BLMP-1 Contributes to Collagen-related Morphogenesis in *C. elegans*

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Abstract: *C. elegans blmp-1* is the homologue of mammalian *Blimp1* (*B Lymphocyte-Induced Maturation Protein-1*) and encodes a zinc finger and SET domain-containing protein. Genetic and molecular analysis of *blmp-1* revealed that downregulation of this gene leads to morphological defects that include a dumpy, uncoordinated phenotype, short rays on male tails, and a weak cuticle. Downregulation of typical collagen genes, such as *dpy-2,-3,-7,-8,* and *-13*, enhance the dumpy phenotype caused by *blmp-1* deletion. Worm cuticle images captured by in cryo scanning electronen microscope demonstrated the annuli defect of *blmp-1(tm548)* mutants. In conclusion, *blmp-1* plays a role in morphogenesis through the effect on cuticle.
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1. Introduction

Blimp1/PRDM1 was originally identified as a zinc finger transcriptional repressor, due to its ability to silence beta-interferon gene expression and act as a key regulator of plasma cell differentiation in mammalian cells(Turner, 1994). In addition, this protein also controls paradigms of gene expression in different cell types, such as T lymphocytes (Kallies, 2006, Martins, 2006), macrophages (Chang, 2000), and the sebaceous gland (Horsley, 2006). Blimp1 orchestrates plasma cell differentiation by inhibiting the mature B cell gene expression program (Shaffer, 2002). Studies in mouse demonstrated that Blimp1 represses the initiation of the somatic program and instead promotes progression toward the germ-cell fate (Ohinata, 2005, Vincent, 2005). Another study in which *blimp-1* was conditionally deleted in mice revealed that Blimp-1 is an important regulator of the transition of keratinocytes from the granular to the cornified layer of the epidermis (Magnusdottir, 2007). Blimp1 may also have a role in the maintenance of early germ cell fate by promoting the transition from a somatic fate and preventing reversion to a pluripotent stem cell state (Hayashi, 2007). Additionally, Blimp1 has a critical role in the foundation of the mouse germ cell lineage, because disruption of *blimp1* causes a block early in the process of primordial germ cell formation (Ohinata, *et al.*, 2005).

C. elegans blmp-1 is the homologue of mammalian *Blimp1* (*B Lymphocyte-Induced Maturation Protein-1*), which encodes a zinc finger and SET domain-containing protein (Andersen, 2007). In mammals, *Blimp-1/PRDM1* is expressed in skin epithelial cell lineages (Horsley, *et al.*, 2006, Magnusdottir, *et al.*, 2007, Chang, 2002), which may be analogous to the hypodermis and seam cells in worms. Indeed, in *C. elegans*, a *blmp-1::gfp* transcriptional fusion protein is expressed from late embryogenesis through adulthood and found in a variety of tissues, including hypodermis, seam cells, gonad distal tip cells and vulva, ventral nerve cord, amphids and phasmids, and rectal muscles (Reece-Hoyes, 2007).

Though much has been published regarding lymphocyte maturation and germ-cell fate decision, little is known about *blmp-1* functions in the morphogenesis and skeleton biosynthesis. *C. elegans* is an excellent model for studying morphogenesis and skeleton biosyntheses because the surface of the worms is covered with a flexible and resilient exoskeleton called a cuticle(Cox, 1981, Cox, 1981). The cuticle facilitate locomotion by muscle attachment, protects the organism from the environment. The cuticle is synthesized once in the embryo and again at the end of each larval stage before molting, which serves to regulate the growth rate and restrict the size of the worms (Kramer, 1988, von Mende, 1988). The cuticle is a highly structured extracellular matrix (ECM) that is primarily composed of cross-linked collagens and insoluble proteins like cuticlins, glycoproteins and lipids. The cuticle collagens are encoded by a large gene family with over 150 members (Johnstone, 2000), which are subject to strict patterns of temporal regulation such that RNAi knockdown of less than 30 of these genes results in an obvious phenotype. Cuticle collagen

synthesis involves translation of the preprocollagen peptide followed by the processing, secretion and crosslinking of the collagen. Procollagen processing occurs after secretion, and crosslinking occurs after collagen processing and assembly. This complex process requires a series of specific enzymes and chaperones (Prockop, 1995, Myllyharju, 2004), several of which are potential therapeutic targets for the treatment of collagen-associated diseases (Myllyharju, 2001). Mutations in individual collagen genes and their biosynthetic pathway components can result in a number of defects, ranging from abnormal morphology (dumpy and blister) to embryonic and larval death, confirming an essential role for this structure and highlighting its potential as an ECM experimental model system.

