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PROSPECTS

RNT-1 Regulation in *C. elegans*

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Abstract RUNXs are important transcription factors, which are involved in animal development and human carcinogenesis. RNT-1, the only homologue of RUNXs, in *Caenorhabditis elegans* (*C. elegans*) has been identified and viable mutant animals of *rnt-1* gene have been isolated and characterized recently. Genetic analyses using *rnt*-1 mutants have shown that RNT-1 is regulated by TGFβ- and Wnt-signaling pathways in the body size regulation and male tail development. Here, we review our current understanding of RNT-1 functions in these signaling pathways. Furthermore, future prospects of RNT-1 and BRO-1 studies in *C. elegans* are discussed in this review. J. Cell. Biochem. 96: 8–15, 2005. © 2005 Wiley-Liss, Inc.

Key words: Runx; *rnt-1*; *bro-1*; TGFβ; Wnt; body size; male tail abnormality

RUNXs are the α subunits of polyomavirus-enhancer binding protein 2/core-binding factor (PEBP2/CBF) [Ogawa et al., 1993a], which are transcription factors containing a highly conserved Runt domain [Kagoshima et al., 1993]. The RUNX has DNA binding activity, and forms a hetero-dimer with the β subunit, PEBP2β/CBFβ, that enhances the DNA-binding activity of the α subunit [Ogawa et al., 1993b].

In mammal, three *RUNX* genes (*RUNX1, RUNX2* and *RUNX3*) and one *CBF*β gene have been identified. The *RUNX1* was initially named as *AML1* since it had been found to be located at the frequent breakpoints of chromosomal translocations of acute myeloid leukemia patients [Miyoshi et al., 1991]. Because of its implication in acute myeloid leukemia, it has been extensively studied after its first identification. In 1996 *Runx1* knockout mice were generated by several groups revealing physiological functions of *Runx1* [Okuda et al., 1996; Wang et al., 1996]. Knock-out studies of *Runx1* have shown that *RUNX1* is one of the important molecules in regulating hematopoiesis, thereby mouse models for the acute myeloid leukemia have been established. *RUNX2* was identified in 1993 by Ogawa et al. [1993a]. Subsequently, *Runx2*-deficient mice were generated by two groups and it has been shown that *Runx2* plays essential roles in the maturation of osteoblast [Komori et al., 1997; Otto et al., 1997]. Moreover, haplo-insufficiency of *RUNX2* causes cleidocranial dysplasia, which is an autosomal dominant bone disease in human [Lee et al., 1997; Otto et al., 1997]. Identification of the third gene, *RUNX3*, was first reported 10 years ago [Levanon et al., 1994]. The *Runx3* knockout mice showed physiological functions of *Runx3* in three different tissues; gastric epithelium [Li et al., 2002], $CD4^-$ $CD8^+$ T cells [Taniuchi et al., 2002], and dorsal root ganglial neuron [Inoue et al., 2002]. *Runx3* regulates the growth of gastric epithelium [Li et al., 2002], and regulates the survival and development of dorsal root ganglia neuron [Inoue et al., 2002]. The *CBF*β gene was identified and cloned in 1992 [Ogawa et al., 1993b], and the phenotypes of *CBF*β null mice are very similar to the phenotypes observed in *Runx1* knockout mice, indicating that *CBF*β also functions in hematopoiesis [Okuda et al., 1996; Niki et al., 1997]. *RUNXs* and *CBF*β are all essential genes for the development of mouse and malfunctions of them cause human diseases. Therefore, greater attention has been drawn to the studies on the

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in vivo functions of *RUNXs* and *CBF*β genes. However, since all the *Runx* knock-out mice are lethal, studies on *Runx* genes using these mice have been limited. Many researchers in this field are now trying to generate conditional knockout mice.

