The Tumor Suppressor BCL7B Functions in the Wnt Signaling Pathway

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Abstract

Human BCL7 gene family consists of BCL7A, BCL7B, and BCL7C. A number of clinical studies have reported that BCL7 family is involved in cancer incidence, progression, and development. Among them, BCL7B, located on chromosome 7q11.23, is one of the deleted genes in patients with Williams-Beuren syndrome. Although several studies have suggested that malignant diseases occurring in patients with Williams-Beuren syndrome are associated with aberrations in BCL7B, little is known regarding the function of this gene at the cellular level. In this study, we focused on bcl-7, which is the only homolog of BCL7 gene family in Caenorhabditis elegans, and analyzed bcl-7 deletion mutants. As a result, we found that bcl-7 is required for the asymmetric differentiation of epithelial seam cells, which have self-renewal properties as stem cells and divide asymmetrically through the WNT pathway. Distal tip cell development, which is regulated by the WNT pathway in Caenorhabditis elegans, was also affected in bcl-7-knockout mutants. Interestingly, bcl-7 mutants exhibited nuclear enlargement, reminiscent of the anaplastic features of malignant cells. Furthermore, in KATOIII human gastric cancer cells, BCL7B knockdown induced nuclear enlargement, promoted the multinuclei phenotype and suppressed cell death. In addition, this study showed that BCL7B negatively regulates the Wnt-signaling pathway and positively regulates the apoptotic pathway. Taken together, our data indicate that BCL7B/BCL-7 has some roles in maintaining the structure of nuclei and is involved in the modulation of multiple pathways, including Wnt and apoptosis. This study may implicate a risk of malignancies with BCL7B-deficiency, such as Williams-Beuren syndrome.

Introduction

Cytogenetic abnormalities of chromosome 7 occur frequently in patients with cancer. Patients with certain types of malignant transformation, such as acute lymphoblastic leukemia, myelodysplastic syndrome, or juvenile myelomonocytic leukemia, frequently have deletions or abnormalities in chromosome 7 [1–4]. Some of the genes located on chromosome 7 are thought to act as tumor-related genes, with roles in cancer initiation and/or progression. However, few studies have investigated the molecular mechanisms controlled by specific genes located on chromosome 7.

One of the most well-known diseases related to chromosome 7 microdeletions is Williams-Beuren syndrome (WBS), a contiguous gene syndrome with a dominant autosomal inheritance pattern. WBS patients show a variety of phenotypes, including elfin face, mental retardation, reduced spatial reasoning capacity, supravalvular aortic stenosis, and peripheral pulmonic stenosis. In the past three decades, several reports have described the occurrence of malignant diseases in WBS patients [5–9]. These reports have shown that patients with WBS are at an increased risk of malignant transformation due to aberrations in candidate genes, such as BCL7B.

BCL7B is a member of the BCL7 gene family; members of this gene family, including BCL7A and BCL7C, located on chromosomes 12 and 16, respectively, have a conserved amino-terminal region as their functional domain [10]. A number of studies have found that BCL7 family members are involved in cancer initiation, progression, and development. For example, decreased expression of BCL7A may be a risk factor for astrocytoma [11], Burkitt lymphoma [12], non-Hodgkin’s lymphoma [13], mycosis fungoides [14], and cutaneous T cell lymphoma [15]. Although the BCL7 gene family is thought to have tumor-associated functions, little is known regarding the specific functional roles of BCL7 genes; this may be attributed to the functional redundancy among BCL7 family members, which makes it difficult to analyze the individual roles of BCL7 genes.

In this study, the functional significance of C28H8.1 (designated here as bcl-7), which shares 41% homology with the amino-terminal region of human BCL7 gene family and is the only
KATOIII cells, a human gastric cancer cell line [16].

...function of the Wnt-signaling pathway and apoptotic pathway. In addition, bcl-7 deletion mutants show enlarged nuclei in epithelium and germ cells. Furthermore, in KATOIII human gastric cancer cells, BCL7B knockout induces nuclear enlargement, as observed in Caenorhabditis elegans, and promotes the multinucleated phenotype, both of which are reminiscent of malignant diseases. BCL7B also negatively regulates the Wnt-signaling pathway and promotes the apoptotic pathway, similar to Caenorhabditis elegans. Altogether, this study may open the door for understanding the function of BCL7 family in cell differentiation and malignancies.

Author Summary

BCL7B, a member of the human BCL7 gene family, is deleted in patients with Williams-Beuren syndrome. Although several clinical studies have suggested that malignant diseases occurring in patients with Williams-Beuren syndrome are associated with aberrations in BCL7B, little is known regarding the physiological function of this gene. Here, we show that bcl-7, the only homolog of BCL7 gene family in Caenorhabditis elegans, regulates asymmetric cell differentiation in somatic "stem-like" seam cells through at least the Wnt pathway and promotes the apoptotic pathway. In addition, bcl-7 deletion mutants show enlarged nuclei in epithelium and germ cells. Furthermore, in KATOIII human gastric cancer cells, BCL7B knockout induces nuclear enlargement, as observed in Caenorhabditis elegans, and promotes the multinucleated phenotype, both of which are reminiscent of malignant diseases. BCL7B also negatively regulates the Wnt-signaling pathway and promotes the apoptotic pathway, similar to Caenorhabditis elegans. Altogether, this study may open the door for understanding the function of BCL7 family in cell differentiation and malignancies.

Results

bcl-7 is required for normal seam cell development in C. elegans

First, we knocked down bcl-7 expression using the feeding RNA interference (RNAi) technique with a bcl-7-specific RNAi clone and observed the phenotypes associated with bcl-7 downregulation in wild-type C. elegans hermaphrodites. Downregulation of bcl-7 in wild-type worms resulted in the egg-laying defective (Egl) phenotype (S1B and S1E Fig.), the protruding vulva (Pvl) phenotype (S1C Fig.), and the burst phenotype (S1D Fig.), reminiscent of defects in epidermal barrier formation [17]. Therefore, we hypothesized that bcl-7 is involved in the development of the epidermis.

Next, to examine the phenotypes produced by bcl-7 knockout, we generated a bcl-7 deletion mutant, tm5268, containing a deletion of 0.7 kbp (Fig. 1A). Because the deletion covered almost all bcl-7 regions, including the amino-terminal domain, which is conserved and considered to be the functional domain [10], tm5268 is practically a null mutant. The tm5268 mutant showed a variety of phenotypes, including Pvl (the rate was 61.4%; S1F-I Fig.), the alae with only one or two ridges) or absent in tm5268 worms in contrast to wild-type worms (Fig. 1B and 1C). The Pvl phenotype and alae malformation are caused by defects in epidermal cells, particularly epidermal stem-like seam cells [17,18]. The presence of these phenotypes in bcl-7 deletion mutants suggests that BCL-7 influences the development of seam cells, which have both self-renewal potential and differentiation capability, in C. elegans.

Then, we investigated whether the number of seam cells was altered in bcl-7 deletion mutants by analyzing transgenic worms carrying the sem::gfp transgene [19] as a marker of seam cell nuclei. In a wild-type L4-stage hermaphrodite, there were 16 seam cells on each side (Fig. 1E). By contrast, the number of seam cells was significantly reduced in the mutant worms (Fig. 1I), and sem::gfp-negative cells were observed more often in the V cell lineage than in the H and T cell lineages (Fig. 1I). Differences between wild-type worms and tm5268 worms were found in most larval stages, except for the early L1 stage (Fig. 1F-I and 1K). The expression pattern of another seam cell marker, edh-3::gfp [20], which is localized to the cytoplasm of seam cells, also revealed that the number of GFP-positive cells was lower in bcl-7 mutants (S2A-D Fig.). Both the defect of alae and the decreased seam cell number were rescued by the introduction of bcl-7 genomic DNA, Pbcl-7::bcl-7::egfp (tm5268;tmEx2966) or Pbcl-7::bcl-7::mCherry (tm5268;tmEx3496) (Fig. 1D and 1J-M). The expression of the rescue constructs was ubiquitous, including in the hypodermis, from the embryonic stage to the adult stage, and BCL-7 was localized to the nuclei (S3A-H Fig.). Therefore, we hypothesized that BCL-7 functions cell-autonomously in seam cells. To test this hypothesis, we analyzed whether expressing a seam cell-specific construct rescues decreased seam cell number. The number of seam cells was significantly increased by the introduction of bcl-7 genomic DNA under a seam cell-specific promoter (tmEx1265::promoter::bcl-7, smp::mCherry) [Fig. 1M]). These results suggest that BCL-7 is involved in the normal development of seam cells and functions cell-autonomously in seam cells.

Next, we addressed whether the observed decrease in seam cells in tm5268 worms resulted from the hyperactivation of apoptosis. To examine this, we analyzed whether the apoptotic pathway was hyperactivated in bcl-7 deletion mutant worms using worms with a mutation in the cod-3 gene, which encodes a member of the caspase family required for the execution of apoptosis in C. elegans [21]. However, bcl-7[III];cod-3[IV] double mutants did not exhibit increased numbers of seam cells compared with bcl-7 single mutants (the average seam cell number in adult, double-mutant hermaphrodites was 3.6 (n = 12)), suggesting that the decrease in the number of seam cells in bcl-7-deficient worms is not caused by hyperactivation of apoptosis.