In this study, we use *C. elegans* as a model to investigate the function of *blmp-1* in collagen-restricted morphogenesis. We found that reduced expression of *blmp-1* leads to morphological defects, such as *Dpy, Unc,* short rays on male tails, and formation of a weak cuticle. Epistasis analysis indicated that *blmp-1* function is independent of the classic collagen genes. Collagen biogenesis and ECM function play a critical role in organogenesis and body morphogenesis in all metazoans. Defects in these processes result in a variety of human osteogenesis imperfecta, including Epidermolysis bullosa, Ehlers-Danlos syndrome, several chondrodysplasias and excessive fibrosis associated with wound healing and liver disease. Additionally, the ability to control and engineer ECM synthesis will be critically important in any future attempts at organ culture. This study contributes to our understanding of the fundamental mechanisms of these diseases and provides insight into additional possible targets for osteogenesis disease therapy.

2. Materials and Methods

2.1 Worm strains

C. elegans strains N2 and *blmp-1(tm548)* were generously provided by Dr. Chonglin Yang. *CB4088 him-5(e1490)V* and *EM733, pkd-2::gfp,him-5(bxIs34)* were generously provided by Prof. Hong Zhang.

2.2 RNA interference

The *blmp-1* RNAi clone was obtained from the Ahringer library and induced with 1 mM IPTG one day before feeding on Nematode Growth Medium (NGM) plates.

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2.3 Lifespan assay

Synchronized eggs grown to the young adult stage were distributed onto RNAi or non-RNAi plates containing 20 μg/ml FUdR to prevent the growth of progeny. RNAi bacterial clones were selected from Ahringer's RNAi feeding bacteria library (Kamath, 2003), and RNAi was induced with 1 mM IPTG. Bacteria carrying empty vector were used as controls in all experiments. The worms were cultured at 20℃. Worms that crawled off were excluded from all experiments. The number of dead worms was counted every other day. All experiments were performed at least twice. The P-value was calculated with a log-rank test on the Kaplan–Meier curves.

2.4 Male tail observation

Worms were cultured with or without *blmp-1* RNAi on plates; male worms were subsequently picked up to check the tail fan and rays. Strains N2; *EM733, pkd-2::gfp, him-5(bxIs34);* and *CB4088 him-5(e1490)V* were used in this assay.

2.5 NaClO lysis assay

Synchronized N2 or *blmp-1* mutant worms were grown on NGM plates until one day after entering adulthood. Next, the worms were transferred into M9 buffer (22 mM KH_2PO_4, 34 mM K_2HPO_4, 86 mM NaCl, 1 mM $MgSO_4$) in a 96-well plate, with each well containing 20-30 worms. An equal volume of bleach buffer (1/10 5 N NaOH, 1/20 NaClO solution) was added to each well to lyse the worms. Images were taken once every minute using a dissecting microscope (Motic) to monitor the lysis.

2.6 cryo scanning electronen microscope (Cryo-SEM)

Cryo-SEM is used for observing cuticle surface of *C. elegans*. SEM involves freezing worms with liquid nitrigon, conductive Pt coating , and imaging on an SEM at -130℃.

3. Results

3.1 Downregulation of blmp-1 causes defects in wild-type N2 worms

The *blmp-1* gene was first identified in our lifespan screen of histone modification-related genes. We used a feeding RNAi approach to knock down endogenenous *blmp-1* expression in *C. elegans*. RNAi targeting of *blmp-1* at the Larval 1 (L1) stage resulted in a significantly decreased lifespan (Figure 1A), which is consistent with previous data (Greer, 2010). To confirm the association between *blmp-1* and aging, we examined the lifespan of worms with *blmp-1* knocked down at adulthood. To our surprise, *blmp-1* knockdown worms from adulthood did not display the short lifespan phenotype (Figure 1B), suggesting that the short lifespan of L1 stage *blmp-1* mutants is caused by a developmental defect. Indeed, we observed a number of defects at developmental stages when RNAi was applied from the L1 stage; the most obvious phenotypes were dumpy (*Dpy*) and

