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Regulation of *rnt-1* expression mediated by the opposing effects of BRO-1 and DBL-1 in the nematode *Caenorhabditis elegans*

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Abstract

During development of *Caenorhabditis elegans*, expression of the RUNX homolog, *rnt-1*, is tightly regulated both spatially and temporally. In this study, we investigated the mechanism underlying the temporal regulation of *rnt-1*. We found that *rnt-1* contained evolutionarily conserved consensus RUNX binding sequences within one of its introns, and that RNT-1 bound to these intronic sequences both *in vitro* and *in vivo* in the presence of BRO-1, suggesting that RNT-1 together with BRO-1 represses its own transcription. Fine deletion and substitution experiments revealed a binding site within the intron that was critical for *rnt-1* regulation. Importantly, we found that the TGFβ homolog, DBL-1, was required for counteracting the repressive activity of BRO-1 at postembryonic stages. Accordingly, ectopic expression of DBL-1 induced transcription of *rnt-1* in the lateral hypodermis and other tissues even at the postembryonic stages. Taken together, our data suggest that *rnt-1* expression is regulated by the balance between DBL-1-mediated activation and BRO-1-mediated repression at the postembryonic stages.

Keywords: *C. elegans*; RNT-1; BRO-1; Autoregulation; DBL-1

RUNX proteins are evolutionarily conserved transcription factors that form heterodimers with the cofactor protein, CBFβ [1]. The DNA binding activity of the RUNX proteins is enhanced by heterodimerization with CBFβ [1–4]. *Caenorhabditis elegans* expresses only one RUNX homolog, RNT-1, which is known to regulate the proliferation of hypodermal seam cells and male tail development [5,6]. *rnt-1* is also involved in the development of male tail seam cells derived from T cells [6–9]. Like *rnt-1*, *bro-1*, a homolog of CBFβ, has been shown to regulate the seam cell division and male specific seam cell fate determination [10,11], suggesting that RNT-1/BRO-1 act as a heterodimer and that their biological function may be conserved. Consistent with this, it was recently reported that BRO-1 physically interacts with RNT-1 and functions together [12]. RNT-1 is the rate-limiting factor controlling seam cell proliferation at L2–L3 transition [6]. Mutations in *rnt-1* lead to a reduced number of the seam cells at subsequent larval stages, and overexpression of *rnt-1* produces extra seam cells [6]. Therefore, regulatory mechanisms must exist to ensure *rnt-1* expression when it is needed and to block *rnt-1* expression when it is no longer necessary. It was recently reported that *bro-1* represses *rnt-1* expression in *C. elegans* [12]. However, there is a discrepancy between the expression patterns of *bro-1* and *rnt-1*: *bro-1* is mainly expressed in the hypodermis constitutively throughout development [11], but the *rnt-1* expression is not constitutively repressed, since its expression is fairly high at the embryonic stages and gradually decreases in the larval stages [5], making it difficult to explain how *rnt-1* is still expressed from embryonic to early postembryonic stages even in the presence of repressing BRO-1 activities. Here, we set out to investigate the mechanism by which *rnt-1* expression is temporally regulated in the lateral

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hypodermis. The questions that we addressed in this study are 2-fold: first, how *bro-1* represses *rnt-1* expression, and second, how *rnt-1* repression is overcome at the embryonic and early larval stages. We here show that, at the postembryonic stages, *rnt-1* expression in the lateral hypodermis is regulated by the balance between an activating mechanism mediated by the TGFβ homolog DBL-1 and by an autorepression mechanism mediated by BRO-1.

Materials and methods

Worm culture. Bristol N2 served as the wild type *C. elegans* strain [13]. N2 and mutant strains were grown at 20 °C on NGM plates. The *rnt-1* (*ok351*) and *dbl-1* (*wk70*) mutant strains were obtained from CGC (Minneapolis, MN), and the *rnt-1* (*tm491*) and *rnt-1* (*tm388*) mutant strains were a generous gift from NBRP (Tokyo, Japan).