The existence of *RUNXs* homologue, RNT-1, in *C. elegans* was firstly identified by Bae and Lee [2000]. Originally, they had named this gene as *run*, but it has been recently re-named as *rnt-1* by the standard *C. elegans* nomenclature. The *C. elegans rnt-1* mutants have been isolated by several groups including the *C. elegans* knockout consortium. Surprisingly, the *rnt-1*(*ok351*) deletion mutants show very mild but distinct phenotypes which are small body size and male tail abnormalities [Ji et al., 2004]. These phenotypes are informative and valuable in dissecting genetic interactions of RNT-1 in *C. elegans*. Indeed, genetic analyses of *rnt-1* in *C. elegans* have revealed that RNT-1 is involved in the conserved signaling pathways such as TGFβ- and Wnt-signaling pathway [Ji et al., 2004; Kagoshima et al., personal communication]. Therefore, *C. elegans* might be a promising animal model system in the field of *RUNXs* studies. In this review, we will discuss the advantages of *C. elegans* as a model organism for the RNT-1 studies and recent findings about *C. elegans* RNT-1.

Caenorhabditis elegans AS A MODEL ORGANISM FOR THE RNT-1 STUDIES

Caenorhabditis elegans is a small, free-living soil worm, which belongs to the phylum Nematoda. The phylum Nematoda includes free-living organisms in most terrestrial and marine environments as well as parasitic organisms to a wide variety of plant and animal hosts. The nematode *C. elegans* has been widely used as a model organism to study animal development and behavior. It has many advantages for the genetic analyses such as a large brood size (300–350 progenies from one hermaphrodite), rapid life cycle (3 days), small size (1.5 mm-long adult), and easy cultivability under laboratory conditions.

C. elegans is the first multi-cellular organism to have its genome completely sequenced [The *C. elegans* Sequencing Consortium, 1998]. Its genome size is approximately 97 Mb in total and encodes total of 19,099 proteins. Approximately 40% of predicted protein products are homologous to those of human counterparts. In addition, informative database, mutant stocks (*C. elegans* Genetic Center) and DNA library as well as active communication among *C. elegans* researchers make the *C. elegans* an excellent and promising animal model system.

Two most prominent features of *C. elegans* related to RNT-1 studies are (1) its simple body anatomy and (2) complete cell-lineage of total somatic cells are known. *C. elegans* body is composed of muscle, hypodermis, neuron, intestine and gonad. This simple anatomy may not provide any direct insight for the study of hematopoiesis or bone formation. But, early development of hypodermis and intestine will provide insight for the RUNX studies. The complete cell lineage of *C. elegans* was discovered by Sulston and White [1988]. This invariant cell lineage provides a unique opportunity to study cell fate determination not only during embryogenesis but also during tissue differentiation.

For the studies of RUNX proteins, in particular, *C. elegans* has additional advantages. First, *C. elegans* has only one homologue of RUNXs, RNT-1, and one binding partner, BRO-1, which is a CBFβ homologue [Lee et al., 2004]. Therefore, it would be much simpler to study RNT-1 in *C. elegans* than in other organisms such as mouse and *Drosophila*, which have more than three isoforms of RUNX homologue. Second, *C. elegans rnt-1* mutants show relatively mild phenotypes compared to those of mouse mutants, but they have specific and distinct phenotypes. These phenotypes, which are small body size and mail tail abnormalities, make *C. elegans* more feasible and desirable model system for genetic dissection of RNT-1 function in vivo [Ji et al., 2004]. Third, the conserved signaling pathways such as TGFβ and Wnt signaling are well characterized and conserved in *C. elegans* [Patterson and Padgett, 2000; Herman, 2002]. These pathways have also been shown to regulate RUNXs in mammal [Miyazono et al., 2004]. Fourth, *C. elegans* is a good model system to conduct a genome-wide screening since microarray analyses of total transcripts and genome-wide RNAi for functional assay are possible [Sugimoto, 2004]. Even though RUNXs studies in *C. elegans* have just begun, it has a promising prospect to provide meaningful insights for higher organisms.