uncoordinated (*Unc*), which were also reflected by an abnormally locomotion trace (Figure 1C). We subsequently investigated whether the function of blmp-1 in germ cell fate decision is conserved from mammals (Hayashi, de Sousa Lopes and Surani, 2007) to *C. elegans*. We found that *blmp-1* RNAi worms had a decreased brood size (Figure 1D), indicating that there is a defect in a germ cell formation-associated process. Additionally, the *blmp-1* RNAi male worms' tails were small with short rays and insufficiently expanded fans, though there was no fusion or loss of the rays (Figure 1E), suggesting that the abnormal tail phenotype is caused by a cuticle defect. Furthermore, *blmp-1* mutants grow more slowly than wild-type worms, reaching adulthood about 12 h later than N2 worms, and some mutants had a protruding vulva. In conclusion, *blmp-1* knockdown contributes to a series of morphological phenotypes that suggest cuticle defects, and these defects consequently contribute to a short lifespan in worms.

3.2 blmp-1 morphological defects are independent of HOX genes

HOX genes have roles in morphogenesis in many species; therefore, we wanted to know if the morphological defects in the *blmp-1* mutant are caused by misregulation of HOX genes or are only a result of the cuticle defect. To test this hypothesis, we examined male tail development. In *C. elegans*, male tail development can be used to examine the HOX gene network, including such genes as *egl-5* (Nicholas, 2009, Toker, 2003, Chisholm, 1991) and *mab-5* (Jungblut, 1998, Gutierrez, 2003). We used a GFP labeled *pkd-2* transgene, which functions as an indicator of HOX gene function and ciliary trafficking and targeting, to trace neuron development and migration in the tail (Bae, 2008, Bae, 2006, Knobel, 2008). RNAi knockdown of *blmp-1* was induced at the L1 stage, and GFP fluorescence was assessed on day 1 of adulthood; we were unable to discern any difference between *blmp-1* RNAi and control worms. This finding suggests that *blmp-1* activity during morphogenesis is not mediated via HOX gene activity (Figure 2A). Therefore, the cuticle defect caused by disrupted expression of collagens or collagen biogenesis genes is probably responsible for the morphological defect in the male tail(Roushdy,2011).

To confirm that an abnormal cuticle causes the morphological defects that we observed in the mutant, we designed an assay to assess the strength of the cuticle by examining the worm's resistance to a strong oxidative solution. To determine the rate at which the worms were lysed, adult wild-type and *blmp-1* mutant worms were immersed in a 50% bleach solution in which NaClO was the main active component. All worms were alive at the time the bleach solution was applied; after 4 minutes, the majority of the *blmp-1* mutants but not the N2 worms had lysed, indicating that the *blmp-1* mutant worms did not have as strong a cuticle wild-type worms (Figure 2B). This indicates that the *blmp-1* mutant cuticle does indeed have defects that could be responsible for the dumpy phenotype.

To further investigate the structure of culticle surface in blmp-1 mutant, we observed both WT and mutant sample in cryo scaning microscopy. Cryo-SEM shows that the surface of *blmp-1* mutant worms in the adult stage is abnormal, the alae and annulus are not as clearly as wild type (Figure 2C). It is the direct evidence that abnormal cuticle cause the morphological defects.

3.3 Knockdown of the typical collagen genes dpy-2,-3,-7,-8, and -13 in blmp-1(tm548) mutant worms enhances the dumpy phenotype.

The cuticle is synthesized several times before molting occurs in the embryo and at the larval stage. The main component of the cuticle is collagen. To date, mutations of more than 30 collagen genes have been found to result in dumpy or other abnormal phenotypes. As the most obvious phenotype resulting from *blmp-1* RNAi or mutation is dumpy, which probably arises from a defect in collagen, we tested whether collagen disruption is responsible for the dumpy phenotype in the *blmp-1* mutant.

