# The gene expression and deficiency phenotypes of Cockayne syndrome B protein in *Caenorhabditis elegans*

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**Abstract The *Caenorhabditis elegans* Cockayne syndrome B protein homologue is encoded by 10 exons of the predicted open reading frame F53H4.1. The gene is expressed in germ cells and all somatic cells of the embryonic to adult stage. Although the gene expression was ubiquitous, its expression level was relatively higher in dividing cells and cells that play fundamental roles in essential physiological functions such as feeding, sensation, and reproduction. RNA interference of the gene hypersensitized *C. elegans* to UV radiation, as observed in enhanced germ cell proliferation arrest and apoptosis, and increased embryonic lethality, suggesting its role in nucleotide excision repair.** 

*Key words:* Nucleotide excision repair; UV sensitivity; Transcription-coupled repair; *Caenorhabditis elegans*

## 1. Introduction

Nucleotide excision repair (NER) is the main pathway involved in the removal of cyclobutane pyrimidine dimers and (6-4) photoproducts produced by UV radiation [1] and other base modifications [2]. Defects in the NER reaction in humans are represented by photosensitive genetic disorders such as xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy. CS shows growth retardation, severe neurological abnormalities, and premature aging, and is characterized at the cellular level by defective coupling of NER with transcription and slow recovery of RNA synthesis following DNA damage [3,4]. CSA, CSB, or either XPB or XPD, both of which are also associated with XP as components of the TFIIH complex, are mutated in CS patients. CSB belongs to the SWI2/SNF2 family and its activity as a chromatin remodeling factor has been demonstrated in vitro [5]. Transcription-coupled repair (TCR) is mediated by the TCRF factor (*mfd*), which replaces RNA polymerase stalled at a damaged DNA site in *Escherichia coli* [6]. Physical and functional interaction of CSB with RNA polymerase II has been reported [7,8], but contrary to the results in *E. coli*, CSB was shown not to displace stalled RNA polymerase II [7]. The CSB protein also physically interacts with TFIIE, TFIIH, XPA, XPG, and p53 but its exact role in TCR is still an enigma [9–11]. Disruption of the *Saccharomyces cerevisiae* CSB homologue, Rad26, leads to defective TCR but not to UV sensitivity, suggesting that TCR may not be as important in lower eukaryotes [12].

Embryonic fibroblasts of CSB knockout mice were defective in TCR, similar to CSB patient cells [4]. However, the knockout mice were distinctive from human CSB patients in showing relatively minor neurological abnormalities, no significant reduction of life span, and predisposition to skin cancer. To further understand the functions of CSB in a model organism, we observed the expressional localization of a *Caenorhabditis elegans* CSB homologue (*Ce*CSB) and analyzed the phenotypes due to gene suppression by RNA interference.

## 2. Materials and methods

### 2.1. C. elegans culture and preparation of embryos

Wild type N2 worms were grown on NGM plates seeded with the *E. coli* strain OP50 at 20°C and early embryos were prepared by lysing the mixed stage worms in an alkaline hypochlorite solution [13].

### 2.2. Amplification of the 5′-end cDNA of Cecsb

mRNA was isolated from mixed stage *C. elegans* as described in our previous work [14]. To obtain the genuine 5′-end cDNA of *Cecsb*, the first cDNA strand was synthesized using a primer of the sequence 5′-TCATTGTCATCTAGCAATCC, complementary to nucleotides (nt) 421–440 in the predicted open reading frame (ORF) F53H4.1. PCR was performed on the first cDNA strand using a primer of the SL1 sequence (5′-GGTTTAATTACCCAAGTTTGAG) and a nested primer (5′-ATCATCTGATGAAGATCGAG), complementary to nt 362–381 of the ORF. The amplified DNA product was inserted into the pGEM-T vector (Promega) and its sequence was determined using the ABI Prism dye terminator cycle sequencing kit (Perkin Elmer) and an automatic sequencer (Perkin Elmer).

