrepancy remains unresolved.

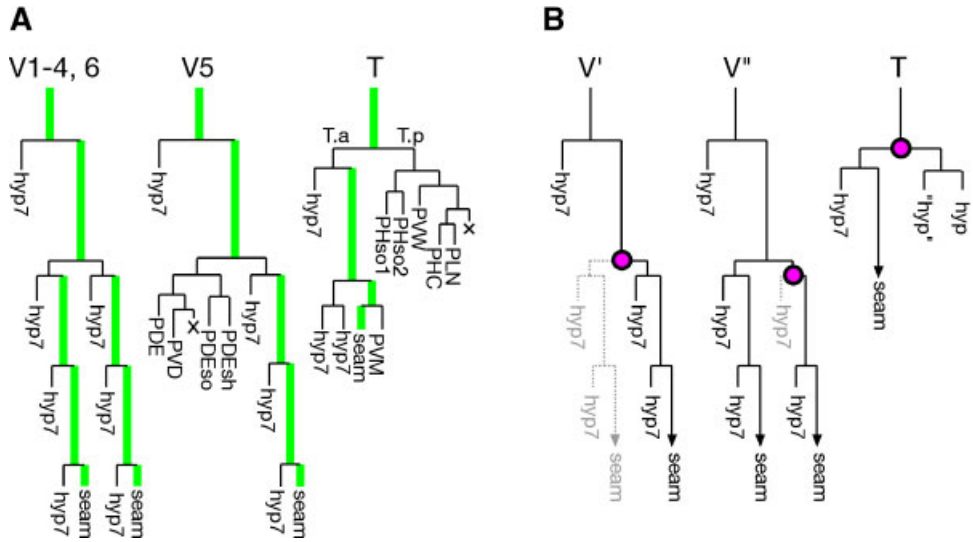

Fig. 2. The seam cell lineages in wild-type and *rnt-1* hermaphrodite *Caenorhabditis elegans*. (**A**) Wild-type V1-6 and T cell lineage in hermaphrodite. The directions of the cell divisions are shown with anterior to the left and posterior to the right. The expression pattern of RNT-1::GFP are indicated in thick lines. The hyp7 cells and the seam cells are hypodermis cells. The hyp7 fuse to form hypodermal syncytium, and the seam cells are specialized lateral hypodermis cells. x indicates programmed cell death. The other cells are neural cells. In the wild-type, the T cells divide asymmetrically and the anterior daughter of each T cell (T.a) produces predominantly hypodermal cells (four hypodermal cells and one neuron), whereas each posterior daughter (T.p) generates five neural cells. (**B**) Typical V and T cell lineages in *rnt-1* mutant hermaphrodites. Abnormal divisions are marked with circles, and missing lineages caused by the mutation are shown in grey hatched lines. The V′ and V″ lineages are representatives of the mutant V1-4 and V6 lineages. In the V′ lineage, a proliferative division failed, causing a reduction in the number of seam and hypodermal nuclei. In the V″ lineage, an asymmetric division failed, leading to loss of a hypodermal nucleus. The T cell underwent an abnormal division, giving rise to two hypodermal (T.a-like) sublineages with a concomitant loss of the neural T.p sublineage. More specifically, the posterior daughter of the T.p cell, T.pp, transformed into hypodermis, whereas the anterior daughter, T.pa, often showed intermediate appearance between neuron and hypodermis (shown as ''hyp'') (H. K. and Woollard unpubl. data). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

rnt-1 IS PREFERENTIALLY EXPRESSED IN THE SEAM CELLS

Earlier studies with *mab-2* male animals clarified that their ray defects result from failures in the seam cells, and that mutant hermaphrodites also share similar defects in seam cell divisions despite their apparently normal gross morphology. The seam cells are post-embryonic epidermal blast cells, which initially consists of H0–2, V1–6, and T as located in single rows on both sides of the worm body. They undergo stem-like asymmetric and symmetric divisions during four larval stages to give rise to extra hypodermal cells, various neuronal assemblages including male-specific ray structures and self-regenerated seam cells. Thus, *rnt-1* as currently equated with *mab-2* must be involved in the regulation of seam cell division and differentiation. Consistent with this notion, reporter analyses using RNT-1::GFP demonstrated that *rnt-1* is preferentially expressed in the seam cells and also some body wall muscle cells [Kagoshima et al., 2005; Nimmo et al., 2005]. Interestingly, RNT-1::GFP expression in the H-, V-, and T-cell lineages was always observed in the seam (self-renewing) cell sublineages, but not in the hypodermal and neuronal descendants.