From Wormbook.org, we already know that *dpy-2,-3,-7,-8,* and *-10* mutants are dumpy and have no cuticular annuli, while *dpy-5,* and *-13* mutants have narrow annuli. Worms that are mutant for both a "no annuli" gene and a "narrow annuli" gene will have a more severe dumpy phenotype, while a worm mutant for two genes within the same group will not. To determine which group of dumpy genes is regulated by *blmp-1*, we used RNAi to knock down each of these genes in N2 worms to confirm the knockdown phenotype for each gene, as well as the effect of the RNAi technique. Next, we performed RNAi for each of the dumpy genes in a *blmp-1(tm548)* genetic background to determine if it resulted in a compound phenotype. We observed that downregulation of each of these genes leads to a dumpy phenotype in N2 worms (Figure 3A) and a compound dumpy phenotype in a *blmp-1(tm548)* background (Figure 3B). This finding suggests two possibilities; *blmp-1* does not regulate these collagen genes, or *blmp-1* regulates these genes redundantly with some other factor(s). If either of these possibilities is true, then these collagen genes are at least partially expressed in the *blmp-1(tm548)* mutant.

Figure 1

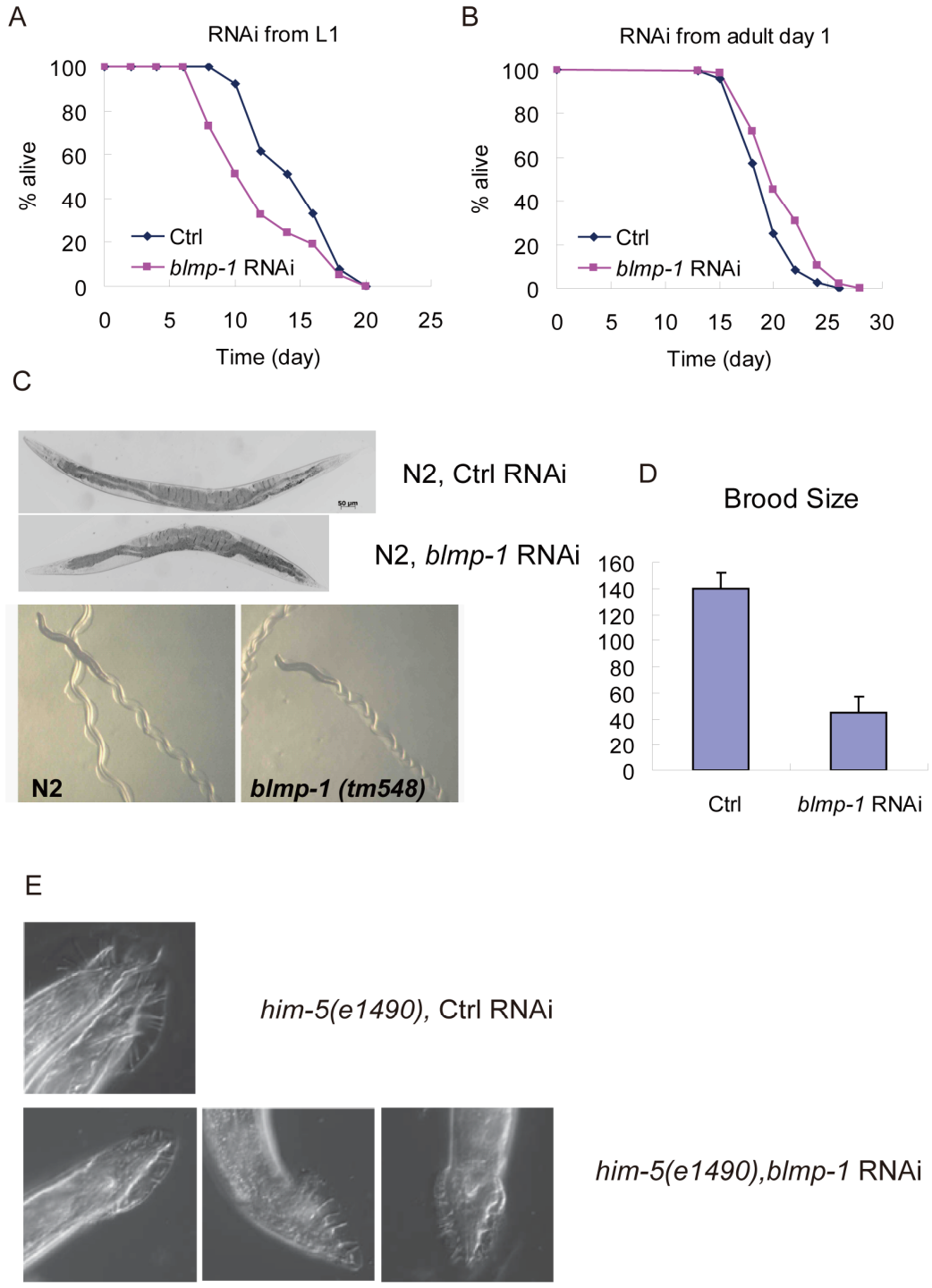

Figure 1. Typical phenotype of *blmp-1* knockdown in *C. elegans*.
(A) *blmp-1* RNAi at the L1 stage decreases lifespan in wild-type N2 worms (vector RNAi, n=39; *blmp-1* RNAi, n=37; long-rank test, p<0.01). (B) *blmp-1* RNAi at adulthood results in a slightly increased lifespan of N2 worms (vector RNAi, n=148; *blmp-1* RNAi, n=134; long-rank test, p<0.01). (C) *blmp-1* RNAi and *blmp-1(tm548)* worms have several morphological defects, such as short body length, dumpy (*Dpy*), uncoordinated (*Unc*), and abnormal movement. (D) *blmp-1* knockdown affects germ cells and results in a decreased brood size. (E) *blmp-1* RNAi worms have abnormal male tail morphology. The rays are all short with insufficiently expanded fans.