IDENTIFICATION OF *RUNXS* AND *CBF-β* HOMOLOG IN *C. elegans*

The *C. elegans* RNT-1 contains a highly conserved runt domain, which is known to have

DNA binding activity and to dimerize with β subunit [Bae and Lee, 2000]. The runt domain of RNT-1 shows approximately 50% similarity in amino acid sequences to that of human RUNXs. Moreover, at the C-terminal region of the runt domain, the conserved motif, "IWRPF" sequence, which is quite similar to mammalian "VWRPY" sequence, is found. This sequence motif is well conserved among all RUNXs and known to be required for binding to transcriptional repressors, TLE (transducin-like enhancer-of-split), which is human orthologue of *Drosophila* Groucho [Levanon et al., 1998]. These sequence similarities suggest that RNT-1 is indeed homologue of RUNXs in *C. elegans*.

The *rnt-1* gene is physically mapped at the center of linkage group I in *C. elegans*, and is located on the cosmid B0414. The genomic structure of *rnt-1* has been shown to be composed of eleven exons which extends over 12.5 kb region [Bae and Lee, 2000]. Several unusual features of *rnt-1* gene are noticed. First, the size of *rnt-1* gene is much greater than the average gene size of *C. elegans* (approximately 5 kb). Second, the third intron of *rnt-1* gene is about 7.2 kb, which is unusually longer than the average intron. The average size of intron is less than 100 bp long in *C. elegans*. Indeed, Nam et al. [2002] have shown that regulatory elements including tissue specific enhancers are located in this intronic region. Third, translation of RNT-1 starts from the fourth exon, which would generate a long 5′-untranslated region (UTR) for *rnt-1* mRNA. However, in *C. elegans* many genes are trans-spliced by the small RNA molecules, SL1 or SL2, therefore the function of this long 5′-UTR of *rnt-1* gene is not understood yet.

The binding partner of RNT-1, CBFβ homolog, in *C. elegans* has been searched but unsuccessful until quite recently. It appears that there is now a CBF-β homolog, BRO-1, in *C. elegans* [Lee et al., 2004], which shows about 18% homology to the mammalian CBFβ and 18% and 14% homology to the *Drosophila* Brother and Big brother proteins, respectively.

EXPRESSION PATTERNS OF RNT-1 AND BRO-1

RNT-1 is expressed in a stage-specific and tissue-specific manner in *C. elegans* [Nam et al., 2002]. From the bean stage of embryogenesis (about 400 min after fertilization) through the L3 stage (the third larval stage), RNT-1 is strongly expressed and translocated to the nuclei of hypodermal seam cells. RNT-1 is also localized in the nuclei of gut cell from the late stage of embryogenesis through the L2 stage (the second larval stage). The highest level of RNT-1 expression in the intestinal cells is detected in the four cells of anterior ring (int-1; the most anterior cells of the intestine) of the intestine. The RNT-1 expression in intestinal cells is greatly diminished in the L3 stage. In addition, RNT-1 is expressed in the ray precursor cells, which eventually differentiates into the male mating structure in the male tail [Ji et al., 2004].

The stage-specific and tissue specific RNT-1 expressions are regulated by the enhancer elements in the third intron [Nam et al., 2002]. As we mentioned, the genomic structure of *rnt-1* is unique in that *rnt-1* has extraordinarily long intron in between the third and fourth exon [Bae and Lee, 2000]. Within this long intron, two regions, 1.7 kb region in upstream and 5.3 kb region in downstream, show enhancer activity for the hypodermal seam cell-specific and intestinal cell-specific expression, respectively [Nam et al., 2002].