RNT-1 FUNCTIONS TO CONTROL ASYMMETRICAL CELL DIVISION OF THE T BLAST CELL

In hermaphrodites, the T blast cells undergo multiple, mostly asymmetric, divisions in the L1 and L2 stage (Fig. 2A). It has been well-established that the asymmetrical cell division of the T blast cells in the L1 stage is regulated by the Wnt signaling genes, that is, *lin-44*/Wnt, *lin-17*/Frizzled, *wrm-1*/β-catenin, and *pop-1*/TCF/LEF-1[Herman and Wu, 2004; Takeshita and Sawa, 2005]. With a hint in mind that RUNX proteins can functionally interacts with LEF-1 at mammalian TCRβ enhancer [Giese et al., 1995], we next asked whether *rnt-1* mutations could affect the asymmetric T-cell division. In wild-type animals, the anterior daughters of

the T cells (T.a) produce primarily hypodermal cells, while the posterior daughters (T.p) generate neural cells (Fig. 2B). In the *rnt-1* mutants, the T blast cells failed to divide asymmetrically and generated two T.a-like (hypodermal) cells, resulting in the loss of T.p-derived neural sublineages (Fig. 2B,C) [Kagoshima et al., 2005].

The symmetrical T-cell division observed in the *rnt-1* mutants resembles the phenotypes of *lin-17*/Frizzled and *pop-1*/LEF/TCF-1 defective animals, lending support to our hypothesis that *rnt-1* is involved in the Wnt signaling pathway [Herman and Wu, 2004]. Furthermore, phenotypic analysis of *rnt-1*/RUNX and *lin-44*/Wnt double mutants demonstrated that *rnt-1* is epistatic to *lin-44* in the Wnt signaling cascade. Similarly, the *rnt-1*/RUNX and *lin-17*/Frizzled double mutants showed synergistic failures in the T-cell division. These data suggest that *rnt-1* has a genetic interaction with this signaling pathway [Kagoshima et al., 2005]. We also examined the expression pattern of the target transcription factors of the Wnt pathway, *pop-1*/LEF/TCF1 and *tlp-1*/Sp [Herman and Wu, 2004]. Whereas the asymmetry of GFP::POP-1 was normal in T.a and T.p in *rnt-1* mutants, the asymmetrical expression of TLP-1::GFP was lost in two T daughter cells [Herman and Wu, 2004; Kagoshima et al., 2005]. These data indicate that RNT-1 functions downstream of (or in parallel to) the primary asymmetry determinant, POP-1, and upstream of the T.p (neural sublineage)-specific factor TLP-1, in the Wnt signaling cascade for the asymmetrical T-cell division (Fig. 3). Taken together, our results suggest that RNT-1 regulates asymmetrical cell division of T blast cell by cooperating with the Wnt signaling pathway in *C. elegans*.

RNT-1 ALSO FUNCTIONS TO CONTROL CELL PROLIFERATION IN THE V AND T LINEAGES FROM THE L2 STAGE ONWARDS

Nimmo et al. [2005] have independently carried out systematic lineage analyses of the V and T blast cells in both males and hermaphrodites. They found that the seam cell number is reduced in *rnt-1* animal. At hatching, wild-type *C. elegans* contains 10 seam cells, H0-2, V1-6, and T, on each side of the animal body at hatching (Fig. 2A). During development, these cells divide to generate 16 seam cells in the adult hermaphrodites (Fig. 2B) [Sulston and Horvitz, 1977]. In *rnt-1* mutant, adult hermaphrodites contain fewer (~13) seam cells [Nimmo et al., 2005]. However, the seam cells in *rnt-1* (albeit reduced in number) maintain the correct cell fate, indicating that *rnt-1* is required for cell proliferation, but not for fate determination. Interestingly, detailed cell lineage analyses in the *rnt-1* mutants revealed that the loss of divisions could occur sporadically in various seam lineages in different mutant animals at almost any larval stages except L1 (Fig. 2C). They also showed that overexpression of *rnt-1* in the seam cells driven by heat shock promoter effected a significant restoration or even a slight increase in the number (from 16 to 19–20) of the seam cells in hermaphrodites [Nimmo et al., 2005]. These results suggest that *rnt-1*