Construction and microinjection of reporter genes. The pattern of *rnt-1* expression was investigated using the pPD-run-FL construct, which encodes the full-length RNT-1 protein fused to GFP (RNT-1::GFP) [5]. Derivatives of the construct containing *rnt-1* intronic deletions have been described previously and are renamed here (pPD-run-ΔMB now rnt-MB, pPD-run-ΔMS now rnt-MS, pPD-run-ΔSB now rnt-SB) [5]. To generate constructs containing serial deletions in the intron four region, we used eight primers to clone seven different constructs (rnt-MS-0 to −7). The rnt-MS-0 construct contains a fragment of pPD-run-FL, from position −3674 from the translation start site to the end of the gene. The rnt-MS-1 construct contains a fragment beginning from −1924, rnt-MS-2 from −1850, rnt-MS-3 from −1802, rnt-MS-4 from −1352, rnt-MS-5 from −744, and rnt-MS-6 from −705. The rnt-MS-7 primer was used as a reverse direction primer for all rnt-MS derivatives, and forward primers were given the same name as the construct. All constructs were amplified from N2 genomic DNA by PCR and fused to pPD95.77 vector. To identify the core consensus site of the intron sequence, we introduced mutations into the rnt-MS-0 construct using the QuickChange Site-Directed Mutagenesis Kit (Stratagene), according to the manufacturer's protocol. The rnt-MS-0-mut construct was generated using rnt-MS-0 plasmid as a template and the primers, *rnt-1* mutagen-1 and *rnt-1* mutagen-2. To study the pattern of *bro-1* expression, we cloned 2.4 kb of *C. elegans bro-1* genomic DNA into the reporter gene expression vector pPD95.77. The *bro-1* PCR fragment was generated using F56A3.5–1 and F56A3.5–2 primers. Microinjection of DNA into the gonads of adult hermaphrodites was carried out according to standard procedures [14]. To visualize transgene expression, fluorescence was observed using an Axioplan2 microscope (Zeiss). Images were captured using an AxioCam camera (Zeiss). All the sequences of the PCR primers used in this study are available upon request.

Protein purification and gel shift assay. *rnt-1* cDNA was generated with rnt-35/36 primers and *bro-1* cDNA with F56A3.5–5/6 primers. Each was then subcloned into pRSET-A vector. Overexpressed RNT-1 was purified using Ni-NTA agarose beads (QIAGEN). Because BRO-1 was primarily present in an insoluble fraction, *in vitro* translation was carried out as described in the Promega technical manual. Purified RNT-1 and *in vitro* translated BRO-1 were used in gel shift assays. Five picomoles of each strand was phosphorylated at the 5′ terminus using γ-32 P-dATP (New England Biolabs) and T4 polynucleotide kinase (MBI). Radiolabeled oligonucleotides were mixed in TEN buffer (10 mM Tris–HCl, pH 8.0; 1 mM EDTA, pH 8.0; 100 mM NaCl), boiled at 100 °C for 5 min, and slowly cooled to the room temperature. The annealed double-stranded probes were separated and eluted in non-denaturing 15% (w/v) polyacrylamide gels. RNT-1 and BRO-1 were pre-incubated with 2 ng of poly [dI-dC] (Sigma) in 40 μl of binding buffer (10 mM Tris–HCl, pH 8.0; 1 mM EDTA; 50 mM NaCl; 5 % [v/v] glycerol) for 10 min on ice. The radioactive probe was then added, and the mixtures were incubated for 15 min at room temperature and run on non-denaturing 6% (w/v) polyacrylamide gels in 1× TBE. Binding was monitored by autoradiography and a Fuji phosphorimager.

Feeding RNAi. *bro-1* cDNA was amplified by PCR from a cDNA library using F56A3.5–3 and F56A3.5–4 primers. The amplified cDNA was subcloned into the bacterial expression vector L4440 (pPD129.36), and the resulting construct was transformed into HT115 (DE3) bacterial cells. The clone was confirmed by sequencing. Worms were administered RNA*i* while at the L4 stage, and the resultant phenotype was observed in embryos and adult worms.