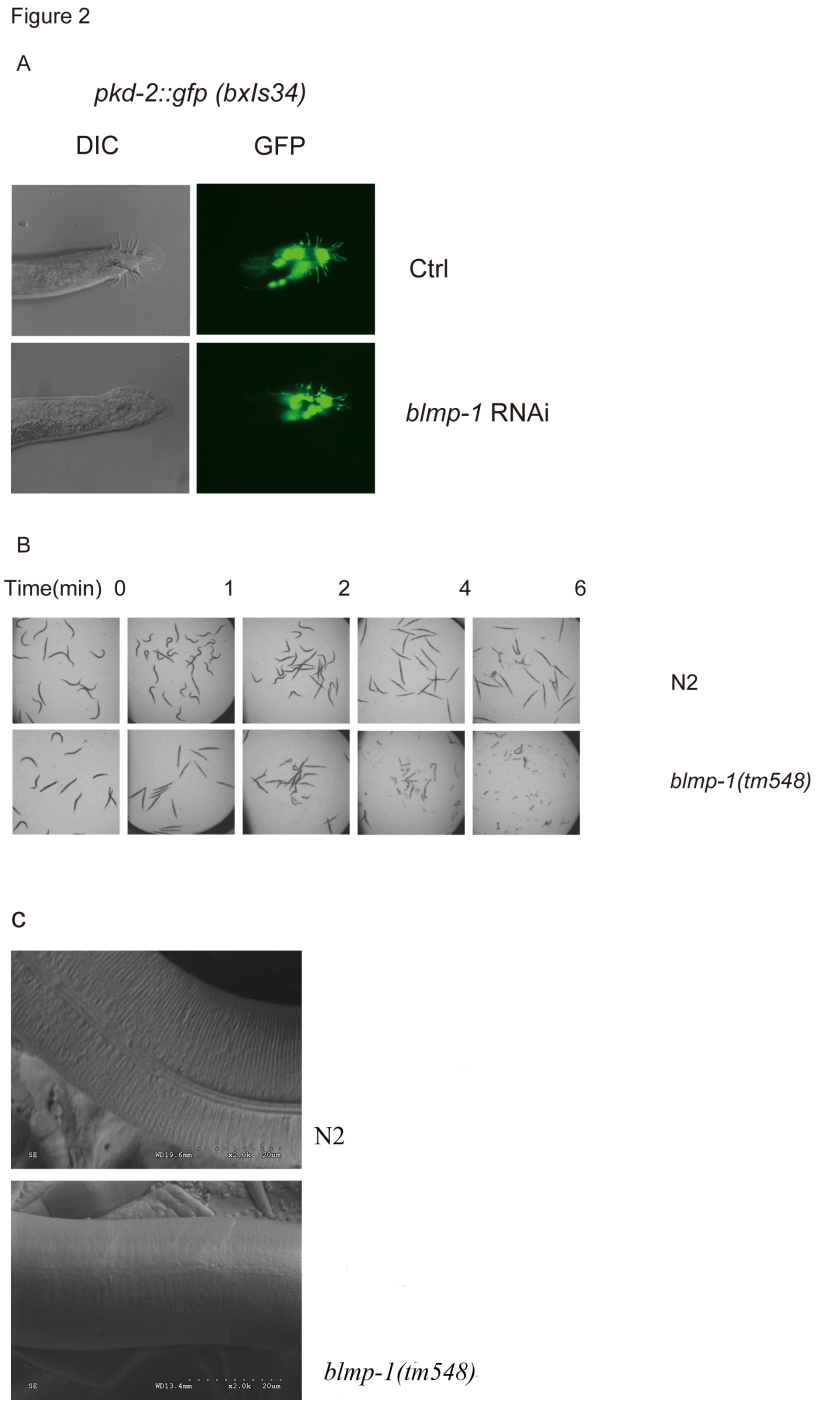

Figure 2. Morphological defects are associated with the cuticle but not Hox genes. (A) Representative observation of the *pkd-2::gfp* labeled male tail. There are no neuron development defects that can be visualized by *pkd-2::gfp*; however, the male tail defect can clearly be seen in the *blmp-1* mutant male worm's tail, indicating that the defect is likely caused by collagen disruption but is independent of HOX gene function in development. More than 30 worms were observed for each group, and typical images are shown here. (B) *blmp-1* mutant worms are more sensitive to the oxidative reagent. In the