Surprisingly, BRO-1::GFP was detected only in the hypodermis throughout development but the GFP signal was not detected in intestine [Lee et al., 2004]. Perhaps, the GFP signal in the intestine was not detect because of its lower level expression. Alternatively, there could be another binding partner of RNT-1 in the intestine, which needs to be confirmed. RNT-1 is found only in nucleus, whereas BRO-1 is localized both in the cytoplasm and nucleus, which is similar to the patterns observed in mammalian cells.

RNT-1 FUNCTIONS IN THE SMA/MAB PATHWAY OF TGFβ SIGNALING

The members of transforming growth factor β (TGFβ) superfamily, which include the TGF-βs, activins, and bone morphogenetic proteins (BMPs), play important roles during development and growth [Irish and Gelbart, 1987; Wozney et al., 1988]. For examples, in *Drosophila*, *dpp*, *Gbb-60A* and *screw*, which encode TGFβ-like ligands, are required during embryonic and/or pupal stages to regulate development [Arora et al., 1994; Khalsa et al., 1998]. *C. elegans* has also divergent TGFβ-like

ligands, which are DBL-1, UNC-129, and DAF-7 [Patterson and Padgett, 2000].

The TGFβ-like ligand DAF-7 in *C. elegans* regulates the formation of the dauer, which is an alternative developmental stage induced by large population, high level of pheromone or food scarceness [Riddle and Albert, 1997]. When this pathway is chosen, worms arrest in the third larval stage as dauer, which have morphological, behavioral and physiological specialization that allow survival and dispersal from conditions in which food resources are inadequate to allow reproduction. Mutants that disrupt normal regulation of the dauer decision are two types: dauer constitutive, which develop as dauer even under conditions that are appropriate for normal reproductive growth, and dauer defective, which fail to develop as dauer under conditions that are inappropriate for normal development. A group of dauer constitutive genes that constitute a TGFβ-like signaling pathway has been identified, as the genes encoding a ligand (*daf-7*), two receptors (*daf-1* and *daf-4*), and two Smads (*daf-8* and *daf-14*), which are cytoplasmic signal transducers [Patterson and Padgett, 2000]. Two dauer defective genes, *daf-3* and *daf-5*, for the TGFβ-like signaling pathway have also been identified. DAF-3 is another type of Smads which antagonizes this pathway, and *daf-5* encodes the onco-proteins Ski (for Sloan Kettering Virus)/Sno (for Ski-related novel sequence) [da Graca et al., 2004].

The *unc-129*, encoding another member of TGF-β superfamily, is required for guidance of pioneer motor-axons along the dorsoventral axis of *C. elegans* [Colavita et al., 1998]. However, this pathway has been largely unknown since no additional receptors or Smads for this pathway have been identified.

The third TGFβ-like ligand, DBL-1, in *C. elegans* (Fig. 1) regulates body size and male tail development [Suzuki et al., 1999]. All the mutants in this pathway show very specific phenotypes, which are small body size and male tail abnormalities. For this pathway, *sma-2*, *sma-3* and *sma-4* encode Smads, and *sma-6* encodes the type I receptor [Savage et al., 1996; Krishna et al., 1999]. As these gene names suggest, "sma" for small, all these mutants show small phenotypes. Interestingly, DAF-4, a type II receptor in TGF-β signaling pathway for the dauer formation, also plays a role as the type II receptor for this pathway. Recently, a transcription cofactor, SMA-9, in this pathway was also identified [Liang et al., 2003].