ChIP assay and real time PCR. To verify *in vivo* binding of RNT-1 to consensus sequences within its own intronic binding sites, we performed real time PCR of the RNT-1 binding region in the fourth intron after chromatin immunoprecipitation. For the ChIP assay, was mixed stage RNT-1::GFP transgenic worms were grown on NGM-lite plates and harvested. After cross-linking the worm extract, the protein–DNA pellet was obtained, washed, and resuspended in HEPES lysis buffer. The pellet was then sonicated more than eight times and centrifuged. The resulting supernatant was precleared with salmon sperm DNA/protein A agarose and incubated with anti-GFP antibody at 4 °C overnight. The remainder of the procedure was performed according to the method of Oh and colleagues [15]. DNA samples isolated using the ChIP assay were subjected to real time PCR as described in TAKARA SYBR premix Ex Taq technical manual. Primers used in RT-PCR are listed in Supplementary Table 1, and all primers were designed using a primer generation program available from http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi primer generation web page.

Heat shock. Hsp::*dbl-1* and RNT-1::GFP transgenic N2 worms at the L3–L4 stage were incubated at 30 °C for 2 h to induce mild heat shock. The worms were then allowed to recover at 20 °C for 2–3 h before analysis.

Results and discussion

bro-1 represses RNT-1 expression more efficiently at late larval stages

It was recently reported that *bro-1* represses RNT-1 expression [12]. To further investigate the biological consequence of the repression of *rnt-1* by *bro-1*, we compared the expression patterns of BRO-1 and RNT-1 at various developmental stages. Examination of *rnt-1* expression using a construct containing the full-length *rnt-1* gene and all the regulatory regions revealed that *rnt-1* expression in the lateral hypodermis was strong at the embryonic stage and gradually decreased after hatching, with expression being still moderately high at L1, but almost undetectable in adults (Fig. 1, left panels). On the contrary, *bro-1* was strongly expressed throughout development from the late embryonic stage (Fig. 1, right panels). The interpretation on this discrepancy in the expression patterns may be that *bro-1* repressed RNT-1 transcription more efficiently at late larval stages than at early larval stages despite its ubiquitous expression throughout development. Two questions were raised. First, how does *bro-1* repress *rnt-1* at the larval stages? Second, how does *rnt-1* escape the repression by *bro-1* at the embryonic stage and the early larval stage?

rnt-1 contains conserved intronic RUNX binding sites, which RNT-1 binds in the presence of BRO-1

To identify the regulatory sites required for *rnt-1* down-regulation *in vivo*, we used a comparative genomics approach to analyze the third intron of *rnt-1*, which con-

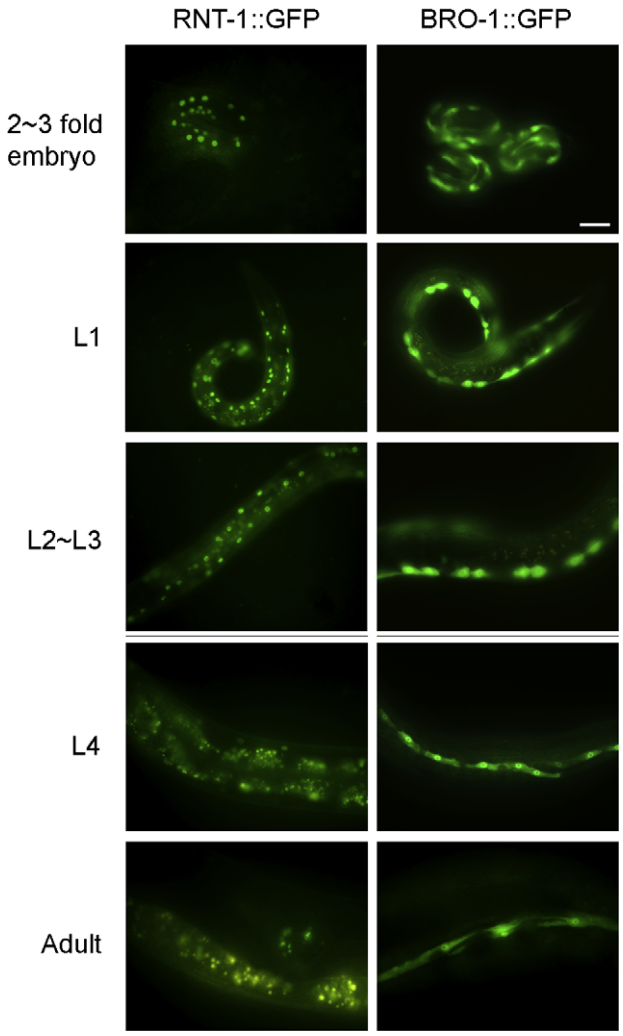

Fig. 1. *bro-1* represses RNT-1 expression more efficiently at late larval stages. The figures show expression patterns of RNT-1 and BRO-1. The left panels are RNT-1::GFP expression. The right panels are BRO-1 expression. While BRO-1 is constitutively expressed, RNT-1 expression is relatively high at the embryonic and early larval stages. The scale bar is 20 μm.