### 2.3. Transgenic GFP expression regulated by the csb gene promoter in C. elegans

*Cecsb* DNA containing the 5′-upstream region and two exons of the N-terminal was amplified from N2 genomic DNA through a PCR reaction using the Expand Long Template PCR system (Roche) and the two primers, 5′-GTACTCTGCAAGTATACATG (1411–1430 nt upstream of the start codon in genomic DNA) and 5′-ATCATCTGATGAAGATCGA (428–409 nt downstream of the start codon in genomic DNA). The amplified DNA fragment was cloned into the pGEM-T vector (Promega), cut out of the recombinant plasmid DNA using the *Sph*I and *Bam*HI (399 nt downstream of the start codon in genomic DNA) restriction enzymes, and then inserted into the *gfp*

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*Abbreviations:* CSB, Cockayne syndrome B; dsRNA, double-stranded RNA; GFP, green fluorescent protein; NER, nucleotide excision repair; ORF, open reading frame; RT-PCR, reverse transcription-polymerase chain reaction; RNA*i*, RNA interference; TCR, transcription-coupled repair; XP, xeroderma pigmentosum

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reporter vector pPD95.69, which contains a nuclear localization signal (supplied by A. Fire, Carnegie Institute). The green fluorescent protein (GFP) fusion construct was injected into the gonads of young adult N2 worms. The transgenic progeny were selected and observed under a fluorescence microscope (DMR HC, Leica).

*2.4. In situ mRNA hybridization*

Hybridization of hermaphrodite gonads was performed as described in our previous work [15]. Briefly, the gonads of N2 adult worms were extruded, fixed, and hybridized with digoxigenin-11-dUTP (Roche)-labeled antisense (or sense) DNA (nt 1–381 in the complete cDNA sequence of the *Cecsb* gene). To visualize the hybridized cDNA probe, the specimen was incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche) and then with the color-developing reagents, 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate.

*2.5. Double-stranded (ds) RNA interference of Cecsb gene expression and measurement of C. elegans sensitivity to UV radiation*

The EST clone yk646a6, containing a partial cDNA sequence corresponding to nt 672–2874 of the predicted ORF F53H4.1 and the 3′-untranslated region, was obtained from Y. Kohara (National Institute of Genetics, Japan) and linearized with the *Spe*I restriction enzyme. The linearized plasmid DNA was used to prepare antisense RNA by in vitro transcription as described in our previous work [15]. Sense RNA was prepared using the yk646a6 plasmid DNA linearized at the *Kpn*I site. An equivalent mixture of antisense and sense RNAs (1 μg/μl) was microinjected into the intestine of N2 young adults. Microinjected worms were placed on an NGM plate seeded with *E. coli* OP50 and transferred to a new plate 12 h after the microinjection. In order to examine the effects of the RNA interference (RNA*i*) [16,17] on *C. elegans* sensitivity to UV radiation, the F1 progeny worms were irradiated with a germicidal lamp ($\lambda_{max} = 254$ nm) at the doses of 25, 50, 100, and 200 J/m$^2$ at the young adult stage. F2 eggs were collected 24 h later for a 12 h period and their viability to L1 larvae was measured. As a control experiment, F1 progeny from *C. elegans* worms that had been microinjected with dsRNA derived from a promoter region (−365 to −45 nt from the *trans*-splicing site) of the E03A3.2 gene were also irradiated and scored for hatching efficiency.

*2.6. SYTO12 staining and microscopic observation of the germ cells in Cecsb(RNAi) C. elegans after UV radiation*

RNA*i* F1 progeny worms were irradiated with UV light (100 J/m$^2$) at the young adult stage. Every 12 h, up to 36 h following UV irradiation, a fraction of the worms were incubated with SYTO12 (Molecular Probes, 33 μM) in M9 buffer for 2 h, transferred to an NGM plate seeded with OP50, and recovered for 1 h. The worms were mounted on an agar pad and observed under a fluorescence microscope with an FITC filter (DMR HC, Leica) to score the SYTO12-positive germ cells. The gonads were also observed under Nomarski optics to examine morphological changes and proliferation of the germ cells.