In wild-type C. elegans, seam cells divide asymmetrically during each larval stage. The anterior daughter cell loses its seam cell properties and differentiates into a hyp7 cell, whereas the posterior cell keeps its self-renewal potential, remaining a seam cell (S4S Fig.) [22,23]. To examine whether the reduction in seam cell numbers in tm5268 is followed by an increase in the hyp7 cell number, we used transgenic worms that expressed an adult-specific hypodermal marker, col-19::gfp [24,25]. In wild-type animals, col-19::gfp was expressed in the hypodermal syncytial hyp7 cells and 16 seam cells (Fig. 2A, 2B, and 2E). The number of GFP-positive hyp7 cells in tm5268 was not increased but tended to decrease compared with the number in the wild-type worms. Additionally, the number of GFP-positive seam cells in tm5268 was significantly decreased compared with the number in the wild-type worms (Fig. 2G-E). Interestingly, the nuclei of hyp7 cells from bcl-7 mutants were significantly enlarged and had an irregular shape compared to those from wild-type animals (7.66 ± 0.10 μm and 6.77 ± 0.14 μm, p < 0.001; S5A-D Fig.).
**Fig. 1. Knockout of bcl-7 inhibits normal seam cell development in Caenorhabditis elegans.** *A*: Structure of bcl-7. Black boxes, exons; bent lines, introns. The region deleted in the strain tm5268 is shown as a dotted line, below. The numbers indicate the location in the cosmID C28H8. *B*-**D**: Nomarski images of adult hermaphrodites. A wild-type hermaphrodite had complete alae (the region between arrows) (*B*); Alae were incomplete in bcl-7 (tm5268) hermaphrodites. A region between dotted arrows indicates partial alae with only two ridges. The area without arrows indicates regions without alae (*C*); a transgenic rescue line carrying *Pbcl-7::bcl-7::egfp* reporters (*tm5268;tmEx2966*) with complete alae (three ridges) (*D*). *E*-**J**: Examples of SCM::GFP localization in wild-type, tm5268, and tm5268;tmEx3496 hermaphrodites with *wls*1 (SCM::GFP). tm5268;tmEx3496 is a transgenic rescue line carrying *Pbcl-7::bcl-7::mCherry* reporters. A wild-type L4 hermaphrodite expressing SCM::GFP in 16 seam cell nuclei (*E*); tm5268 hermaphrodites carrying *scm::gfp* reporters (*H*, *G*), and a tm5268;tmEx3496 hermaphrodite expressing SCM::gfp reporters (*J*). Inserts show the Nomarski images of the same animals of the fluorescence images. *K*: Bar chart showing the average number of seam cells (about the worms at each larval stage, except for the middle L2 stage, and the young adult stage) expressing GFP in wild-type and tm5268 hermaphrodites (*n* = 21–46). *L*: Percentages of worms with complete alae in wild-type, tm5268, and tm5268;tmEx2966 adult hermaphrodites (*n* = 20–30). *M*: Bar chart showing the average number of seam cells expressing GFP in wild-type, tm5268, tm5268;tmEx3496, and tm5268;tmEx4126[SCMp::bcl-7, SCMp::mCherry] adult hermaphrodites with *wls*1 (SCM::GFP) (*n* = 15–23). Error bars indicate the standard error of the mean (SEM). Asterisks indicate statistical significance compared with each other. *p* < 0.05. **p** < 0.005. ***p** < 0.001. N.S.: no significance. Scale bar = 25 μm.

to determine whether the somatic stem-like cells acquire another fate in the cell lineage, we analyzed neural cells, including sensory PVD neurons, PDE neurons, and phasmids, derived from V- and T-cell lineages. PVD and PDE neurons originate from an asymmetric division of the V5 cell (*s4* Fig.) [23], whereas phasmid cells are generated by the asymmetric T-cell division when the posterior daughter cell maintains the seam cell phenotype and the anterior daughter cell commits to the neural fate, giving rise to the phasmid (*s4t* Fig.) [23]. We analyzed the cell fates of PVD and PDE in transgenic worms carrying the PVD and PDE markers *des-2::gfp* and *dat-1p::gfp*, respectively [20,27]. No extra PVD or PDE cells were found in any of the *bcl-7* mutants (*n* = 10–15, *s4a-j* Fig.). In wild-type worms, phasmids can be detected by the uptake of fluorescent dye [20]. Similarly, in all *bcl-7* mutants, both socket cells in the phasmid, but not other cells, absorbed the fluorescent dye in dye-filling assays (*n* = 10, *s4k-n* Fig.). These results suggest that stem-like cells fail to acquire a terminal differentiation fate in *bcl-7* mutants.

Next, we hypothesized that more undifferentiated cells are present in *bcl-7* deletion mutants because we observed an increase in the number of cells with enlarged nuclei, as well as the loss of differentiated-cell markers. To test this hypothesis, we analyzed the expression patterns of an undifferentiated state marker, *egl-27*/*mit* [29], in wild type and tm5268 worms carrying the *egl-27p::his-24::mCherry* transgene. The expression pattern of *egl-27* was different between wild type and *bcl-7* mutant worms. In wild type worms, *egl-27* was strongly expressed in the nuclei of intestinal cells (*s6a-b* Fig.) and weakly expressed in the nuclei of epidermis (*s6c-d* Fig.) during the L4 stage (*n* = 10). By contrast, *egl-27* was strongly expressed ubiquitously in particular in the epidermal cells including both seam cells and hyp7 cells in *bcl-7* mutant worms (*n* = 10, *s6e-h* Fig.). Furthermore, we analyzed the expression levels of undifferentiated cell markers using a quantitative real-time polymerase chain reaction (qRT-PCR) analysis. Both *egl-27* and *ceh-6*, markers of the undifferentiated state in *C. elegans*, were significantly increased in the tm5268 mutants compared with the wild-type worms (*s6j* Fig.). These results suggest that stem-like cells fail to acquire a terminal differentiation fate in *bcl-7* mutants.

**Loss of BCL-7 function affects gonadal size and germ cell differentiation**

The Ste phenotype was observed in *bcl-7* deletion mutants (*Fig. 3M*); specifically, no oocytes were detected in the homozygous mutants, and their gonads were shortened (*Fig. 3D*). In addition, the brood size of *bcl-7* heterozygotes was significantly reduced compared with that of wild-type worms (*Fig. 3M*), which indicates that the genetic trait of *bcl-7* mutation in *C. elegans* is haploinsufficiency, similar to that of human diseases. These results revealed that the loss of BCL-7 function affects not only seam cells but also the development of somatic gonads and/or germ cells. To
further investigate the gonadal and germ cell phenotypes in *tm5268* worms, we performed diamidino-phenylindol (DAPI)-staining and fluorescence immunostaining with an anti-phospho-histone H3 (PH3) antibody as a mitotic marker [30]. In wild-type *C. elegans* hermaphrodites, the mitotic region covered approximately 10–15 cell diameters as previously reported (Fig. 3A) [31]. In *bcl-7* mutant worms, PH3-positive cells were found, but tended to decrease compared with the wild type worms (Fig. 3D). In addition, they were occasionally observed farther from the distal tip cells (DTCs) than in wild type worms. These results suggest that the shortened gonad observed in *tm5268* mutants was not because of the absence of mitosis but rather because of the decrease of mitosis and may be due to the defects of cell differentiation after mitosis. In addition, the average length of the major axis of germ cell nuclei was 3.17±0.03 μm in wild type worms (Fig. 3A–C, S2A Fig.). By contrast, *bcl-7* mutants exhibited irregularly shaped and significantly larger germ cell nuclei measuring 4.42±0.08 μm (Fig. 3D–F, S7B Fig.). Furthermore, the number of germ cells in *tm5268* worms was decreased compared with that in wild-type worms (Fig. 3D–F). Thus, BCL-7 is necessary for gonadal development, particularly for gonadal arm elongation, and for germ cell entry into meiosis. The Ste phenotype, shortened gonads, and enlarged germ cell nuclei in *tm5268* worms were rescued by the introduction of *bcl-7* genomic DNA under the *bcl-7* promoter (Fig. 3G–I, 3M and S7C). The rescue construct was strongly expressed in the nuclei of germ cells and gonadal sheath cells (S3I–L Fig.). These results suggest that the expression of BCL-7 in DTCs, germ cells, and/or gonadal sheath cells is necessary for its function.

To further examine the observed phenotypes, we introduced *bcl-7* genomic DNA under the DTC-specific *lag-2* promoter (Plag-2::*bcl-7::*egfp). The Ste phenotype of *tm5268* worms was partially rescued by the expression of the *bcl-7* gene in DTCs (Fig. 3M). In addition, thin gonads and irregularly shaped nuclei of germ cells were also partially rescued by the expression of this construct (Fig. 3J–L and S7D). These results suggest that BCL-7 expression in DTCs, germ cells, and/or gonadal sheath cells is partially rescued by the expression of the *tm5268* genomic DNA under a sheath cell-specific sequence (*tm5268*::*bcl-7::egfp, *tmEx4104*). Our study showed that BCL-7 is necessary for gonadal development, particularly for gonadal arm elongation, and for germ cell entry into meiosis. The Ste phenotype, shortened gonads, and enlarged germ cell nuclei in *tm5268* worms were rescued by the introduction of the *tm5268* genomic DNA under the *bcl-7* promoter (Fig. 3G–I, 3M and S7C). The rescue construct was strongly expressed in the nuclei of germ cells and gonadal sheath cells (S3I–L Fig.). Thus, BCL-7 is necessary for gonadal development, particularly for gonadal arm elongation, and for germ cell entry into meiosis. The Ste phenotype, shortened gonads, and enlarged germ cell nuclei in *tm5268* worms were rescued by the introduction of the *bcl-7* genomic DNA under the *bcl-7* promoter (Fig. 3G–I, 3M and S7C). The rescue construct was strongly expressed in the nuclei of germ cells and gonadal sheath cells (S3I–L Fig.). These results suggest that the expression of BCL-7 in DTCs, germ cells, and/or gonadal sheath cells is necessary for its function.

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One of the factors that regulates the size of the gonads and the timing of the entry into meiosis is LAG-2, a homolog of the Notch receptor ligand that is secreted by DTCs [35,36]. We therefore hypothesized that DTC functions, including LAG-2 secretion, may be impaired in *bcl-7* mutants. We analyzed DTCs using worms carrying the *lag-2p::gfp* transgene (Fig. 3E) [37]. Two GFP-positive DTCs were detected in 100% (17/17) of wild-type worms (S8A and B Fig.), whereas only 19.5% (25/128) of *bcl-7* mutant worms were positive for GFP in both DTCs. The remaining 80.5% (103/128) of *bcl-7* mutant worms were positive for GFP in only one DTC. Furthermore, 12.5% (16/128) of *bcl-7* mutants showed mispositioning of the DTCs with the expression of *lag-2p::gfp* (S8E–H Fig. and S1 Table). Interestingly, heterozygous *bcl-7* mutants exhibited the same phenotypes; i.e., 30% (6/20) of heterozygous *bcl-7* mutants had expression of *lag-2p::gfp* on only one DTC and 50% (10/20) showed mispositioning of the DTC with GFP expression (S1 Table, middle row). These differences between homozygous and heterozygous mutants further demonstrate that the phenotype is semi-dominant. These results suggest that BCL-7 partially controls the differentiation of DTCs.