tains most of the tissue-specific enhancers [5]. A comparison of sequences of *C. elegans* and *C. briggsae* revealed small blocks of conserved sequences within this region (Fig. 2A). As shown in Fig. 2A, these conserved regions contained four putative consensus RUNX binding sequences (sites a–d). The binding sites were present as two pairs (a, b and c, d) separated by about 100 nucleotides, making these sequences good candidates for regulatory elements. *In vitro* gel shift assay and chromatin IP were performed to determine whether RNT-1 binds to the conserved consensus sequences. Gel shift assays showed that RNT-1 alone was unable to bind these sequences. Next, we tested the possibility that RNT-1 may require BRO-1 to bind to these sites. As shown in Fig. 2B, RNT-1 strongly bound two of the intronic sites (site b and c) in the presence of BRO-1. The site b sequence, in particular, was efficiently bound by RNT-1 and BRO-1. Chromatin IP followed by quantitative PCR revealed that *bro-1* RNA*i* decreased RNT-1 binding to these sequences *in vivo* (Fig. 2C).

One of the intronic binding sites is essential for rnt-1 autorepression in vivo

Next, serial deletion and substitution experiments were performed to pinpoint the regulatory element required for *rnt-1* autoregulation. We confirmed that *bro-1* RNA*i* efficiently upregulated RNT-1 (Fig. 2B, left uppermost panels). The rnt-MS-0 construct containing all four binding sites was also upregulated by *bro-1* RNA*i*. A similar effect was seen in the absence of the site a (rnt-MS-2). However, deletion of both site a and b completely abolished induction of RNT-1::GFP (rnt-MS-3), suggesting that site b is essential for *bro-1* RNA*i*-mediated derepression (Fig. 3). Consistently, deletion of only site b (rnt-MS-mut) also abolished the effect of *bro-1* RNA*i*. These data suggest that site b constitutes the *in vivo* target for *rnt-1* regulation. Taken together, it is strongly suggested that RNT-1 represses its own transcription through intronic sequences in the presence of BRO-1, although it is also possible that *bro-1* RNA*i* may have reduced RNT-1 binding to the promoter region of other *rnt-1* repressor genes. Consistent with our findings, the mammalian RUNX2 gene is known to be autoregulated by negative feedback on its own promoter and 5′ UTR [16].

The TGFβ homolog, DBL-1, is required for counteracting rnt-1 autorepression

Because *bro-1* is constitutively expressed, the repressive effect of BRO-1 on *rnt-1* transcription should be counteracted by an activator to allow for *rnt-1* expression during early larval stages. In search of the activators, we explored whether the TGFβ pathway may be involved, as *rnt-1* has been shown to regulate body size within or in parallel to this pathway [17]. We found that, in the *dbl-1(wk70)* mutant animals, RNT-1::GFP expression was not induced by *bro-1* RNA*i*, suggesting that DBL-1 acts to activate *rnt-1* expression (Fig. 4A; c). Furthermore, during the larval stages, basal *rnt-1* expression was lower in the *dbl-1* mutant background than in the wild type background (Fig. 4A; a, b), suggesting that the TGFβ pathway is required for *rnt-1* activation during this time.

Ectopic expression of dbl-1 drives rnt-1 expression

DBL-1 is expressed and secreted by neurons to act upon neighboring cells [18]. Moreover, the dosage of *dbl-1* is a critical factor in the regulation of the TGFβ pathway [18]. Similar to *rnt-1* expression, *dbl-1* expression is higher at early larval stages and gradually diminishes at later stages. Therefore, we tested whether DBL-1 was sufficient to drive *rnt-1* expression through ectopic expression of *dbl-1* under the control of a heat shock promoter. After