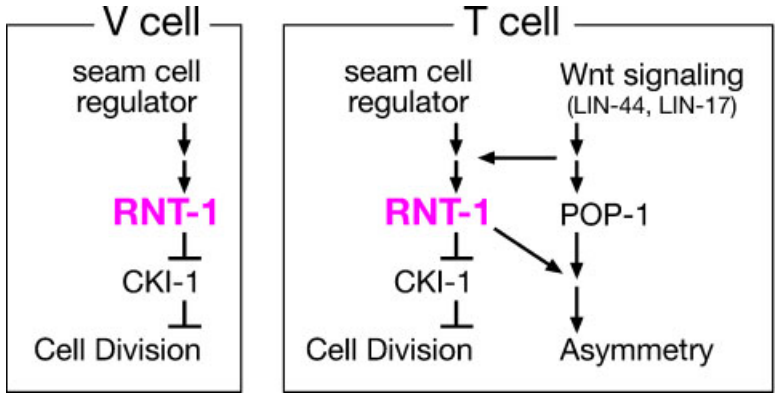

Fig. 3. Genetic models for the regulation of the seam cell division.: In V blast cells, RNT-1 downregulates the expression level of CKI-1, promoting cell division of the seam cells. In T blast cells, in addition to the CKI-1 pathway, RNT-1 is also involved in the Wnt signaling pathway to regulate asymmetrical cell division. In this regulatory cascade, *rnt-1* functions upstream of *tlp-1* and probably in parallel to *pop-1*. Specific and recurrent expression of *rnt-1* in the T and V cells and their seam cell progeny might be regulated by an unknown seam lineage-specific regulatory mechanism. The Wnt signaling is supposed to affect indirectly the spatial pattern of expression of *rnt-1* among the T-cell descendants. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

prevalently stimulates seam cell proliferation from the L2 stage onwards.

They also examined genetic interaction between *rnt-1* and *cki-1* [Nimmo et al., 2005]. *cki-1* encodes a homolog of the mammalian cyclin-dependent kinase (CDK) inhibitor family, KIP/CIP. KIP/CIP CDK inhibitors function to link developmental programmes to cell-cycle progression, acting by inhibiting the activity of the cyclin E/CDK2 complex during G1 [Boxem and van den Heuvel, 2001; Fukuyama et al., 2003]. *cki-1(RNAi)* in wild-type hermaphrodites causes an increase in the seam cell number (from 16 to 20), demonstrating that *cki-1* normally acts to limit seam cell proliferation. Interestingly, when *rnt-1* animals (which normally have reduced seam cell proliferation) are subjected to *cki-1(RNAi)*, the seam cell number is recovered to almost the wild-type level. Furthermore, CKI-1::GFP expression was shown to be upregulated in *rnt-1* mutants, suggesting that RNT-1 may function in G1 to promote seam cell division via the downregulation of *cki-1* (Fig. 3) [Nimmo et al., 2005].

Of another point of interest, the reduced seam cell proliferation in *rnt-1* mutants was consistently accompanied by a small decrease (~5%) in their body size [Nimmo et al., 2005]. This feature is presumed to result from 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]. Another group has also observed a similar, somewhat greater, reduction (~20%) in the body size in *rnt-1* mutant animals, though they failed to notice any change in the seam cell number [Ji et al., 2004]. Instead, they found that the *rnt-1(ok351)* mutant shows synergistic effects with the TGFβ signaling mutants, such as *sma-2*, *sma-3*, *sma-4*, and *sma-6*, which cause marked reductions (~40%) in the body size as well as various male tail abnormalities. Thus, they proposed the view that *rnt-1* acts as a downstream target of the TGFβ signaling pathway in the regulation of the body size and male tail development. However, Nimmo et al. [2005] claimed that *rnt-1* and the TGFβ signaling mutants exhibited different phenotypes in the male tail (ray missing and ray fusion, respectively), being suggestive of their differing mechanisms of function. Further detailed studies would be needed to settle this controversy.