Next, we attempted to determine the time course of BCL-7 function using a chromophore-assisted light inactivation (CALI) assay. We created a light-inactivatable BCL-7::KillerRed fusion protein by substituting *egfp* of the rescue construct with *KillerRed*. We generated transgenic worms (*tmEx3878*) that expressed the *bcl-7* promoter-driven BCL-7::*KillerRed* construct (*Plag-2::*bcl-7::*KillerRed*) in the *bcl-7*-deficient background (i.e., *tm5268*). In the absence of green-light illumination, this transgene rescued the Ste phenotype (similar results were observed independently in three transgenic lines). When the animals were illuminated with a green light, the BCL-7 protein fused to *KillerRed* was inactivated [38,39], allowing inactivation of BCL-7 function. We exposed *tm5268*::*tmEx3878* to a green light starting from the comma stage of the embryo, the early larval L1 stage, or the L2 stage to the young adult stage and analyzed the Ste phenotype. All worms exposed to the green light at the L2 stage were fertile; however, approximately 90% (70/79) of worms illuminated at the comma stage and 54.5% (18/33) of worms illuminated at the early L1 stage retained the Ste phenotype (S7E Fig.). In addition, we performed a pulse experiment to identify the most critical stage in development for the function of BCL-7. We exposed the same transgenic worms to a green light during the comma and early L1 stages and found that only 20% of the worms were fertile (S7E Fig.). These results imply that the expression and function of BCL-7 in DTCs begin during the early L1 stage, which corresponds to the timing of asymmetric cell division in somatic DTCs.

**RNAi-based screening for suppressors of the bcl-7 mutant phenotypes**

Loss of BCL-7 function resulted in defects in both seam cells and the gonads, suggesting that BCL-7 controls the asymmetric division of cells in *C. elegans*. More than one genetic pathway is involved in the asymmetric division and differentiation of these cells. Therefore, we next sought to determine the role of BCL-7 in these pathways by screening for genes that could suppress the phenotypes of the *tm5268* mutant (*tm5268*). Using *bcl-7* mutants, with wild-type worms as the control, we carried out a feeding RNAi screen of 96 genes involved in the development of seam cells and/or gonads, including genes in the WNT/β-catenin and Notch pathways, heterochronic genes, and other genes that regulate the division and differentiation of seam cells and/or somatic gonadal precursors (SGPs) (functionally classified in S2 Table). We found that downregulation of either *wrm-1* or *lsy-22* suppressed the phenotypes of the *bcl-7* mutant *tm5268* (Fig. 4A–I and S3 Table). Downregulation of *WRM-1*, a homolog of β-catenin [40–42], suppressed both seam cell reduction and Ste phenotypes. Although
Fig. 3. Knockout of bcl-7 affects gonadal development and germ cell proliferation in Caenorhabditis elegans. A–C: A dissected adult germline of a wild-type adult hermaphrodite. A dissected gonad stained with DAPI (blue) and an anti-PH3 antibody (pink) as a mitotic cell-specific marker (A), and the regions of mitosis (B) and meiosis (C) of the gonad stained with DAPI. The arrowhead indicates a distal tip cell (DTC). The white arrows indicate the corresponding position of the gonad.

D–F: A dissected germline of a tm5268 adult hermaphrodite. A dissected gonad stained with DAPI (blue) and an anti-PH3 antibody (pink) as a mitotic cell-specific marker (D), and the regions of mitosis (E) and meiosis (F) of the gonad stained with DAPI. The arrowhead indicates a DTC. The white circles indicate the corresponding position of the gonad.

G–I: A dissected germline of a tm5268;tmEx2966 adult hermaphrodite. A dissected gonad stained with DAPI (blue) and an anti-PH3 antibody (pink) as a mitotic cell-specific marker (G), and the regions of mitosis (H) and meiosis (I) of the gonad stained with DAPI. The arrowhead indicates a DTC. The white rhomboid indicates the corresponding position of the gonad.

J–L: A dissected germline of a tm5268;tmEx3873 adult hermaphrodite carrying Plag-2::bcl-7::egfp as a DTC-specific rescue construct. A dissected gonad stained with DAPI (blue) (J), and the regions of mitosis (K) and meiosis (L) of the gonad stained with DAPI.
LSY-22 is considered a homolog of Groucho-like protein [43] and is also a member of the noncanonical Wnt pathway, bsg-22-specific RNAi only partially suppressed the Ste phenotype and did not affect the number of seam cells. Subsequently, we focused on WRM-1, which exhibited potent effects in both somatic cells and germ cells and was expressed in both seam cells and SGP s. Interestingly, the suppressor effect of wrm-1 RNAi appeared to be dependent on the strength of the RNAi clone (S3 Table). The effect of the wrm-1c RNAi clone (constructed with a cDNA generated from wild-type worms) on N2 was stronger than that of wrm-1 RNAi (from the Ahringer library) and comparable with the phenotypes of the wrm-1 mutant strains (WormBase; http://www.wormbase.org/). The effect of diluted wrm-1c RNAi seemed comparable to RNAi by a genomic clone on suppressing the bcl-7 mutant’s phenotypes (Fig. 4E, S3 Table).

Based on the results of the suppressor screening, we hypothesized that BCL-7 negatively regulates WRM-1 expression. To test this hypothesis, we analyzed the expression levels of wrm-1 mRNA using qRT-PCR. Additionally, because WRM-1 is one of the β-catenin homologs in C. elegans, we also analyzed the expression levels of the other β-catenin homologs, BAR-1 and SYS-1. Compared with wild-type worms, bar-1 and sys-1 mRNAs were significantly increased in bcl-7 deletion mutants (S9A Fig.). There was also a trend for increased wrm-1 in the mutants (S9A Fig.). These results suggest that BCL-7 functions as a negative regulator of the expression of three β-catenin homologs in C. elegans.

The phenotypes of the bcl-7 deletion mutants were different from the pop-1 deletion mutants and wrm-1(gf) mutants, which are associated with hyperactivation of the Wnt pathway. Therefore, we hypothesized that BCL-7 not only functions as a negative regulator but also affects the Wnt pathway in a different mechanism. Because WRM-1/β-catenin regulates the levels of POP-1 in the nuclei of target cells during asymmetric cell division, we assumed that the cellular distribution of WRM-1 or POP-1 may be altered in bcl-7 mutants, and therefore we analyzed the localization of WRM-1 and POP-1 using WRM-1::GFP [44] and GFP::POP-1 [45] fusion reporter proteins. In wild-type worms, the cellular localization of WRM-1::GFP in mother seam cells at the L2 stage was observed near the cell cortex in the anterior half of the cells, as shown by previous reports (Fig. 5A–B) [44]. Furthermore, after division, the fluorescence intensity of WRM-1::GFP was stronger in posterior daughter cells than in anterior cells (Fig. 5E–F). In bcl-7 mutants, the cellular localization of WRM-1 before cell division was similar to that in wild-type worms (Fig. 5C–D). However, in daughter cells, the fluorescence intensity in the anterior cells was equal to or stronger than that in the posterior cells in approximately 50% of tm5268 mutant worms (Fig. 5G–I). The asymmetric localization pattern of GFP::POP-1 in tm5268 worms was also different from that in wild-type worms (Fig. S3J–N), as expected due to the change in WRM-1 localization.

We also analyzed the localization of POP-1 in somatic gonadal precursor Z1 and Z4 cells and their daughter cells using gfp::pop-1 reporter transgenes. In wild-type worms, GFP expression was stronger in the nuclei of Z1.p and Z4.a cells than in Z1.a and Z4.p cells (Fig. 5O–P). By contrast, approximately 60% of tm5268 mutants showed aberrant POP-1 localization (Fig. 5Q–S). In addition, 35.7% of the tm5268 mutants showed both wild-type and aberrant POP-1 asymmetry within the same gonad, as shown in Fig. 5T.

Next, we determined whether the impaired nuclear localization of POP-1 disturbs the asymmetric localization of gene products downstream of POP-1 in DTCs. HLH-2 is a downstream factor in the POP-1/TCF pathway [46,47] and an important transcription factor regulating LAG-2; therefore, it is a determining factor in DTC development. We analyzed the localization of HLH-2 using an hlh-2p::gfp::hlh-2 reporter construct (qyIs174) and found that only 28.2% (13/46) of bcl-7 mutants showed hlh-2p::gfp::hlh-2 expression in two DTCs, whereas 100% (10/10) of wild-type worms were positive for GFP in two DTCs (S8L–J Fig., and S4 Table). The remaining mutants showed decreased hlh-2p::gfp::hlh-2 expression, with either no expression observed or expression observed in only one DTC. In addition, 46% (21/46) of the bcl-7 mutants showed mispositioning of the DTCs (S8K–N Fig. and S4 Table). This result is consistent with the expression pattern of lag-2p::gfp in the mutant worms (S1C–H Fig.). HLH-2 is an important transcription factor that regulates LAG-2 and is thus a determining factor for DTCs; therefore, this result may also partially explain the defects in gonadal development in the bcl-7 mutant (tm5268). Taken together, our data suggest that BCL-7 regulates POP-1 distribution in the Z-cell lineage by controlling WRM-1 activity and therefore affects the expression pattern of HLH-2.

BCL-7 affects the apoptotic pathway in C. elegans

BCL-7 has several functions in C. elegans. Therefore, we examined whether BCL-7 regulates the apoptotic pathway by analyzing the number of PLM neurons using transgenic worms carrying the Pmec-4::gfp transgene (bzIs8) [48] as a marker of PLM nuclei. The number of PLM neurons should either decrease or increase if the apoptotic pathway is activated or suppressed, respectively, in tm5268 mutant worms, because one cell in the PLM lineage undergoes to apoptosis. In wild type worms, two PLM neurons were observed in each worm as shown in S4O–P Fig. (n = 10). Similarly, in bcl-7 deletion mutants, two PLM neurons were exhibited in the tail of each worm (n = 12, S4Q–R Fig.). This result showed that extra neurons, which are caused by the suppression of the apoptotic pathway, were not found in bcl-7 mutants. Next, we analyzed whether the levels of apoptosis-related factors were increased in tm5268 mutant worms. The expression of the anti-apoptotic factor, ced-9, was significantly increased in tm5268 mutant worms compared with wild-type worms (S9B Fig.). These results suggest that the apoptotic pathway is suppressed in tm5268 mutant worms.