## 3. Results and discussion

*3.1. The C. elegans csb gene structure*

The ORF F53H4.1 was predicted (by the *C. elegans* Sequencing Consortium using the Gene Finder Program) to consist of 20 exons and was annotated as a putative orthologue of human CSB protein (ERCC6). Although several EST clones for this ORF have been reported, they did not include the predicted start codon. Therefore, the exact 5′-end cDNA sequence was obtained by RT-PCR of N2 mRNA using gene-specific primers and a primer of the SL1 *trans*-splice leader sequence. The *trans*-splice leader sequence of 22 nucleotides was used in the PCR reaction since it is found in two thirds of *C. elegans* mRNAs, indicating that the precursor mRNAs have been *trans*-spliced with SL1 RNA [18]. The 5′-end cDNA clone thus obtained contained six more nucleotides (5′-GTAAAA) at the 5′-end compared with the predicted

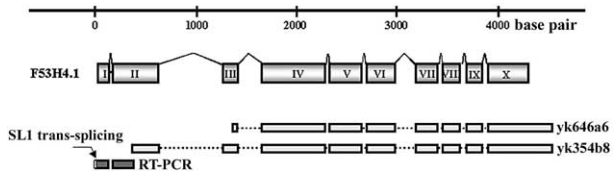


Fig. 1. The gene structure of the *C. elegans* CSB protein homologue. Analysis of EST clones and comparison of the ORF with other CSB homologues suggested that the *csb* gene consists of only the first 10 exons among the predicted 20 exons of the ORF F53H4.1. The 5′-end cDNA sequence was obtained by RT-PCR using an SL1 primer, which indicated *trans*-splicing of the pre-mRNA with SL1 RNA.

ORF. This supports the prevailing belief that one of the purposes of *trans*-splicing is to put a 5′-mRNA end close to the start codon for translation efficiency. The ORF of 20 exons encodes 1785 amino acids, which is much larger than the human CSB protein (1493) and the *S. cerevisiae* homologue RAD26 (1085) [12]. The long ORF and the following analysis of EST clones reported for this ORF suggested that the ORF probably had been mispredicted. Four EST clones, yk354b8, yk646a6, yk658b, and yk260c6, have their 3′-end cDNA sequences ending after the 10th exon (yk646a6 and yk354b8 in Fig. 1), while one cDNA clone (yk339g11) covers only exons 11–13. No EST clones have been reported for exons 14–20. Therefore, it can be conjectured that only the first 10 exons of the F53H4.1 ORF belong to the *C. elegans csb* gene. The full-length cDNA sequence of 3205 nt encodes 957 amino acids and contains a 5′-untranslated region of 6 nt and a 3′-untranslated region of 328 nt. The deduced amino acid sequence of *Ce*CSB shares 37% and 34% identities with human and *S. cerevisiae* homologues, respectively, over the 525 amino acid stretch containing the seven helicase motifs.

*3.2. Cecsb gene expression*

A GFP fusion construct containing 1.4 kb of the 5′-upstream region of the *Cecsb* gene was made, with GFP translationally fused to the second exon of the *Cecsb* gene. In the transgenic embryos, GFP first appeared during the 50–100 cell stage and was detected throughout the following embryonic development as shown in Fig. 2A. GFP expression was also observed in all of the somatic cells up to the L3 larval stage. However, GFP expression was relatively stronger in pi and P lineage vulval cells at the L3 larval stage, where active cell division and differentiation take place (Fig. 2B). At the L4 larval stage, GFP was relatively stronger in amphid and tail neurons, and vulva and somatic gonad cells than in other body regions (Fig. 2C,D). In adult hermaphrodites, the overall GFP expression weakened, but strong expression was still observed in intestine, head, and tail regions (data not shown). In L4 and adult males, the tail region, including the spicule, protractor, and PC sensilla, showed intense fluorescence (Fig. 2E). The cells that showed relatively stronger GFP expression were dividing cells and cells that play fundamental roles in essential physiological functions such as feeding, sensation, and reproduction. Since multicopy transgene expression is silent in the *C. elegans* germline, *Cecsb* gene expression in the gonad was detected by in situ mRNA hybridization instead of GFP expression, as depicted in Fig. 3. The antisense cDNA probe of *Cecsb* stained the whole region of the gonad including oocytes, while the sense probe did not produce any signal.