THE PRIMARY TARGET OF ACTION FOR RNT-1 IN THE T-CELL LINEAGE: WHICH COMES FIRST, ASYMMETRY OR PROLIFERATION?

Our model (referred to as "asymmetry model") deduced from T-cell lineage analysis posits that *rnt-1* is involved in asymmetrical cell division, determining the fate of the T daughter cells [Kagoshima et al., 2005]. However, the finding by Nimmo et al. raises the possibility of an alternative explanation (referred to as "proliferation model") that the loss of neural fates in the T.p sublineage in the *rnt-1* mutant might be caused by an irregular arrest of cell division prior to the completion of differentiation, rather than a failure in the asymmetric cell fate execution per se [Nimmo et al., 2005]. However, it should be remarked that the reduced and symmetrized expression of a representative neural marker, TLP-1::GFP, between T.a and T.p in *rnt-1* mutants preceded the actual arrest of cell division as observed with the daughters of the latter, T.pa and T.pp, which normally undergo one or more divisions. Moreover, the loss of neural cell fate in the T.p lineage concomitant with its premature division arrests was also reported to occur in animals mutant for *tlp-1* [Herman and Wu, 2004]. It thus seems likely that the reduction in the TLP-1 activity resulting from the *rnt-1* mutation, rather than the defect of RNT-1 itself, is directly responsible for the reduced division potential of the T.p lineage. On these accounts, we prefer the asymmetry model to the proliferation model as far as the primary regulatory role of RNT-1 is concerned. Nevertheless, the proliferation model may remain valid to understand the role of TLP-1. To further resolve the issue in point, it would be worth asking whether the blocked proliferation and differentiation of the T-cell descendants in *rnt-1* or *tlp-1* mutants could be rescued by *cki-1(RNAi)* as in the above-noted case of V- and T-cell divisions during L2–L4 stages. If the answer would be positive, the proliferation model should be more appropriate than the asymmetry model, and if not, vice versa.

The asymmetry model is obviously unable to explain the *rnt-1*-induced failures of cell proliferation observed with V- and T-cell lineages, in which symmetric divisions (self-renewal expansion) were predominantly affected. Of interest, *rnt-1* had no inhibitory influence on the particular event of asymmetric division in

the V5 lineage that takes place at the L2 stage to generate a neural assemblage, postdeirid [Nimmo et al., 2005]. This asymmetric division is known to be controlled by a different Wnt signaling gene, *egl-20* [Emmons and Sternberg, 1997]. Accordingly, the regulatory linkage of *rnt-1* with asymmetric division may be specific to the T lineage that is positioned at the posterior end of the seam cell row and controlled by *lin-44*.

FUNCTIONAL CONSERVATION IN RUNX-DEPENDENT GENE REGULATIONS

The dual roles the *C. elegans rnt-1* as described above are remarkably concordant with, and provide further insights into, the emerging proposition that the most fundamental and well-conserved role of RUNX genes is to regulate the balance between proliferation and differentiation [Coffman, 2003]. We would like to elaborate this issue in some detail below.