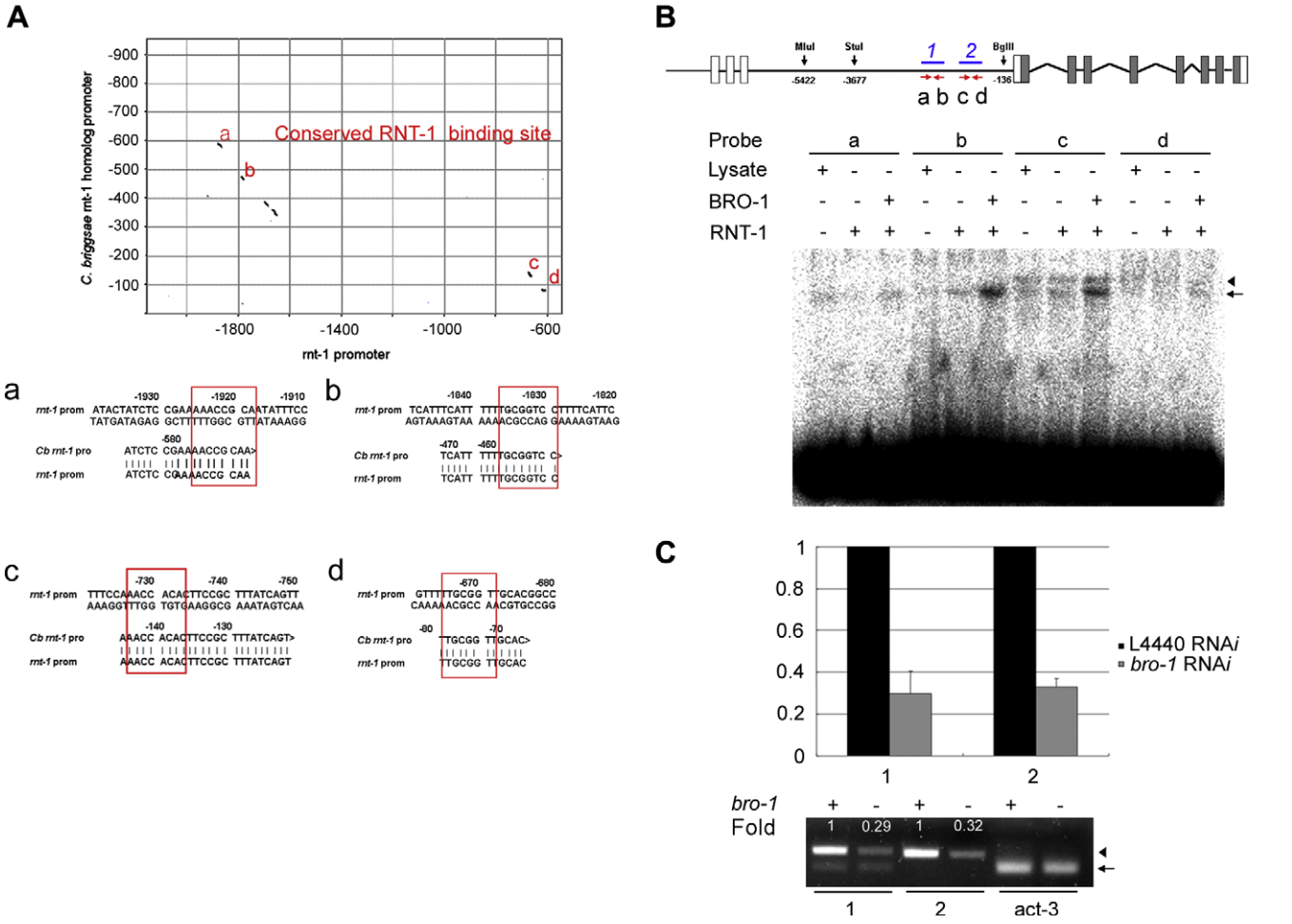

Fig. 2. *rnt-1* contains conserved intronic RUNX binding sites, which RNT-1 binds in the presence of BRO-1. (A) The nucleotide sequences of sites a–d conserved in *C. elegans* and *C. briggsae rnt-1* genes. Sequence alignment with *C. brigssae* is shown in a–d. Red boxes indicate RUNX consensus sequences. (B) The genomic structure of the *rnt-1* gene is shown in the upper panel. Four RUNX consensus binding sequences within the third intron are labeled (sites a–d). The bottom panel shows the *in vitro* binding of RNT-1 to the indicated binding site sequence in the presence or absence of BRO-1. Lysate indicates the negative control containing the *in vitro* translation system used for *bro-1* expression. The arrowhead indicates nonspecific binding of lysate protein, and the arrow shows specific binding of RNT-1. (C) Endogenous RNT-1 binds to intronic RUNX binding sites. Quantification of the ChIP RT-PCR products, as measured by the C_t value, is shown. Agarose gel electrophoresis of the ChIP-PCR products is also shown. The locations of the PCR products within the regulatory regions are indicated with blue lines in the upper panel of (B). Real time PCR samples were loaded after the 15th cycle. The arrowhead indicates a specific PCR product of ChIP-PCR and the arrow indicates the PCR primers.