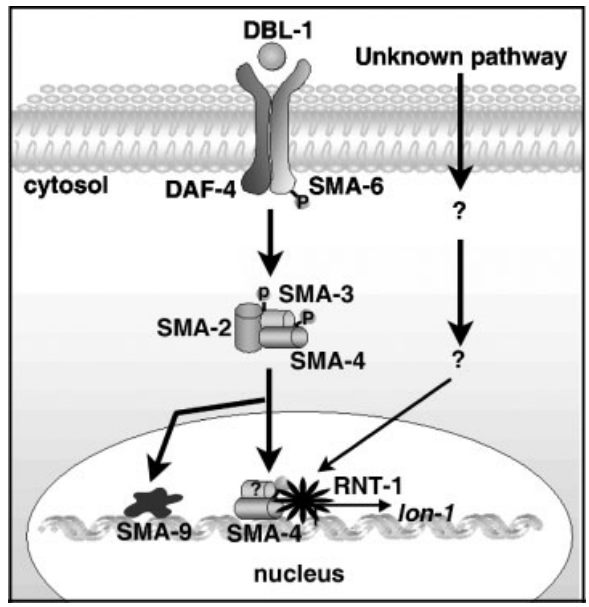

Fig. 1. The DBL-1 pathway of TGF-β signaling in *C. elegans*. DBL-1, a TGFβ-like ligand, binds to the receptor complexes which are composed of SMA-6, a type I receptor, and DAF-4, a type II receptor. Then, the activated receptor complexes transduce signals to the downstream transcription factor, RNT-1 or SMA-9, through the Smad complexes which are composed of SMA-2, SMA-3, and/or SMA-4. The downstream target gene, *lon-1*, may be regulated by RNT-1, but not SMA-9. Unknown pathway may also regulate the RNT-1 function for body size regulation and male tail development in parallel to DBL-1 pathway of TGF-β signaling.

The phenotypic similarities of *rnt-1*(*ok351*) mutants with the DBL-1 pathway mutants initially suggest us the possibility of RNT-1 involvements to that pathway (Fig. 1) [Ji et al., 2004]. In addition, RNT-1 protein has been shown to interact with SMA-4, which is a cytoplasmic signal transducer in the TGF-β signaling pathway. Moreover, the 5'-upstream region of *lon-1* gene, a downstream target gene of *dbl-1* pathway of TGF-β signaling [Morita et al., 2002], is found to have conserved binding sites for RNT-1, which are identical to the mammalian RUNXs consensus sequences at the upstream of promotor [Shim and Lee, personal communication]. These findings suggest that RNT-1 may play a role as a transcription factor for this pathway. However, double mutants between *rnt-1*(*ok351*) and other mutants of this pathway show synergistic effects rather than clear epistasis. These genetic data suggest the possibility that there may be a parallel pathway

that regulates body-size and male tail development other than TGF-β pathway (Fig. 1). Therefore, it would be very interesting to uncover the relationship between RNT-1 and TGF-β signaling and to identify the other pathways.

RNT-1 REGULATES THE ASYMMETRIC DIVISION OF T CELL IN WNT SIGNALING

The Wnt signaling pathway, which uses "Wnt proteins" as signals, is one of the signaling pathways controlling animal development [Cadigan and Nusse, 1997]. For example, wg, porcupine (porc), dishevelled (dsh), armadillo (arm; the *Drosophila* homolog of β-catenin), and pangolin (pan, *Drosophila* homolog of Tcf), which are the components in Wnt signaling pathway in *Drosophila*, regulate the segment polarity [Siegfried et al., 1994]. In the canonical Wnt signaling pathway, a Wnt ligand transduces a signal to the Dishevelled (Dsh) through a Frizzled (Fz) receptor on the cell surface. The Dsh inhibits degradation of β-catenin by the protein complex, which is composed of glycogen synthase kinase-3 (GSK-3), the adenomatous polyposis coli protein (APC), Axin and others. Therefore, β-catenin is stabilized and it accumulates both in the cytoplasm and the nucleus. In the neuclus, β-catenin interacts with Tcf factors and activates target genes [Cadigan and Nusse, 1997; Wodarz and Nusse, 1998]. The other Wnt signaling pathway, that does not utilize β-catenin molecules, is known as "noncanonical" Wnt pathway [Wallingford et al., 2001].