RUNX and Proliferation

In vertebrates, the role of RUNX factors to promote or suppress proliferation has generally been envisaged from the fact that the three known paralogs are required for the generation and maintenance of tissues like blood, bone, and gastric epithelia, in which differentiated cells have to be continually and actively replenished from stem cells. Recent studies begin to provide clues to the molecular mechanisms by which RUNX genes mediate such roles. As one most clear-cut example, RUNX1 has been reported to regulate proliferation of hematopoietic stem cell (HSC) under the Notch signaling pathway [Barnes et al., 2004]. In zebrafish, transient expression of activated form of Notch (NICD: Notch intracellular domain) greatly expanded HSC number. Morpholino oligonucleotide-mediated knock-down of RUNX1 function completely abolished this increase, indicating that the expansion of HSC is dependent on RUNX1. Various markers of stem and progenitor cells, including RUNX1, are upregulated in the NICD-induced HSC population, suggesting that RUNX1 is a downstream target of Notch signaling to regulate the proliferation of HSC. Furthermore, accumulating evidence indicates that RUNX proteins play direct and multimodal roles in proliferation control. RUNX1 and RUNX2 were found to promote cell proliferation by repressing the expression of CDK inhibitor p21Waf1/Cip1 in various tissues including myeloid cells [Lutterbach et al., 2000]. These observations closely parallel the putative repression of *cki-1* by the *C. elegans rnt-1* as mentioned in the preceding section [Nimmo et al., 2005]. RUNX1 was also shown to stimulate G1 to S cell-cycle progression by inducing cyclin D3 gene expression in immature hematopoietic cells [Bernardin-Fried et al., 2004]. On the other hand, RUNX2 has conversely been reported to mediate cell cycle withdrawal in terminally differentiating osteoblasts through induction of another CDK inhibitor, p27KIP1 [Thomas et al., 2004]. In a different mode of growth control, RUNX3 has been implicated in TGFβ-mediated apoptosis of gastric epithelial cells [Li et al., 2002]. As suggested by Ji et al. [2004], a similar functional cooperation between *rnt-1* and the TGFβ shignaling appears to occur in *C. elegans* as well with respect to the body size control (however, see Nimmo et al., 2005 for a cautionary view).

RUNX and Differentiation

RUNX proteins are also essential for cell differentiation in specific tissues as noted above. Mutational or artificial disruptions of RUNX genes generally block differentiation of stem cells or progenitor cells, which is often accompanied by their aberrant proliferations to result in various human diseases: RUNX1, acute myeloid leukemia [Wang et al., 1996; Takakura et al., 2000]; RUNX2, cleidocranial dysplasia [Komori et al., 1997]; RUNX3, gastric cancer [Inoue et al., 2002; Li et al., 2004b]. Extensive studies made thus far have identified or suggested a large number of genes that serve as downstream targets of different RUNX proteins as well as several signaling pathways that control RUNX activity [see, e.g., reviews by Otto et al., 2003; Ito, 2004]. Among them, here we focus attention to information related to the Wnt signaling, with which the *C. elegans rnt-1* was found to cooperate in controlling the asymmetric T-cell division as described above. Transcription factor TCF/LEF-1, the crucial downstream target of the Wnt signaling, has long been known to act as an important interacting partner for RUNX factors in activating the transcription of TCRβ and IFN-β [Giese et al., 1995; Carey, 1998]. More recently, the Wnt signaling pathway has been suggested to play a broader role in promoting the establishment and proliferation of HSCs [Reya et al.,

2003]. These factors were also reported to effect synergic transactivation of the ELA2 neutrophil elastase gene [Li et al., 2004a]. In addition, canonical Wnt signaling has recently been revealed to regulate skeletogenesis by inducing the expression of RUNX2 as its direct downstream target [Bodine et al., 2005; Gaur et al., 2005, 2006]. Taken together, these observations indicate that the regulatory linkage between the Wnt signaling and the RUNX family is well-conserved in diverse regulatory contexts from at least nematodes to mammals. Furthermore, it would be tempting to speculate that RUNX genes in vertebrate might also play a role in dictating asymmetrical cell division of the stem cells or progenitor cells as in the case of the *C. elegans rnt-1*.