BCL7B regulates the size of nuclei in human gastric cancer cells

As described above, BCL-7 likely affects the morphology of nuclei and functions in the Wnt-signaling pathway in the development of C. elegans. Because bel-7 is a homolog of the human BCL7B gene, we wondered whether BCL7B has similar roles in humans. To examine this, we used KATOIII cells, which are derived from gastric signet-ring cell cancer and express only BCL7B of the BCL7 family members. First, we examined whether siRNA-mediated BCL7B knockdown results in nuclear enlargement,
as was observed in *C. elegans* bcl-7 mutants. As expected, KATOIII cells transfected with *BCL7B* siRNA showed enlarged nuclei compared with control cells transfected with nontargeting siRNA (170.8 ± 7.8 μm² and 98.0 ± 4.5 μm²; Fig. 6A–D, and S10A–B Fig.). In addition, the occurrence of multinucleated cells (containing two or more nuclei) was increased in *BCL7B*-knockdown cells compared with control cells (the rates of multinucleated cells were 18.8% and 5.7%, respectively; Fig. 6E–H). The average number of nuclei per cell was 1.13 in control cells and 1.41 in *BCL7B*-knockdown cells (*p*, 0.05, Student’s *t*-test). In general, cell nuclear enlargement and multinucleated cells are observed in undifferentiated cells, such as cancer cells. Therefore, we hypothesized that the downregulation of

![Fig. 4. Downregulation of *wrm-1* or *lsy-22* suppresses the phenotypes of *bcl-7* mutants. A–D: Nomarski (A, C) and GFP images (B, D) of *tm5268* adult hermaphrodites carrying an *scm::gfp* reporter treated with empty vector (L4440) (A, B) or *wrm-1*-specific RNA (C, D). E: Bar chart representing the average number of seam cells in wild-type, *tm5268*, *tm5268*L4440 (RNAi), or *tm5268; wrm-1* (RNAi) hermaphrodites at the L1, late L2, late L3, or young adult stages. F–H: Nomarski images of *tm5268* treated with L4440 (RNAi), *wrm-1* (RNAi), or *lsy-22* (RNAi). Eggs in the uterus are outlined with dotted lines (G, H). I: Percentages of the Ste phenotypes in wild-type and *tm5268* adult hermaphrodites treated with L4440 (RNAi), *wrm-1* (RNAi), or *lsy-22* (RNAi). Numbers of examined animals were more than 60 for all strains. Error bars indicate SEM. Asterisks indicate statistical significance. *p*, 0.05. **p*, 0.005. N.S.: no significance. Scale bar = 50 μm. doi:10.1371/journal.pgen.1004921.g004]
Fig. 5. BCL-7 is involved in the asymmetric localization of Wnt components in Caenorhabditis elegans. A–D: Nomarski (A, C) and GFP (B, D) images of wild type (A, B) and tm5268 (C, D) L2-stage hermaphrodites carrying a wrm-1:gfp reporter (osIs5) at pre-division states. Seam cells are traced with dotted ovals (A, C). E–H: Nomarski (E, G) and GFP (F, H) images showing the localization of WRM-1::GFP in two daughter cells of wild-type or tm5268 L2 hermaphrodites at post-divisions. The pairs of daughter cells are outlined with dotted ovals. I: Frequency of the localization patterns of WRM-1::GFP. The equal and unequal signs indicate the relative intensities of nuclear WRM-1::GFP between two daughter cells. J–M: Nomarski (J, L) and GFP (K, M) images showing the localization of GFP::POP-1 (qIs74) in two daughter cells of wild-type (J, K) or tm5268 (L, M) L1 hermaphrodites in post-division states. The pairs of daughter cells are outlined with dotted ovals. Asterisks indicate nuclei with stronger expression of GFP. N: The frequency of the localization patterns of GFP::POP-1. The equal and unequal signs indicate the relative intensities of nuclear GFP::POP-1.
BCL7B is involved in cell differentiation. To test this hypothesis, we analyzed the expression levels of undifferentiated markers of human cells using qRT-PCR. We found that the stem cell markers, Nanog, Oct3/4, and Sox2, were increased in BCL7B-knockdown KATOIII cells (S1A Fig.). This result suggests that BCL7B affects cell differentiation in KATOIII cells, similar to its role in C. elegans. Because enlarged nuclei are generally a result of uncontrolled DNA synthesis [49], we next tested whether BCL7B-knockdown cells exhibited aneuploidy or cell cycle defects. According to the results of a cell cycle assay, BCL7B knockdown did not induce aneuploidy (S10C-D Fig.) but did result in a significant accumulation of cells in the G0/G1 phase and a decrease in cells in the S phase (Fig. 6J and S10C-E Fig.). Because the observed nuclear enlargement was not induced by an alteration in DNA synthesis, we hypothesized that this phenomenon was caused by increased RNA levels. To test this hypothesis, we analyzed the RNA content of the nucleus by determining the fluorescence intensity of ethidium bromide-stained cells with or without RNase. We found that the fluorescence intensity of ethidium bromide in cells transfected with BCL7B siRNA was significantly stronger than in control cells. Furthermore, the addition of RNase eliminated the difference between the control cells and BCL7B-knockdown cells (S12A–I Fig.). The expression of nuclear paraspeckle assembly transcript 1 (NEAT1), a noncoding RNA and the core molecule of nuclear paraspeckles [50], was increased in BCL7B-knockdown cells compared with control cells (S12J Fig.). These results suggest that BCL7B plays a role in cell cycle progression and in the maintenance of the nuclear structure.

BCL7B regulates several signaling pathways including Wnt and apoptosis

Next, we examined whether BCL7B is involved in the Wnt signaling pathway in human cells, as observed with BCL-7 in C. elegans. We analyzed the expression of β-catenin, an important member of the Wnt signaling pathway, and high-mobility group A1 (HMGA1), one of the target genes of the Wnt pathway [51]. According to a qRT-PCR analysis, the expression levels of β-catenin and HMGA1 were significantly increased in BCL7B-knockdown cells (Fig. 6K, S10F and S10G Fig.). These results suggest that BCL7B functions as a negative regulator of the Wnt pathway in KATOIII cells.

We then analyzed the effects of BCL7B overexpression by transfecting KATOIII cells with a BCL7B_EGFP fusion construct or EGFP alone. Our experiments demonstrated that BCL7B overexpression modulated cell proliferation (S13A Fig.). Specifically, 25.5% of BCL7B-overexpressing cells died two days after transfection with BCL7B_EGFP, compared with 12.3% of control EGFP cells (Fig. 6M). Therefore, we hypothesized that overexpression of BCL7B may promote apoptosis. To test this hypothesis, we analyzed the number of apoptotic cells using flow cytometry. Indeed, overexpression of BCL7B resulted in increased apoptosis compared with the control (Fig. 6N, S13B–C Fig.). To determine whether the apoptotic pathway was conversely inhibited in BCL7B-knockdown cells, we analyzed the survival of BCL7B-knockdown cells and control cells following treatment with actinomycin D. The rate of surviving cells was increased in BCL7B-knockdown cells compared with control cells (Fig. 6I). We next analyzed the expression of apoptosis inhibitors. qRT-PCR analysis showed that the expression of cellular FLICE-like inhibitory protein (c-FLIP), which inhibits apoptosis by antagonizing caspase-8 and caspase-10 [52,53], was significantly upregulated in BCL7B-knockdown cells (Fig. 6L). Another apoptosis inhibitor, Bcl2, was also significantly increased but the apoptotic factor, PTEN, was not increased in BCL7B-knockdown KATOIII cells. Interestingly, Bax, which binds Bcl2 and functions as a positive regulator of apoptosis, was increased in BCL7B-knockdown KATOIII cells, similar to what has been observed in other apoptosis-resistant cells [54]. Collectively, these results suggest that BCL7B is a positive regulator of apoptosis in KATOIII cells and support the hypothesis that BCL7B contributes to the apoptotic pathway.

Discussion

Malignant diseases are caused by defects in cell differentiation, cell cycle progression, DNA repair mechanisms, or apoptotic pathways [55–57]. Although previous studies have revealed the genetic backgrounds of certain types of cancer—for example, the MSH2 gene in familial nonpolyposis colon cancer [58], the BRCAl gene in familial breast cancer [59–61], the APC gene in hereditary adenomatous polyposis [62,63], and the RB gene in retinoblastoma [64,65]—the functional mechanisms leading to cancer initiation, progression and development remain to be elucidated. BCL7B, located on chromosome 7, is a member of the BCL7 family of genes and is thought to be a tumor-associated gene [7–9]. In this study, we investigated the functional roles of the bcl7 and BCL7 genes in the Wnt signaling pathway and apoptosis in C. elegans and in human gastric cancer cells, respectively. Our results revealed that BCL7, located on chromosome 7, is a member of the BCL7 family of genes and is thought to be a tumor-associated gene [7–9]. In this study, we investigated the functional roles of the bcl7 and BCL7 genes in the Wnt signaling pathway and apoptosis in C. elegans and in human gastric cancer cells, respectively. Our results revealed that BCL7, located on chromosome 7, is a member of the BCL7 family of genes and is thought to be a tumor-associated gene [7–9]. In this study, we investigated the functional roles of the bcl7 and BCL7 genes in the Wnt signaling pathway and apoptosis in C. elegans and in human gastric cancer cells, respectively. Our results revealed that BCL7, located on chromosome 7, is a member of the BCL7 family of genes and is thought to be a tumor-associated gene [7–9].
Fig. 6. BCL7B functions as a tumor suppressor via multiple pathways. A–D: Nomarski (A, C) and DAPI (B, D) images of KATOIII cells transfected with control siRNA (A, B) and BCL7B-specific siRNA (C, D). E–H: Fluorescence images of KATOIII cells stained with WGA-Alexa Fluor 488 (E, G) and DAPI (F, H). These cells were transfected with control siRNA (E, F) or BCL7B-specific siRNA (G, H). The arrowheads indicate the presence of three nuclei in one cell. I: The percentages of the surviving KATOIII cells after transfection with control siRNA or BCL7B-specific siRNA and addition of actinomycin D. The number of living cells was counted 5 h after the addition of actinomycin D using trypan blue dye exclusion; this number was then divided by the total cell number. J: Percentages of KATOIII cells transfected with control siRNA or BCL7B-specific siRNA in the G0/G1, S, and G2 phases. More than twenty thousand cells were counted for each analysis. K, L: The expression levels of β-catenin (K) and c-FLIP (L) by the qRT-PCR analysis. M: Survival rates of KATOIII cells transfected with EGFP, as a control, or BCL7B_EGFP. The number of living cells was counted using trypan blue dye exclusion, and this number was then divided by the total cell number. N: The rates of apoptotic KATOIII cells transfected with EGFP, as a control, or BCL7B_EGFP. Apoptotic cells were stained for Annexin-V and/or 7-AAD and monitored by flow cytometric analysis. More than ten thousand cells were counted. Each experiment was performed three times independently. Error bars indicate SEM. The asterisks indicate the statistical significance of differences between the two groups. *p<0.05, **p<0.005, ***p<0.001. N.S.: no significance. Scale bar = 25 μm.