In *C. elegans*, Wnt signaling controls cell fate decision, cell migration, and cell polarity [Herman, 2002]. As in most animals, *C. elegans* has both canonical and noncanonical Wnt singaling pathways. The canonical Wnt pathway in *C. elegans* regulates migration of the QL neuroblast descendants, which express the Hox gene *mab-5*. This pathway is composed of EGL-20/Wnt, MIG-5/Dsh, SGG-1/GSK-3, BAR-1/β-catenin, PRY-1/Axin, and POP-1/Tcf [Guo, 1995; Harris et al., 1996; Korswagen et al., 2000; Herman, 2001; Korswagen et al., 2002]. In addition, this pathway is also involved in controlling the fates of the P12 ectoblasts and the vulval precursor cells (VPCs) with the Ras pathway [Eisenmann et al., 1998; Jiang and Sternberg, 1998].

At least three noncanonical Wnt pathways have been identified in *C. elegans* [Herman, 2002], they regulate EMS cell polarity, T cell polarity, and Z1/Z4 cell polarity. For example, an asymmetric cell division of T cell, which is positioned at the tail region of animal, is regulated by noncanonical Wnt signal [Herman, 2001]. In wild-type animals, T cell divides into the anterior daughter, T.a, which generates primarily epidermal cells and the posterior daughter, T.p, which produces primarily neural cells. The anterior daughter of T cell eventually forms sensory rays in the male tail, which are chemosensory copulatory organs. Higher level of POP-1/Tcf induces the epidermal T.a cell fate, whereas lower level of POP-1/Tcf promotes the neural T.p cell fate (Fig. 2). In a current model of T cell polarity (Fig. 2), LIN-44/Wnt binds to LIN-17/Fz receptor on the posterior surface of the T cell before its division [Herman et al., 1995; Sawa et al., 1996]. Then, the activated receptor transduces signals to the unknown factors, which will lead to the activation of LIT-1/NLK in the nuclei. The activated LIT-1/NLK phosphorylates downstream POP-1, and the phosphorylated POP-1 can interact with PAR-5, which is a homologue of mammalian 14-3-3

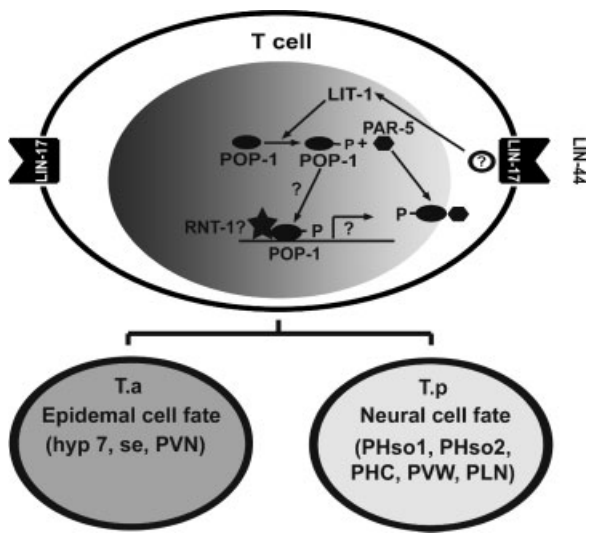

Fig. 2. The Wnt signaling of T cell polarity. LIN-44, a Wnt signal, binds to LIN-17, a Frizzled receptor, at the posterior surface of T cell before its division. Then, the signal activates LIT-1 through unknown factors. The activated LIT-1 phosphorylates POP-1, which can interact with PAR-5. The POP-1/PAR-5 complex can be exported from the nucleus, so that the POP-1 level in the T.p cell is lower than in that of T.a. In this figure, gray color in the T cell represents gradient levels of POP-1. RNT-1 may be involved in the regulation of gene expression with POP-1. After division, anterior descendents of T cell become mostly hypodermal seam cell 7 (hyp7), and posterior descendents become phasmidial support cells (PHso1 and PHso2), tail spike neuron (PHC), and lumbar ganglia (PLN and PVW).

proteins. The POP-1/PAR-5 complex is then being exported from the nucleus. Therefore, POP-1 level in the posterior T cell descendent is low, which could repress epidermal-specific genes in T.p. The *tlp-1* gene might be one of the target genes in the T. p. On the other hand, higher level of POP-1 is maintained at the anterior portion of T cell, which will result in epidermal T.a cell [Herman, 2001; Lo et al., 2004].