RUNX as a Global Coordinator of Proliferation and Differentiation

In reflection of the composite regulatory functions ascribed to *rnt-1*, its deficiency results in a slight reduction in the body size together with a frequent loss of the chemosensory apparatus, phasmids, for both males and hermaphrodites, being additionally accompanied by defects in the copulatory rays specific to males. In this overall context, *rnt-1* may well qualify its suggested role as a global coordinator of seam cell proliferation and differentiation. The observed defects are rather benign and tolerable for survival of mutant animals per se, so far as they are kept under experimental conditions with ample nutrients and an optimal temperature. This makes an apparent contrast with the well-established indispensability of the RUNX family genes in higher animals such as *Drosophila* and vertebrates. Nevertheless, we speculate that *rnt-1* could be essential for the long-term survival, reproduction and evolutionary adaptability of the nematode species under natural environmental conditions, because *rnt-1* mutants would be ready to lose competitions with wild-type animals for their inferior physique, mating ability, and chemo-sensitivity. Accordingly, we may well draw commonality and conservation among the RUNX family members from at least nematodes to vertebrates in that they are pivotal for sound and prolific postnatal lives.

An additional interesting feature of the *rnt-1* function in *C. elegans* is that even its complete mutational loss (as effected by *os11*, e.g.) occasionally allows normal patterns of division at low but noticeable rates (20%–30%, on average) in terms of either the differentiation of T cells or the proliferation of seam cells. This means that *rnt-1* acts on these processes in a permissive, rather than instructive, manner. Given such a mode of functionality, the primary significance of *rnt-1*, and perhaps also RUNX genes in vertebrates, may be to fine-tune, but not to directly drive, its multifarious target processes. In this regard as well, the RUNX family appears to fit with the proposed role in globally coordinating proliferation and differentiation.

CONCLUSIONS AND FUTURE PROSPECTS

How can the RUNX proteins coordinate their diverse and often opposing functions? A key to understand this puzzle is to elucidate the downstream target genes of the RUNX proteins. Although scores of such target genes have already been reported thus far [Otto et al., 2003], further extensive efforts toward that goal are warranted to understand the exact mechanisms and biological significances of RUNX proteins in controlling proliferation, differentiation, and asymmetrical cell division. It should provide bases for further elucidation of molecular mechanisms of carcinogenesis in vertebrates. Another key for future study will be to identify the interacting partners of RUNX proteins. RUNX proteins are known to interact with numerous transcription factors, transcriptional co-activators and co-suppressors, such as LEF-1/TCF, Ets-1, AP-1, p53, p300/CBP, Groucho/TLE, etc. RUNX proteins would form complexes with different proteins in different cell lineages. This would be the reason why RUNX proteins act in versatile ways, on both proliferation and differentiation, depending upon cellular contexts. Perhaps RUNX forms a specialized complex for each function, say, differentiation complex, proliferation complex, etc, and each complex would bind to a distinctive combination of cis-regulatory elements including one or more of RUNX compatible sites. Thus, it would be hard to understand their exact functions by looking at RUNX proteins alone. We have to be prepared for daunting challenges to identify and analyze innumerable transcriptional complexes in their entirety.

For such approaches, *C. elegans* will provide unique advantages as a model organism. First, *C. elegans* genome contains only one RUNX

gene, whereas most of other genomes contain multiple RUNX orthologs. Second, in *C. elegans*, cell divisions are almost completely invariant and well characterized [Sulston and Horvitz, 1977], allowing us to analyze the effects of mutations on cell proliferation and differentiation at single cell resolution. Third, *C. elegans* is one of the simplest animal among the RUNX containing organisms during development. *rnt-1* in *C. elegans* would represent the primitive RUNX function, which probably provides clues to understand fundamental roles of RUNX family proteins. *C. elegans* has only 20 seam cells at hatching (36 in adult) and they can be easily isolated, for example, by fluorescence-activated cell sorting (FACS) of primary culture of *C. elegans* embryo carrying seam::GFP reporter transgene. The seam cells can serve to facilitate biochemical screening of downstream gene and interacting partners.

As noted before, *C. elegans* also bears a CBFβ homolog, brother homolog (*bro-1*). Preliminary data from our ongoing analysis of *bro-1* have indicated that its expression pattern as well as its mutational effect are very similar to those observed with *rnt-1*, providing additional evidence for the prevalent evolutionary conservation of obligatory functional interdependency between RUNX and CBFβ (H.K. and A. Woollard, unpublished observation). Further analysis of the RNT-1/BRO-1 complex in *C. elegans* will provide new insights into the mechanisms organizing both proliferation and differentiation in developmental processes.

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