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Wnt/β-catenin pathway in *C. elegans*. In humans, BCL7B is a component of the SWI/SNF complex [66], which has multiple functions, including regulation of the Wnt pathway through transcriptional control of related signaling molecules [67]. In *C. elegans*, the SWI/SNF complex regulates asymmetric cell division and may be associated with Wnt signaling [68,69]. Although there is currently no evidence of an association between BCL-7 and the SWI/SNF complex in *C. elegans*, our data are consistent with a recent report [68]. Therefore, the SWI/SNF complex may represent the link between BCL-7 and the Wnt pathway.

The Wnt pathway is known to regulate the asymmetric division of most somatic cells in *C. elegans* through the asymmetric localization of Wnt components [70]. Our results showed that *bcl-7* knockout also induced defects in the localization of WRM-1 and POP-1 in the target cell nuclei. Specifically, *bcl-7* deletion caused a reversal of cell polarity and occasionally a loss of cell polarity in seam cells and SGPs. Yamamoto et al. (2011) [71] found that the knockout of multiple Wnt ligands resulted in the randomization, but rarely the loss, of cell polarity. By contrast, knockout of *apr-1/APC* resulted in the symmetrical localization of WRM-1 to both the anterior and posterior nuclei by inhibiting the export of WRM-1 from the anterior nuclei [72,73]. Thus, the functions of BCL-7 and multiple Wnt ligands or *apr-1* may be similar. BCL-7 may affect the translocation of WRM-1 from the cortex to the nucleus or the export of WRM-1 from the nucleus. In addition, multiple molecules, including WRM-1 itself, regulate the asymmetric localization of WRM-1. Therefore, BCL-7 may also interact, either directly or indirectly, with WRM-1 itself to regulate its asymmetric localization in the nuclei of target cells and maintain both nuclear WRM-1 and nuclear POP-1 at appropriate levels in *C. elegans*.

As described above, the suppressor screening supported the idea that BCL-7 functions mostly as a negative regulator of the Wnt pathway in *C. elegans*. A weak downregulation of WRM-1 had little effect on wild type worms but was sufficient to partially suppress the phenotype induced by *bcl-7* deficiency (S3 Table). In general, activating the expression of Wnt-related molecules, such as *wrm-1* and *pop-1* mutants, results in an increased number of seam cells, as indicated by downregulation of terminal differentiation markers and upregulation of stem cell markers (Fig. 2, S2 Fig., S6 Fig., S11 Fig.) [74,75]. However, knockout of BCL-7, which is thought to result in the activation of the Wnt pathway, results in a decreased number of seam cells. This discrepancy may be caused by an additional function of BCL-7 other than its role in the Wnt pathway. For example, BCL-7 can affect seam cell development not only by suppressing the Wnt pathway but also by regulating the terminal cell differentiation of seam cells. The phenotype of fewer seam cells observed in *tm5268* mutants is caused by the disturbance of these two mechanisms.

In this study, we show that BCL-7 suppresses the expression of the β-catenin homologs, *bar-1*, *sys-1*, and *wrm-1*. However, RNAi-based screening showed that only *wrm-1* was a suppressor of the *tm5268* mutant phenotype. This discrepancy may be the result of differences in the degree of BCL-7’s effect on the mRNA expression level of these genes in *tm5268* mutants. The expression of both *bar-1* and *sys-1* is markedly increased in *tm5268* mutant worms (S9A Fig.); therefore, the moderate downregulation of these genes caused by feeding RNAi may not be enough to affect the phenotypes in *bcl-7* deletion mutants. By contrast, the mRNA level of *wrm-1* is only approximately doubled in *tm5268* mutants, therefore, even weak downregulation of *wrm-1* may be sufficient to partially suppress the phenotypes of *tm5268* mutants.

Interestingly, our findings showed that knockdown of *ly-22* also partially suppressed the Ste phenotype in *bcl-7*-knockout mutants. LSY-22 is a homolog of the Groucho-like protein and is thought to promote expression of the Groucho homolog UNC-37 [43], thereby repressing the transcription of target genes in *C. elegans*. In our experiments, knockdown of *ly-22* by RNAi partially suppressed the phenotype of the *bcl-7* mutant (*tm5268*). This result is inconsistent with a previous study [43] and suggests that the downregulation of *ly-22* inhibits the Wnt pathway.

### BCL-7 may affect the differentiation of DTCs and the development of gonads

LAG-2/Notch is secreted from differentiated DTCs and regulates gonadal development. The Wnt signaling pathway is an important regulator of DTC differentiation. In this study, the DTC-specific expression of BCL-7 rescued both the Ste phenotype and the defects in gonadal development observed in *bcl-7* mutant worms. Although lag-2 temperature-sensitive (ts) mutants or *glp-1* (ls) mutants exhibited defects in gonadal development, these mutants had only meioetic cells (no mitotic cells) in their gonads and regular germ cell sizes, unlike the *bcl-7* mutant (*tm5268*) [76–78]. This result suggests that BCL-7 is involved in the normal cell differentiation of SGPs and subsequent gonadal development in *bcl-7* mutants is affected by the impairment of normal LAG-2 secretion from differentiated DTCs.

Our study found that BCL-7 was also expressed in gonadal sheath cells, as shown in S3I–L Fig. Differentiated gonadal sheath cells are crucial for gonadal elongation, meioetic maturation of germ cells, and embryogenesis [32]. In this study, gonadal sheath cell-specific expression of BCL-7 partially rescued the Ste phenotype of the *bcl-7* deletion mutant. This result suggests that BCL-7 functions not only in DTCs but also in gonadal sheath cells as a regulator of terminal cell differentiation. Furthermore, BCL-7 was also expressed in germ cells, although we did not demonstrate any cell-autonomous functions of BCL-7 in germ cells (Fig. 3M).

Taken together, these data suggest that BCL-7 functions cell-autonomously at least in DTCs and gonadal sheath cells, similar to in seam cells, and its functions are necessary for terminal cell differentiation and normal gonadal development.

### bcl-7/BCL7B shares characteristics with other tumor-suppressor genes

In this study, we show that BCL-7 and BCL7B positively regulate the apoptotic pathway and negatively regulate the Wnt signaling pathway. These characteristics are common with certain tumor-suppressor genes. For example, p53, one of the most well-studied tumor suppressors, inhibits cancer initiation and progression through the induction of apoptosis in abnormal cells [79–83]. The results of our qRT-PCR analysis revealed that BCL-7 may function as a positive regulator of the apoptotic pathway in *C. elegans* (S9B Fig.). However, there was a discrepancy between the results of qRT-PCR and PLM number analysis, possibly because suppression of apoptosis may occur in the limited cell population or because the remaining cells that are inhibited apoptosis may not undergo terminal differentiation. BCL7B also functions as a positive regulator of apoptosis by repressing the anti-apoptotic factor Bcl2, much stronger than its repression of the pro-apoptotic factor Bax (Fig. 6L, and S11A Fig.). The function of BCL-7 in the apoptotic pathway is in some ways similar to the function of p53, which activates the apoptotic pathway of target cells [54,79–83]. In addition, BCL-7 and BCL7B also negatively regulate the Wnt signaling pathway through suppressing the expression of β-catenin. Downregulation of *ape*, which promotes the degradation of β-catenin (thereby affecting Wnt signaling), induces hyperactivation of the Wnt pathway and is involved in the development of...
The role of BCL7B in the Wnt pathway is similar to APC. However, there are some differences between BCL7B and other tumor suppressors. For example, the RB gene, which encodes the retinoblastoma (RB) protein, primarily functions to modulate the G1/S-phase cell cycle checkpoint [64,65,85], whereas BCL7B knockdown increased the rate of G1 arrest. This dissimilarity is further reflected by the finding that BCL7B knockdown does not induce hyperproliferation. Furthermore, the accumulation of cells in the G0/G1 phase observed in BCL7B-knockdown KATOIII cells is similar to the specific profile of quiescent cancer stem cells, which also accumulate in the G0/G1 phase [86]. Collectively, these findings suggest that BCL7B is a novel tumor suppressor gene and is required for the terminal cell differentiation.

Downregulation of BCL7B in KATOIII cells induced nuclear enlargement, which is considered a hallmark of undifferentiated cells, such as cancer cells, and is associated with the grade of malignancies in neoplastic diseases [49]. This phenotype was similar to the phenotype of the bcl-7 mutant (tm5268) in C. elegans. Because the mechanisms mediating nuclear enlargement are not clearly understood, it is difficult to compare the function of BCL7 with the functions of other tumor-related genes. However, we demonstrated that the mRNA expression of specific genes, e.g., NEAT1, is significantly increased in BCL7B-knockdown cells. Although a direct interaction between nuclear enlargement and an increase in RNA has not been clearly established, a variety of noncoding RNAs have been shown to play various roles in many types of diseases, including cancer. Additionally, the increase in mRNA expression may indicate that transcriptional hyperactivity occurs in BCL7B-knockdown cells, potentially through the loosening of chromatin structure [87–90]. Although our experiments did not evaluate the chromatin structure of BCL7B-knockdown cells, changes in the chromatin structure may explain the observed nuclear alterations in these cells. Furthermore, the mRNA expression levels of undifferentiated markers were significantly increased in both bcl-7 knockout worms and BCL7B-knockdown cells, similar to observations made in some types of malignant cancer cells [91]. Thus, understanding the roles of BCL7B may provide insights into malignant alterations in nuclei.