heat shock treatment of the transgenic animals containing hs-DBL-1 and RNT-1-GFP constructs, we observed a Lon phenotype, which is a well-known phenotype observed in the *dbl-1*-overexpressing animals [18]. Importantly, *rnt-1* was expressed in the lateral hypodermis at late larval stages (Fig. 4B; a, b). In addition, a subset of neurons in the head region expressed *rnt-1* (Fig. 4C; c, d). These data suggest that *dbl-1* expression is solely capable of driving *rnt-1* expression in the seam cells and neurons at inappropriate times.

The factors downstream of DBL-1 that directly activate transcription of *rnt-1* during the larval stages are not known. Interestingly, mutation of *sma-6* or *daf-4*, which encode two DBL-1 receptor subunits [19], did not cause the same phenotype as *dbl-1(wk70)* mutation (Shim and Lee, unpublished observation). Also, mutation of *sma-2, -3* and *-4* single did not result in any defects in *rnt-1* autoregulation (data not shown). Based on these findings, it is possible that DBL-1 acts via as yet unidentified receptors and effectors. Other SMAD cofactors have been identified in mammals, *Drosophila,* and *Xenopus*, which can affect DNA binding affinity or directly bind to the SMAD complex to regulate TGFβ pathway [20]. It would be of interest to identify downstream effectors of DBL-1, including the transcription factor directly acting on the *rnt-1* promoter.

dbl-1 is not required for embryonic expression of rnt-1

We found that *dbl-1* mutant embryos exhibited levels of *rnt-1* expression comparable to that of wild type embryos (Fig. 4C), while hatched larvae showed considerably lower expression of *rnt-1* (Fig. 4A; b), suggesting that *dbl-1* is not required for embryonic expression of *rnt-1*. Consequently, there must be other activators that counteract the