The *rnt-1*(*tm388*) mutants show mail tail abnormalities which are similar to the defects observed in *lin-17*(*n3091*) mutants. Indeed, *rnt-1*(*tm388*) mutants show defects in asymmetric division of T cell, suggesting that *rnt-1* is involved in regulating T cell polarity with Wnt signal [Kagoshima et al., personal communication]. Therefore, it would be interesting to elucidate the further relationship between *rnt-1* and Wnt signaling pathway.

FUNCTIONS OF CBFβ HOMOLOGUE, BRO-1, IN *C. elegans*

Studies on the CBFβ homologue, BRO-1, in *C. elegans* have just started, and only a few clues for the BRO-1 functions have been coming out recently. For examples, BRO-1 might negatively regulate the RNT-1 expression level in hypodermal seam cells, since RNT-1::GFP expression is up-regulated in the worms treated with *bro-1* RNAi [Shim and Lee, personal communication]. However, BRO-1 functions are largely unknown.

First, the binding assay of BRO-1 with RNT-1 would be critical since *C. elegans* BRO-1 shows relatively low homology with *Drosophila* Brother or Big Brother, which are 18% and 14%, respectively [Lee et al., 2004]. Second, the phenotype comparison between *bro-1* mutant and *rnt-1* mutant is needed. Mutant allele of *bro-1*(*tm658*) was recently isolated by National Bioresource Project in Japan. If BRO-1 has other functions in addition to enhancing DNA-binding activity of RNT-1, some phenotypes of *bro-1*(*tm658*) may be different from those of *rnt-1*(*ok351*) mutants. Third, BRO-1 is localized in the cytoplasm whereas RNT-1 is localized in the nuclei even though BRO-1 is the binding partner of RNT-1 [Lee et al., 2004]. Different sub-cellular localization of RNT-1 and BRO-1 suggests that their dimerization is controlled by a specific mechanism. Revealing this mechanism would also be interesting. Fourth, dimerization of RUNX-1 with CBFβ is known to protect RUNX1 from ubiquitin-proteasome-mediated degradation [Huang et al., 2001]. This is why RUNX1 is rarely detected in CBFβ knockout mice. However, RNT-1 expression is increased in the *bro-1* RNAi treated animals in *C. elegans* [Shim and Lee, personal communication]. Therefore, it is possible that BRO-1 regulates RNT-1 function differently in *C. elegans*, which remains to be elucidated.

FUTURE DIRECTIONS

Although studies on RNT-1 and BRO-1 in *C. elegans* are at their early stages, many advantages of *C. elegans* as a model system may accelerate the research in RUNXs field. For example, numerous binding partners of RNT-1 for chromatin remodeling and their downstream target genes could be easily identified by a high-troughput screening using the entire *C. elegans* genome or proteom. Indeed, efforts to identify RNT-1 interacting molecules using genome-wide RNAi screening have been made and interesting candidates are emerging from this screening [Shim et al., personal communication].

Until now, two signaling pathways, TGFβ and Wnt, have been proposed to regulate the RNT-1 since *rnt-1* mutant phenotypes are very similar to the phenotypes of mutants in these signaling pathway [Ji et al., 2004; Kagoshima et al., personal communication]. Therefore, it would be interesting to understand whether these two signaling pathways cross talk with each other or not. If not, two signaling pathways regulate the RNT-1 function in different tissues, meaning that each pathway functions independently and tissue specifically. It would be most interesting part of RNT-1 studies in near future.

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