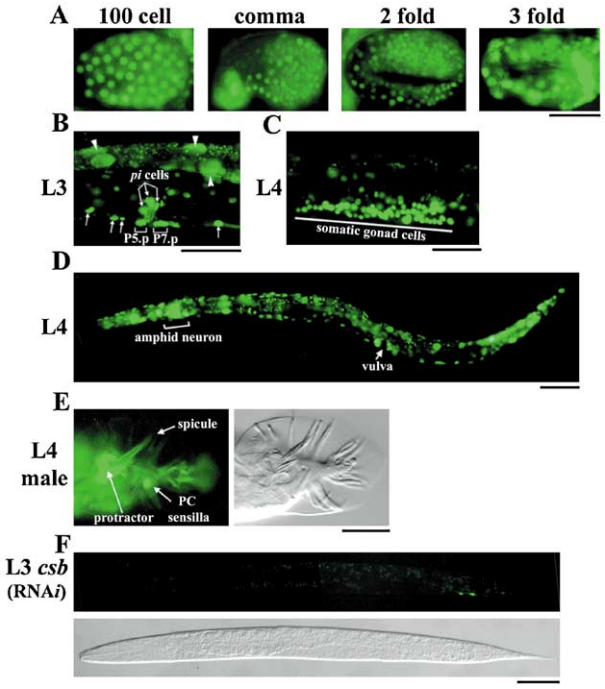


Fig. 2. GFP expression induced by the 5′-upstream DNA of the *csb* gene in *C. elegans* at various developmental stages. The *csb* gene containing 1.4 kb of the 5′-upstream DNA sequence and N-terminal exons was fused with GFP using the pPD95.69 reporter plasmid DNA. The transgenic worms and embryos were observed under a fluorescence microscope with differential interference contrast (DIC) optics. A: Embryos. B: The vulva region of an L3 hermaphrodite larva showed GFP expression in pi and P lineage cells (arrows), and intestinal cells (arrowheads). C: GFP expression in the somatic gonad cells of an L4 hermaphrodite larva. D: At the L4 larval stage, GFP was also observed in amphid and tail neurons, and the vulva. E: GFP expression in the tail of an L4 male (left panel) and the DIC image (right panel). The spicule, protractor, and PC sensilla are marked with arrows. F: Disappearance of GFP expression following RNA*i* targeting the 5′-end mRNA of the *csb* gene (upper panel) and the DIC image (lower panel). Scale bars are 20 μm (A–C,E), and 50 μm (D,F).

### 3.3. Effects of csb RNAi on the UV sensitivity of C. elegans

In order to investigate the role of the CSB protein in *C. elegans*, *Cecsb* gene expression was suppressed by RNA*i*, and developmental growth and UV sensitivity were examined. dsRNA synthesized from the yk646a6 EST clone (Fig. 1) was microinjected into N2 worms at the young adult stage. The number of embryos laid by the microinjected P0 worms was not significantly different from that of wild type worms, and F1 larvae did not show any defects in further development into adults. This result suggested either that CSB is not essential under normal growth conditions despite its ubiquitous gene expression or that another protein can replace the CSB function. The possibility that endogenous *csb* expression was not sufficiently inhibited by RNA*i* was excluded by the almost complete disappearance (Fig. 2F) of *Cecsb::gfp* expression following RNA*i* targeting the 5′-mRNA end of the *csb* gene.

F1 progeny of *csb*(RNA*i*) worms were irradiated at the young adult stage with various doses of UV and their embryos were scored for hatching efficiency. Embryos laid 24–36 h after irradiation were collected and scored, as these were estimated to have been in the mitotic phase during the UV irradiation. The F2 embryos were much more sensitive to UV

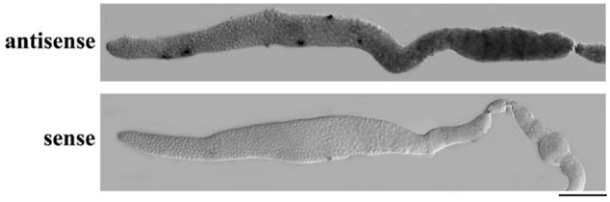


Fig. 3. *csb* mRNA localization in *C. elegans* gonads. Hermaphrodite *C. elegans* gonads were incubated with digoxigenin-labeled antisense (or sense) cDNA probe of *Cecsb*, followed by a visualization process of the hybridized probe DNA. Scale bar is 50 μm.

radiation than control embryos obtained from worms that had been microinjected with dsRNA of a promoter sequence, as shown in Fig. 4. L4 stage larvae could not be used to measure the effects of UV irradiation on hatching efficiency of embryos, since 90% of wild type L4 worms were arrested in their growth on UV irradiation of 100 J/m$^2$, and consequently did not produce any embryos. However, both wild type and *Cecsb*(RNA*i*) young adult worms did not show any lethality for the first 3 days following UV irradiation and showed only a minor lethality of 10% after 5 days (at 100 J/m$^2$). The fact that RNA*i* of the *Cecsb* gene significantly increased the sensitivity of embryos to UV radiation supported its expected role in NER. The increased sensitivity of embryos to UV radiation upon *Cecsb* RNA*i* was also observed when F1 embryos were irradiated with UV, suggesting that the CSB protein plays its role in somatic cells as well as in germ cells (data not shown).