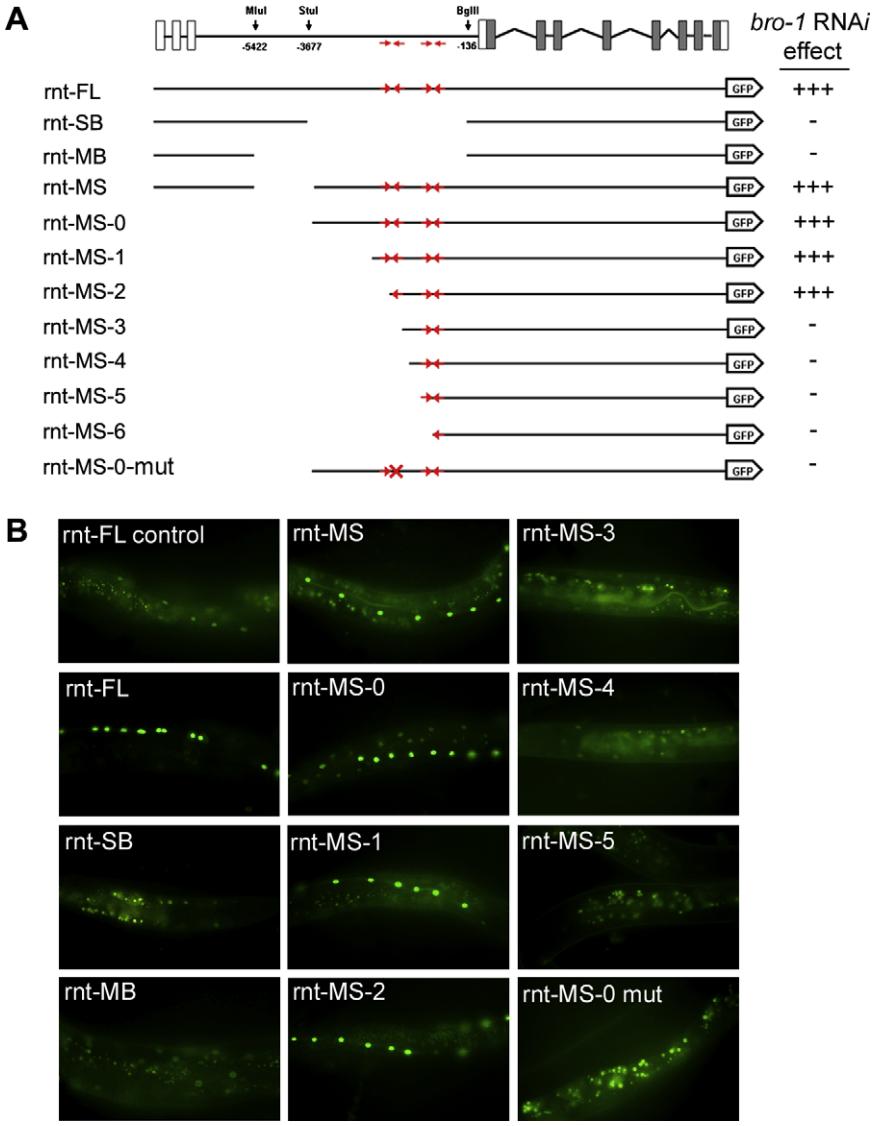

Fig. 3. Identification of the regulatory site required for *rnt-1* autoregulation *in vivo*. (A) A schematic of serially deleted derivatives of the *rnt-1* reporter construct and their corresponding expression in the presence of *bro-1* RNA*i*. The deleted regions in the rnt-SB, MB, and MS constructs are indicated. The RUNX binding sites were sequentially deleted in the rnt-MS constructs (−0 to −6). Induction of RNT-1::GFP by *bro-1* RNA*i* is shown to the right. "+++" Indicates strong derepression, and "−" indicates no derepression. (B) Representative images showing RNT-1::GFP expression by worms transgenic for the indicated deletion construct. rnt-FL control means RNT-1 expression without *bro-1* RNAi.

repressive activity of *bro-1* at the embryonic stages. Since the *rnt-1* gene contains consensus GATA binding sites, it is possible that GATA factors such as *elt-1, -5*, and *-6*, which are expressed in the embryonic hypodermis, may act on the *rnt-1* promoter to activate its expression. However, introduction of single RNA*i* for these factors did not alter *rnt-1* expression in early embryos (data not shown), suggesting that these factors may act redundantly on the *rnt-1* promoter. It will be interesting to investigate whether any combination of these proteins is responsible for *rnt-1* transcription at the embryonic stage.

Our data suggest that the dynamic expression of *rnt-1* at the larval stage is achieved by the balance between DBL-1-mediated activation and BRO-1-mediated repression. *dbl-1* expression is highest after hatching and then gradually diminishes. On the contrary, *bro-1* is constitutively expressed throughout development. Therefore, at a certain point after the larval stage, activation by DBL-1 is counteracted by BRO-1 repressive activity, resulting in a decrease in *rnt-1* transcription. This working model can account for the phenomena reported here. That is, in the presence of *bro-1* RNA*i*, repressive activity is removed, and DBL-1-mediated *rnt-1* transcription persists. On the other hand, in the presence of *bro-1* RNA*i* in the *dbl-1(wk70)* background, *rnt-1* transcription is neither activated nor repressed, resulting in no *rnt-1* transcription. In accordance with this model, BRO-1-mediated repression observed at late larval stages could be overcome by overexpression of DBL-1. Interestingly, ectopic expression of DBL-1 induced by heat shock elicited *rnt-1* transcription in cells that