oxidative reagent NaClO, *blmp-1* mutants are lysed faster than wild-type N2 worms. (C) The surface of all *blmp-1* mutant worms(n=38) don't have clearly alae in the adult stage while N2 (n=36) have clearly alae at 2000x.

Figure 3

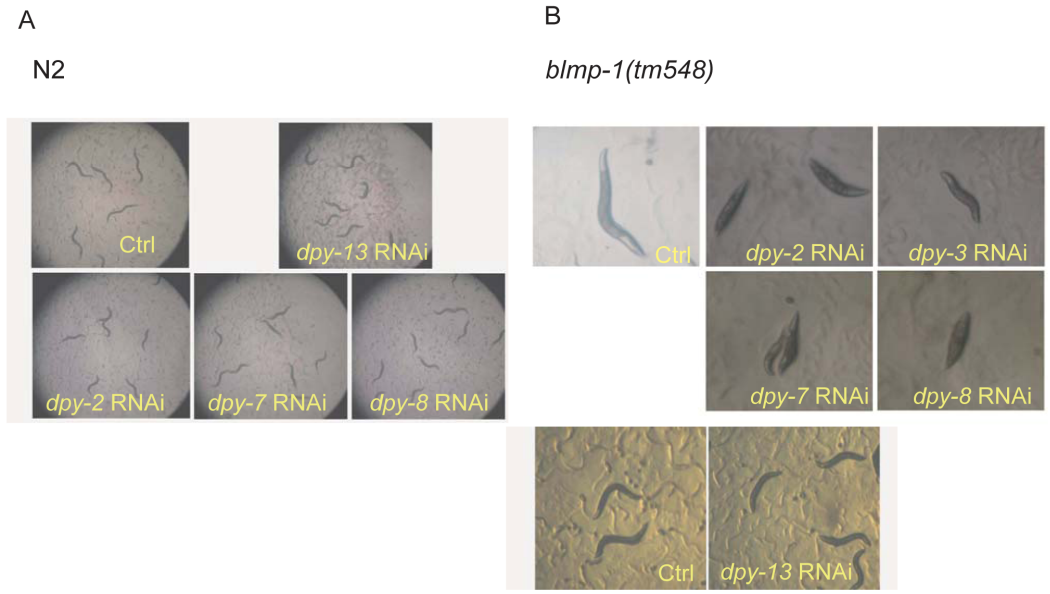

Figure 3. Typical collagen genes that cause *Dpy* phenotype in N2 worms also induce a more severe dumpy phenotype in *blmp-1(tm548)* mutants.
(A) *Dpy-2 -7*, and *-8* ("no annuli" genes) and *dpy-13* ("narrow annuli" gene) RNAi in N2 worms all lead to *Dpy* phenotype. (B) *dpy-2,-3,-7,* and *-8* RNAi and *dpy-13* RNAi in *blmp-1(tm548)* worms all lead to a compound dumpy phenotype.