It should be noted that nuclear changes are not the only alterations that occur in malignant diseases. Poor differentiation, autonomous growth, unlimited proliferation, immortalization, metastatic ability, angiogenic ability, and other phenotypes also contribute significantly to the development of malignancies. Our study demonstrated that BCL7 is involved in mediating nuclear enlargement, which is considered a hallmark of undifferentiated cell cycle arrest. However, our study did not provide evidence of an association between BCL7 and other malignant phenotypes. Furthermore, BCL7B-knockdown cells did not show hyperproliferation compared with control cells, but they did demonstrate phenotypes similar to cancer stem cells [86]. These results suggest that BCL7B activity contributes only to some malignant phenotypes and that cancer initiation may be caused by a combination of aberrations of BCL7 and abnormalities in other tumor-related genes. Therefore, further investigation of the role of BCL7 in malignant transformation and cancer progression and of molecules that associate with BCL7 family proteins is necessary.

**Materials and Methods**

**Strains**

All strains of *C. elegans* were seeded with *Escherichia coli* OP50. All experiments were performed at 20°C using standard techniques [92]. The wild-type strain Bristol N2 and some transgenic animals (wls1, mls3, osb5, qh74, arls51, hims4, bls4, bs608, sxs310165, qh56 and qh174) were obtained from the Caenorhabditis Genetics Center (CGC, Minneapolis, MN). Strains carrying the observed mutations were obtained from the trimethylpsoralen/ultraviolet-mutagenized library, as described previously [93]. Mutated strains were identified by polymerase chain reaction (PCR) amplification with primers spanning the deletion regions: bcl-7 (tm5268) III and ced-3 (tm1196) IV [48]. Strain tm5268 was backcrossed twice with N2 and balanced with hT2 [bli-1(e937) let-7(q782) bgl41]/[III]. The primers used in this study for nested PCR screening were as follows: tm5268-1st round; 5'-TCC GGA TGA GGT GGA TTG TC-3', 5'-TGT CAT TTC AGC GTC GCG CA-3'; 2nd round; 5'-GCT CAC CAT GCG GCC GCA TGG TTG TTG TTA GGT CAT TTC A-3'.

**Constrasts and transgenic worms**

To determine the expression pattern of the rescue plasmid in the wild-type hermaphrodite, the bcl-7 genomic sequence, comprising a 0.3-kbp sequence upstream from the ATG initiation codon of bcl-7, as well as the full-length bcl-7 (0.7 kbp), was PCR amplified from N2 genomic DNA using the following primers: bcl-7_sense, 5'-GGT TCC GCG TGG ATC CCA TTT TGA CGC AAG ATT TGA GAG-3'; bcl-7_antisense, 5'-GGT CAC CAT GGC GCC GCA TGG TTG TTG TTA GGT CAT TTC A-3'.

For the somatic DTC rescue experiment, the full-length bcl-7 (0.7 kbp) was amplified from N2 genomic DNA; the pPD95.77_pFmCherry expression vector was digested with BamHI and NotI, and the full-length bcl-7 fragment was cloned into the distinct vectors to generate Plag-7::bcl-7::egfp and Plag-7::bcl-7::mCherry, respectively [94].

For the nematode-cell rescue experiment, full-length bcl-7 (0.7 kbp) was amplified from N2 genomic DNA; the pPD95.77_pSym::mCherry expression vector was digested with SmaI and NotI, and the full-length bcl-7 sequence was cloned into the vector to generate pSym::bcl-7.

For the germ cell rescue experiment, the first intron of bcl-7 and the full-length bcl-7 (0.7 kbp) were amplified from N2 genomic DNA using the following primers: lim-7(1st intron)_sense, 5'-TTC TGG TTC CGC GTG GAT CCG TGA TGG TTG TTT TTA TAA TTA GGT-3' and lim-7(1st intron)_antisense, 5'-ATT TGC TGA GTA CAT ACG TTC TGA AAA ATG AAA GCT CGA-3'; bcl-7_sense, 5'-TCCA TTT TTA TGG TAT GTT CTA ATC AGG AAA TAG AAT-3' and bcl-7_antisense, 5'-GCG GCC GCA TGG TTG TTG TTA GGT CAT TTC A-3'. The pFX_eggfp or the pFX_mCherry expression vector was digested with BamHI and NotI, and the full-length bcl-7 sequence was cloned into the vector to generate pSym::bcl-7.
For the chromophore-assisted light inactivation (CALI) of BCL-7, we prepared Phel-7:-bcl-7::KillerRed. To generate this construct, a bcl-7 genomic sequence, composed of a 0.3-kbp sequence upstream from the ATG initiation codon of bcl-7 and the full-length bcl-7 (0.7 kbp), was PCR amplified from N2 genomic DNA using the following primers: bcl-7-KR_sense, 5'-GTT TCC GGC TGG ATC CCA TTT TGA CGC AAG ATT TGA GAG-3', and bcl-7-KR_antisense, 5'-GAA GAG CAG GGC GGG GCC GCC CTC CAT TTA TGG TTG-3'. The KillerRed coding sequence was amplified from the commercially available pHKillerRed-C vector (Evrogen, Moscow, Russia) using the following primers: KillerRed_sense, 5'-TAA ATG AGG GGC GGG CCC GC-3', and KillerRed_antisense, 5'-GCT CAC CAT GGC GGT CCT CGT CGC TAC CGA TGA TGG GC-3'. Phel-7:-bcl-7 and KillerRed were cloned into the pxF vector at the BamHI and NotI sites to produce Phel-7:-bcl-7::KillerRed.

To generate the transgenic lines, constructs were injected into worms at 20–100 ng/μL along with Pmyo-2::DsRed, Pmec-4::gfp, or scmp::mCherry as an injection marker (100 ng/μL). The transgenic strains constructed for this study were tmEx2966 [Phel-7:-bcl-7::gfp, PyIs51, tmsEx3496 [Phel-7:-bcl-7::gfp, PmIs96], tmIs3873 [Plag-2::bcl-7::gfp, PyIs56, tmsEx3875 [PpTE5::bcl-7::gfp, PyIs51]; PmIs96], tmIs3878 [PpmIs101, Phel-7:-bcl-7::gfp, PmIs81], tmIs4126 [scmp::bcl-7, scmp::mCherry, tmsEx4116 [Phel-7:-bcl-7::gfp, PyIs51, tmsEx4121 [PmIs101, Phel-7:-bcl-7::gfp, PmIs96, Pmyo-2::DsRed]].
The integrated arrays wIs51, containing scmp::gfp, maIs105, containing col-19::gfp, and arIs51, containing edh-3::gfp, were used to assay the numbers of seam cells and hyp7 cells as well as the differentiation of seam cells. The integrated arrays qIs74, containing pop-1::gfp, and oIs5, containing scmp::wom-1::gfp, were used to assay the distribution of POP-1 and WRM-1, respectively, in the seam cells. The integrated arrays arIs51, containing Pdes-2::gfp, and balIs4, containing Plat-1::gfp, were used to assay the formation of PVD and PDE sensory neurons, respectively. The integrated array balIs8, containing Pmec-4::gfp, was used to assay the number of PLM neurons. The integrated array slIs10165, containing eggl-27p::his-24::mCherry, was used to analyze the expression pattern of eggl-27 as an undifferentiated marker. The integrated array qIs56, containing Plag-2::gfp, and qIs174, containing Phlh-2::gfp::huh-2, was used to assay development of DTCs. These transgenic strains were obtained through CGC.

Microscopy

Differential interference contrast and fluorescence images were obtained using a BX51 microscope equipped with a DP30BW CCD camera (Olympus Optical Co., Ltd, Tokyo, Japan).

Analysis of seam cells

To characterize seam cell phenotypes, we scored the number of seam cell nuclei and observed alae formation. The numbers of GFP-positive seam cell nuclei were counted at each larval stage (early L1, middle L2, late L3, and L4) and the young adult stage, and staging was assessed by vulval shapes and gonadal morphologies. Alae formation was observed through a Nomarski microscope at the young adult stage.

Dye-filling assay in C. elegans

We performed a dye-filling assay to observe the phenotype of the plasmid in bcl-7 mutants as described in a previous report [95]. The assay was performed by incubating the wild-type and bcl-7 (tm5268) hermaphrodites in the DiI solution (10 μg/mL DM in M9 buffer) for 2 h at room temperature. Thereafter, these specimens were observed under a fluorescence microscope as above.

Self-brood size of C. elegans

To determine the average number of progeny produced by each strain, as well as by the transgenic worms, L4 worms were placed on individual NGM plates. Worms were transferred daily until egg laying ceased, and the total number of produced live progeny was then counted.

DAPI staining and immunostaining in C. elegans

Immunostaining of the gonads was performed as described previously [96]. Transgenic or wild-type animals were placed on a subbed slide in 5.0 μL of M9 buffer containing 1 mM levamisole. The heads or tails of these worms were cut off using a 27-gauge needle to extrude their gonads. The dissected gonads were fixed with 1.0% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min and permeabilized with PBS containing 0.1% Triton X-100 for 3 min. Samples were blocked in PBS containing 2.5% bovine serum albumin (BSA) for at least 30 min. Fixed worms were incubated with an anti-PH3 antibody overnight at 4°C. Samples were washed three times with PBS containing 0.5% BSA, incubated with a secondary antibody conjugated to Alexa 594 (Invitrogen, San Diego, CA) for 1 h, and washed at least twice. Then, samples were suspended in 0.1 μg/mL Prolong Gold containing DAPI (Invitrogen) for more than 15 min and observed under a fluorescence microscope.

Chromophore-Assisted Light Inactivation (CALI) of BCL-7

For CALI of BCL-7, bcl-7 (tm5268) mutants expressing Phel-7:-bcl-7::KillerRed were exposed to a light-emitting diode array (572 nm) from the comma-stage, early L1 stage, and L2 stage. At the adult stage, we observed the worms through a microscope and calculated the rates of worms exhibiting the Ste phenotype. In addition, to determine when bcl-7 function is most critical during development, we also performed the pulse experiments using the CALI method. bcl-7 (tm5268) mutants expressing Phel-7:-bcl-7::KillerRed were exposed to a light-emitting diode array (572 nm) only during the comma and early L1 stages (the exposure period was 6 hrs). The mutants grew in a dark room at all other stages until they became adults. At the adult stage, the percent of worms with Ste phenotypes was calculated.