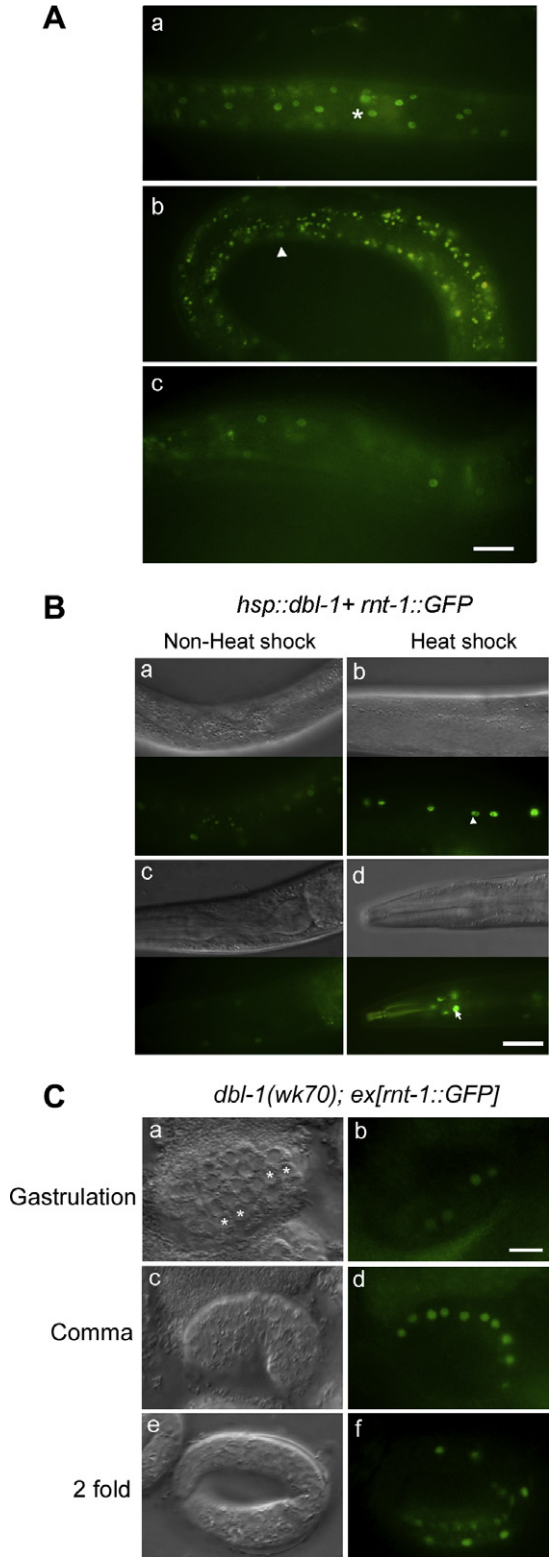

Fig. 4. *dbl-1* is required for transcriptional activation of *rnt-1* at the larval stages, but not at the embryonic stage. (A) RNT-1::GFP expression by (a) a wild type animal, (b) a *dbl-1(wk70)* mutant animal, and (c) a *dbl-1(wk70)* mutant animal treated with *bro-1* RNA*i*. The asterisk in (a) and arrowhead in (b) indicate hypodermal cells expressing relatively high or low level of RNT-1, respectively. All images were captured using identical exposure times. The scale bar is 20 μm. (B) Heat shock-induced expression of *dbl-1* is sufficient to activate *rnt-1* expression in compatible cell types. Images show RNT-1::GFP expression in hypodermal cells (a and b) and sensory neurons (c and d) before and after heat shock. The arrowhead in (b) indicates a hypodermal cell, the arrow in (d) a sensory neuron. The scale bar is 25 μm. (C) *dbl-1* is not required for *rnt-1* transcription in the embryo. Nomarski and fluorescence images of the *dbl-1(wk70); ex[rnt-1::GFP]* strain during gastrulation (a and b), the comma stage (c and d) and the 2-fold embryo stage (e and f) are shown. The asterisks mark the cells expressing RNT-1::GFP. The scale bar is 10 μm.

previously did not express detectable levels of RNT-1, but not in all cells. It is possible that DBL-1 expression driven by the heat shock promoter may not have reached all cells. Alternatively, these neurons may be competent to respond to TGFβ signaling, while the other cells may not. If this is the case, then neurons may not be targets of TGFβ signaling under normal conditions, but may retain the potential to respond to this signaling. Certain conditions such as environmental stress may induce *rnt-1* transcription in these neurons. It would be of interest to determine the conditions at which *rnt-1* can be induced in these cells and the function of *rnt-1* induction under these conditions.

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