Because the mature cuticle is formed by several processing steps after the collagen peptides are translated, any defect in the collagen biosynthesis pathway should be considered in further investigation.Collagen biosynthesis is a complex multi-step process in vertebrates. The main process involves prolyl 4-hydroxylation, procollagen registration and trimerization, transit from the ER, procollagen processing and crosslinking, N-terminal processing, C-terminal processing, and structural crosslinking。

A genome-wide study examining targets of BLMP-1 by ChIP-seq in *C. elegans* has recently become available. The peak significant values for several of the identified gene targets are 1.48E-269 for *bli-4* (NM_001026370), 8.36E-52 for *dpy-10* (NM_062965), and 1.20E-51 for *dpy-2* (NM_062966). This study demonstrates that the *bli-4* locus has a very high signal of BLMP-1 occupation (Niu, 2011), which suggested multiple factors, including collagen and collagen biosynthesis genes are targets of BLMP-1.

4. Discussion

In this study, we investigated the function of *blmp-1* in morphogenesis using *C. elegans* as a model system. Knockdown of *blmp-1* by RNAi or deletion by the mutation *blmp-1(tm548)* results in a number of phenotypes, including dumpy, uncoordinated, a reduced brood size, small tail rays and short lifespan; however, the most remarkable phenotype is the abnormal morphology of the mutants. Using a *pkd-2::gfp* transgene, which allows you to visualize Hox gene function, and an oxidative resistance assay, we ruled out Hox gene dysfunction as a cause of the morphogenesis phenotype and instead identified an abnormal cuticle as being primarily responsible for the morphological defects seen in the *blmp-1* mutants., and further supported by SEM result. The dumpy phenotype could be caused by a collagen biosynthesis defect or a disruption in a collagen gene; however, it was not clear which of these events was downstream of *blmp-1* and causing the mutant phenotype. Because collagen gene expression fluctuates during development, it is not suitable for quantitative PCR analysis, so we were unable to precisely assess changes in the transcription of these genes upon *blmp-1* knockdown. Our data show that knockdown of typical collagen genes in the *blmp(tm548)* mutant lead to a more severe dumpy phenotype, which indicates that collagen biosynthesis may be disrupted in the *blmp-1* mutant.

The abnormal male tail phenotype has also been

found in a genome-wide RNAi screen (Nelson, 2011), which suggests that male tail morphogenesis is governed by a gene regulatory network with a bow-tie architecture that functions to orchestrate regulation of Hox genes, the TGF-beta pathway, nuclear hormone receptors, GATA transcription factors and *blmp-1*, supporting our findings in this study. Our studies suggest that the *blmp-1* mutant male tail defect is caused by abnormal cuticle morphogenesis.

The SET domain is present in numerous histone lysine methyltransferases that are involved in chromatin remodeling (Dillon, 2005). Though there is no data suggesting *Blimp-1* acts on its own to modify histones, this protein may have multiple binding partners such as the H3K9 methytransferase G9a (Gyory, 2004), which directly binds to LSD1 and plays a key role in gene silencing and the mediation of plasma cell differentiation (Su, 2009). This possibility suggests that BLMP-1 may function in epigenetic regulation. In the context of aging, *blmp-1* was identified in a screen for lifespan regulators and, consistent with this finding, RNAi targeting *blmp-1* at the developmental stage shortened the lifespan significantly (Greer*, et al.*, 2010). In our study, we further examined this shortened lifespan phenotype by inducing RNAi at different developmental stages. *Blmp-1* RNAi at adulthood does not affect lifespan and may even increase lifespan slightly, which highlights the multiple functions of BLMP-1 in developmental and post-developmental stages.

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