RNA interference

RNA interference analyses (RNAi) were performed by feeding animals with dsRNA-producing bacteria as described previously [97]. Briefly, the RNAi clones were transformed into E. coli HT115(DE3), and then, approximately 10–20 P0 animals at the early L1 stage were transferred to plates containing RNAi bacteria grown on 100 μg/mL ampicillin and 1 mmol/L isopropyl-beta-D-thiogalactopyranoside (IPTG).

For the analysis of the phenotypes of bcl-7-knockdown worms, the Egl-grade was scored in the F1 generation at the young adult larval stage. More specifically, we observed the worms under a microscope and categorized them as follows: stage 1: 1- to 8-cell stage embryos present in the uterus; stage 2: 16-cell stage to comma-stage embryos present in the uterus; or stage 3: postcomma-stage embryos present in the uterus [98]. We compared the frequencies of bcl-7-knockdown and control worms in each category.

For suppressor screening, the numbers of seam cells and of eggs were counted in the F1 generation at the L4 larval stage. We compared bcl-7 mutants to wild-type worms, and if there was any...
remission of the mutant phenotype, we identified the corresponding gene as a candidate suppressor gene.

All RNAi clones, except for sys-1, glp-1, chi-1, bro-1, lin-17, mig-14, and cwn-1, were taken from the Ahringer RNAi library. The sys-1, glp-1, chi-1, bro-1, lin-17, mig-14, and cwn-1 RNAi clones were constructed with the cDNAs generated from wild-type worms using the primers listed in S5 Table. These cDNA fragments were cloned into the L4440 (wild-type worms) vector.

Cell culture and transfection
KATOIII cells obtained through ATCC were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 20% fetal bovine serum at 37°C in a humidified 5% CO2 incubator. Transfection of 100 nM siRNA (ON-TARGETplus SMARTpool L-017228-00 or ON-TARGET plus siCONTROL non-targeting siRNA; Dharmacon RNAi Technologies, Lafayette, CO) into cells was performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s protocol.

Nuclear size assays
KATOIII cells were grown on 2-well chamber slides (Lab-Tek, Campbel, CA) at 1 × 10^5 cells/well for approximately 24 h prior to transfection. Forty-eight hours after transfection with control-siRNA or BCL7B-siRNA, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature and permeabilized with 0.1% Triton X-100 for 3 min. Then, the cells were washed and incubated in 0.1 μg/mL DAPI stain (Life Technology, Carlsbad, CA) overnight at room temperature in the dark. Samples were observed under a fluorescence microscope (with a UV filter), and acquired images were digitally analyzed with ImageJ software (National Institutes of Health, Bethesda, MA).

Analysis of the number of the nuclei
KATOIII cells were grown on 2-well chamber slides (Lab-Tek) at 1 × 10^5 cells/well for approximately 24 h prior to transfection. Forty-eight hours after transfection of control-siRNA or BCL7B-siRNA, 1 mg/l wheat germ agglutinin (WGA)-Alexa Fluor 488 (Invitrogen) was added to the cells to stain the plasma membrane, which were then incubated for 10 min at 37°C in the dark. Subsequently, the cells were washed twice, fixed with 4% paraformaldehyde for 10 min at room temperature, and permeabilized with 0.1% Triton X-100 for 3 min. Then, the cells were washed and suspended in 0.1 μg/mL DAPI stain at room temperature in the dark. The samples were then observed under a fluorescence microscope, and the numbers of the cells and the nuclei were counted; the number of nuclei per cell was also calculated for each sample.

RNA detection assays in KATOIII cells
KATOIII cells were grown on 2-well chamber slides (Lab-Tek) at 1 × 10^5 cells/well for approximately 24 h prior to transfection. Forty-eight hours after transfection with control-siRNA (slide-a and slide-b) or BCL7B-siRNA (slide-a and slide-b), the cells were fixed with 4% paraformaldehyde for 10 min at room temperature and permeabilized with 0.1% Triton X-100 for 3 min. Thereafter, 0.3 mg/mL RNase (Qiagen, Valencia, CA) was added to the cells on slide-a for all samples, while the cells on slide-b were not treated with RNase; all cells were incubated overnight at 4°C. Then, the cells were washed, incubated with the ethidium bromide (Life Technologies, Gaithersburg, MD) for more than 1 h, washed twice, and suspended in 0.1 μg/mL DAPI stain at room temperature in the dark. Samples were observed under a fluorescence microscope (DAPI: UV filter; ethidium bromide: 594 nm filter). To adjust the focus of the DAPI image, a 25% neutral density (ND) filter was used, and the exposure time was 500 msec at each data acquisition point; for the EtBr image, a 25% ND filter was also used, and the exposure time was 100 msec at each data point. Thereafter, acquired images were digitally analyzed with ImageJ software (National Institutes of Health).

Cell viability assays
To analyze the effects of BCL7B knockdown on cell viability, KATOIII cells were transfected with BCL7B-siRNA or control-siRNA (day 0) and incubated until day 2 at 37°C. Then, 0.1% actinomycin D was added to the medium, and the cells were incubated for more than 5 h at 37°C. Cells were then collected, supplemented with 0.2% trypan blue, transferred to a plastic disposable counting chamber, and counted with an automated cell counter (TC20, Bio-Rad laboratories, Hercules, CA).

qRT-PCR analysis
For worms, total RNA was isolated from young adult animals using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. For KATOIII cells, total RNA was extracted using TRIzol (Invitrogen). Total RNA was used for reverse transcription with the Superscript III reverse transcriptase (Invitrogen) using an oligo(dT) primer (for worms) or random primers (for KATOIII cells), according to the manufacturer’s instructions, and was subsequently diluted with nuclease-free water (Sigma-Aldrich, St. Louis, MO). qRT-PCR amplification mixtures (25 μL) contained 25 ng of template cDNA, 12.5 μL of 2× SYBR Green I Master Mix buffer (Applied Biosystems, Framingham, MA), and 300 nM of each forward and reverse primers. Reactions were performed on an ABI PRISM 7500 Sequence Detector (Applied Biosystems). The cycling conditions comprised 10 min polymerase activation at 95°C. All data was normalized to amo-1 gene (for the worms) or GAPDH gene (for KATOIII cells). The primer pairs used in this study are listed in S6 Table and S7.

Cell cycle analysis
After 48 h of BCBL7B-siRNA or control-siRNA transfection, cells were applied to a CycleTEST PLUS DNA Reagent Kit (Becton Dickinson Biosciences, San Diego, CA) according to the manufacturer’s instructions. Thereafter, cells were subjected to flow cytometry with a Cell Lab Quanta SC flow cytometer (Beckman Coulter, Fullerton, CA). For the cell cycle analysis, unstained non-treated samples were used to set the EV gain (set at 0.25) and the FL1 photomultiplier tube (PMT) voltages (set at 3.26). These experiments were repeated three times independently.

Analysis of apoptotic cells
After 48 h of BCBL7B-siRNA or control-siRNA transfection, cells were applied to an Annexin V/PE/7-AAD kit (BD Biosciences) according to the manufacturer’s instructions. Thereafter, cells were subjected to flow cytometry. FL1 was used to measure the Annexin V fluorescence, and 7-AAD fluorescence was measured using FL3. Annexin V-positive and 7-AAD-positive cells were considered to be late apoptotic cells, and Annexin V-positive and 7-AAD-negative cells were considered to be early apoptotic cells [99,100]. The gating lines were drawn based on the viable cells as the negative control, which were Annexin V-negative and 7-AAD-negative cells. Unstained and single-stained samples were used to set the EV gain (at 0.25), FL1 and FL3 PMT-voltages (at 4.08 and 4.50, respectively), and to compensate for Annexin V spillover into the 7-AAD channel. These experiments were repeated three times independently.
Statistical analyses

All the data were compared using Student’s t-test.

Supporting Information

S1 Fig Decrease or loss of BCL-7 results in a variety of phenotypes, including Egl, Pvl, and Burst in Caenorhabditis elegans. A: A schematic diagram of protein homology of human BCL7 family (hsBCL7A, hsBCL7B, and hsBCL7C), Mus musculus homolog of BCL7B (Mm-Bcl7b), and C. elegans homolog (Ce-Bcl-7) adapted from Ref.10. Numbers indicate amino acid positions; gray boxes indicate high homologous sequences; white boxes indicate the regions that are not conserved in C. elegans. B–D: Nomarski images of adult hermaphrodites treated with bel-7-RNAi. Arrowheads indicate the Pvl phenotype. An arrow indicates the Burst phenotype. E: A bar chart representing the frequency of worms with the Egl phenotype (n = 50–70, and these experiments were repeated five times independently). Egg-laying behavior was assayed as follows. The developmental stage of eggs inside the uterus of the worms, determined by microscopy, was categorized as the 1–8-cell stage (stage 1; 1); 16-cell stage to precomma stage (stage 2; 2); or comma to postcomma stage (stage 3; 3). F–H: Nomarski images of a wild-type (F) and tm5268 hermaphrodites (G) at the L4 stage and at the adult stage (H). I: Percentages of the Pvl phenotypes in wild type and tm5268 mutant worms (n=80). Error bars indicate SEM. Asterisks indicate the statistical significance of differences between groups. *p<0.05, ***p<0.001. N.S.: no significance. Scale bar = 50 μm. (TIFF)

S2 Fig Knockout of bel-7 inhibits normal epidermal development in Caenorhabditis elegans. A–D: Examples of GFP localization in wild-type and tm5268 hermaphrodites carrying the cdh-3::gfp reporter. Nomarski (A, C) and GFP (B, D) images of wild-type (A, B) and tm5268 (C, D) adult hermaphrodites carrying the cdh-3::gfp reporter. A white arrow indicates the absence of a seam cell. Scale bar = 25 μm. (TIFF)

S3 Fig BCL-7 is ubiquitously expressed in wild-type (n = 10) and tm5268 (n = 12) hermaphrodites carrying the des-2::gfp reporter (hmlIs4). Nomarski (A, C) and GFP (B, D, E, F) images of wild-type (A, B, E) and tm5268 (C, D, F) adult hermaphrodites. Two PVD neurons (white arrows) without an ectopic cell are found in both a wild type (B) and a tm5268 mutant (D). A PVD neuron shows characteristic branching dendrites in both N2 (E) and tm5268 (F) worms. Insets show Nomarski images of the same areas. G–J: Expression patterns of DAT-1::gfp in wild-type (n = 10) and tm5268 (n = 15) worms carrying the dat-1p::gfp reporter (hmlIs4). Nomarski (G, I) and GFP (H, J) images of wild-type (G, H) and tm5268 (I, J) adult hermaphrodites. White arrows indicate PDEs. K–N: Patterns of absorbance of fluorescent dye in dye-filling assays. Nomarski (K, M) and Dil (L, N) images of wild-type (K, L) (n = 10) and tm5268 (M, N) (n = 10) adult hermaphrodites. Arrowheads indicate a pair of socket cells in the phasmid. O–R: Expression patterns of mec-4p::gfp in wild-type (n = 10) and tm5268 (n = 12) worms carrying the mec-4p::gfp reporter (hmlIs8). Nomarski (O, Q) and GFP (P, R) images of wild-type (O, P) and tm5268 (Q, R) adult hermaphrodites. Asterisks indicate PLMs. S, T: The images of V1–V6 cells and T cells in wild-type hermaphrodites (Sulston & Horvitz, 1977). The directions of the cell divisions are shown with the anterior to the left and the posterior to the right. PHs91 and PHs92 are socket cells that support phasmid sensory neurons. PDE, PVD, PVN, PVW, PHC, and PLN are neurons. Circles indicate hyp7 cells, double circles indicate adult seam cells, and x indicates programmed cell death. Scale bar = 50 μm. (TIFF)

S5 Fig Knockout of bel-7 induces nuclear enlargement of epidermal cells. A, B: Examples of GFP localization in hyp7 cells of wild-type (A) and tm5268 (B) hermaphrodites carrying the col-19::gfp reporter. C, D: Histograms of the length of the major axis of hyp7 cell nuclei in wild-type (C) and tm5268 (D) hermaphrodites. Counted cells of wild-type and tm5268 were more than 300. Scale bar = 10 μm. (TIFF)

S6 Fig BCL-7 affects cell differentiation in C. elegans. A–H: Nomarski (A, C, E, G) and RFP (B, D, F, H) images of wild-type (n = 10) and tm5268 (n = 16) L4-stage hermaphrodites carrying the egl-27p::HIS-24::mCherry reporter (stkIs10165). The exposure time was 800 msec for mCherry pictures. A wild-type hermaphrodite expressing EGL-27p::HIS-24::mCherry strongly in intestinal cells (white arrowheads indicate a part of the intestinal cells expressing mCherry) and weakly in hypodermis (‘s’ indicates a seam cell; ‘h’ indicates a hyp7 cell (B, D) and a tm5268 hermaphrodite expressing mCherry strongly in seam cells and hyp7 cells (F, H). I: mRNA expression of egl-27 as assessed by qRT-PCR analysis. J: mRNA expression of ceh-6 as assessed by qRT-PCR analysis. All experiments were performed more than three times independently. The mRNA expression levels of tm5268 mutants were normalized by that of wild type worms. Error bars indicate SEM. The asterisks indicate the statistical significance of the differences between groups. **p<0.005, ***p<0.001. Scale bar = 50 μm. (TIFF)

S7 Fig Knockout of bel-7 affects the normal development of germ cells. A–D: Histograms of the length of the major axis of germ cell nuclei in wild-type (A) and tm5268 (B). tm5268;tmEx2966 carrying the Pbehel-7::bel-7::egfp reporter as a rescue construct (C), and tm5268;tmEx3873 carrying the Pbeg-2::bel-7::egfp reporter as a DTC-specific rescue construct (D) adult hermaphrodites. E: A graph showing the percentages of Ste phenotypes in adult hermaphrodites of bel-7 worms with Pbehel-7::bel-7::KillerRed (tm5268;tmEx3873) with or without green light illumination. Thick black lines indicate the exposed periods. A black circle indicates worms without exposure. Figs. in parentheses indicate the number of treated worms. (TIFF)

S8 Fig Knockout of bel-7 inhibits normal differentiation of distal tip cells (DTCs) in Caenorhabditis elegans. A–H: Nomarski (A, C, E, G) and GFP (B, D, F, H) images of wild-type (A, B) and tm5268 (C–F) adult hermaphrodites carrying a lag-2p::gfp reporter (qfIs36). A higher magnification view of the white square
is presented in the lower panel of the images (G, H). I–N: Nomarski (I, K, M) and GFP (J, L, N) images of wild-type (I, J) and tm5268 (K–N) L3-stage hermaphrodites carrying the bllh-2:p::gfp::hkl-2 reporter (qy1714). A DTC is outlined with dotted lines [I–N]. Arrowheads indicate GFP expression in DTCs (B, D, F, H, J, N). Asterisks indicate mispositioning of DTCs with Nomarski images and GFP expression (F–H, M, N). VC, vulval cell (B, L). Scale bar = 50 μm.

(S1F)

S9 Fig Knockout of bcl-7 affects several pathways in C. elegans. A: mRNA expression of bar-1, wrm-1, and sys-1, C. elegans homologues of human β-catenin, as assessed by qRT-PCR analysis. B: mRNA expression of ed-9, a C. elegans homolog of a human anti-apoptotic factor, Bcl2, as assessed by qRT-PCR analysis. All experiments were performed more than three times independently. Each data was normalized to the expression level of wild type worms. Error bars indicate SEM. The asterisks indicate the statistical significance of the differences between groups. *p<0.05, **p<0.01.

(TIFF)

S10 Fig BCL7B downregulation shows various phenotypes but not aneuploidy. A, B: Histograms of the area of nuclei in KATOIII cells transfected with control-siRNA (A) or BCL7B-siRNA (B). C, D: Cell cycle profiles of transfected with control-siRNA (C) or BCL7B-siRNA (D). E: Percentages and numbers of KATOIII cells present in each stage of the cell cycle. F, G: mRNA expression of BCL7B (F) and HMGAL1 (G), as assessed by qRT-PCR analysis. The experiments were performed three times independently. The relative mRNA level of BCL7B-downregulated KATOIII cells were normalized by that of the control. Error bars indicate SEM. The asterisks indicate the statistical significance of the differences between groups. *p<0.05, **p<0.01.

(TIFF)

S11 Fig Line graphs of relative mRNA levels in KATOIII cells. A: mRNA expression of Oct4, Nanog, and Sox2, as assessed by qRT-PCR analysis. B: mRNA expression of PTEN, Box, and Bcl2, as assessed by qRT-PCR analysis. All experiments were performed three times independently. The relative mRNA levels of BCL7B-knockdown KATOIII cells were normalized by that of the control. Error bars indicate SEM. The asterisks indicate the statistical significance of the differences between groups. *p<0.05, **p<0.01.

(TIFF)

S12 Fig mRNA is increased in the KATOIII cells with the downregulation of BCL7B. A–H: KATOIII cells stained with DAPI (A–D) or ethidium bromide (E–H) with (C, D, G, H) or without RNase (A, B, E, F, I. A line graph of the ratio of the fluorescence intensity in KATOIII cells transfected with control-siRNA or BCL7B-siRNA stained with DAPI or ethidium bromide (EtBr). More than one hundred cells were counted. J: A line graph of the RNA expression of NEAT1, as analyzed by qRT-PCR. The experiments were performed three times independently. Error bars indicate SEM. The asterisks indicate the statistical significance of the differences between groups. *p<0.05, **p<0.005. N.S.: no significance. Scale bar = 25 μm.

(TIFF)

S13 Fig Overexpression of BCL7B may regulate the apoptotic pathway positively. A: Total cell count of cells transfected with pEGFP_N3 (black line) as a control or BCL7B_EGFP (red line). B, C: Example data of apoptosis assays. KATOIII cells transfected with pEGFP_N3 (B) or BCL7B_EGFP (C) were stained with Annexin V (AV)/7-AAD (7A) and analyzed using a flow cytometer. More than ten thousand cells were counted, and these experiments were repeated three times independently. The AV+/7A− population is shown in the R2 region of the left upper quadrant and represents early-apoptotic cells. The AV−/7A− population is shown in the R3 region of the right upper quadrant and represents late-apoptotic cells. The gating lines were drawn based on the viable cells as the negative control, which belongs to the left lower quadrant. Error bars indicate SEM.

(TIFF)

S14 Fig A schematic model of the functions of BCL-7/BCL7B. BCL7B and its C. elegans homologous protein, BCL-7, may function as a tumor suppressor through multiple pathways, including the apoptosis and the Wnt signaling pathway, which inhibits and promotes cancer, respectively. BCL-7 and BCL7B also promote terminal cell differentiation, which is important for cancer malignancy.

(TIFF)

S1 Table Number of DTC expressing lag-2:p::gfp in wild-type worms or bcl-7 mutants. "mispositioning" means worms with GFP-positive mispositioned DTC with or without normal positioning of DTC (such as S8 Fig.E–H). All examined animals were mounted on slide-glasses and observed using a fluorescence microscope.

(DOC)

S2 Table Lists of genes for the RNAi screening.

(XLS)

S3 Table Average number of GFP-positive seam cells in RNAi experiments. "wrm-1c RNAi" means RNAi experiments using with wrm-1 RNAi clone constructed with a cDNA of wild-type worms. "1/10 wrm-1c RNAi" means diluted RNAi as described in the text.

(DOC)

S4 Table Number of DTCs expressing GFP::HLH-2 in wild-type worms or bcl-7 mutants. "mispositioning" means worms with GFP-positive mispositioned DTC with or without normal positioning of DTC (such as S8 M Fig. and S8 N Fig.). All examined animals were mounted on slide-glasses and observed using a fluorescence microscope.

(DOC)

S5 Table List of primers used for producing RNAi clones.

(XLS)

S6 Table List of primers used for the qRT-PCR experiments in C. elegans.

(XLS)

S7 Table List of primers used for the qRT-PCR experiments in KATOIII cells.

(XLS)

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Author Contributions

Performed the experiments: TU. Provided instruction for experiments: EKN SY RI SM. Designed the research: TU SM. Wrote the paper: TU SM.
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