More detailed phenotypes of the germ cells due to UV irradiation were observed under a fluorescence microscope with Nomarski optics after staining the worms with SYTO12 dye, which has been used to stain apoptotic germ

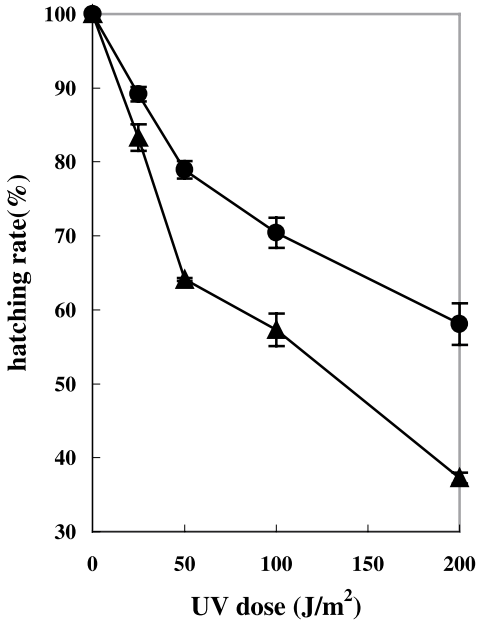


Fig. 4. Effects of *Cecsb*(RNA*i*) on the UV sensitivity of hatching efficiency. Wild type N2 worms were microinjected with dsRNA synthesized from the *Cecsb* cDNA (triangles) or an unrelated promoter DNA (circles), and the F1 progeny were irradiated with UV at the young adult stage. F2 embryos laid during the time period of 24–36 h after the UV irradiation were scored for hatching efficiency. Over 200 embryos were tested at each UV dosage in a single set of experiments, and each experiment was repeated three times. Error bars indicate standard deviations.

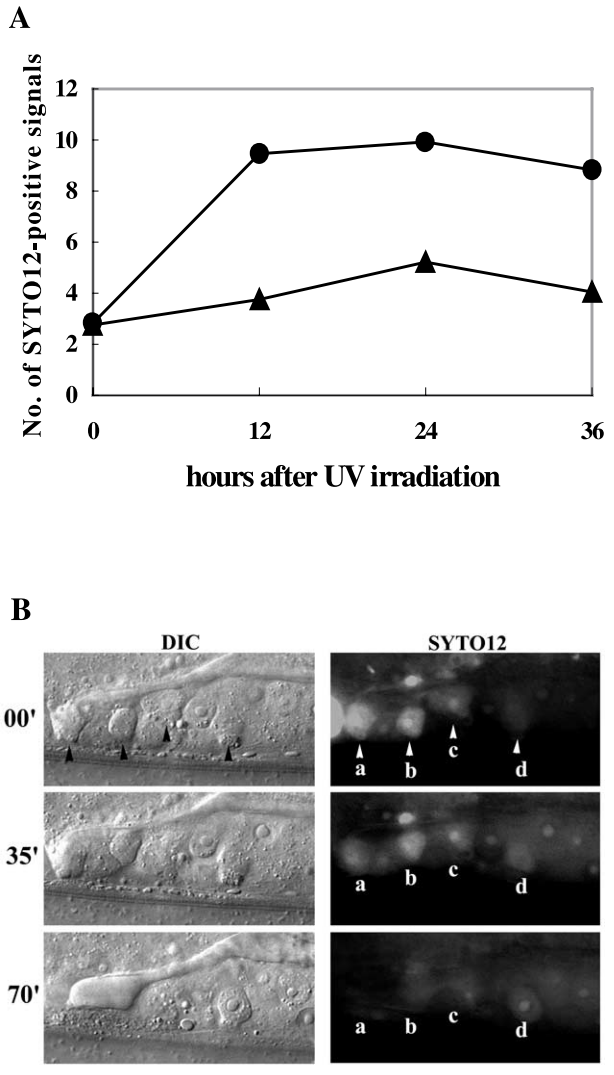


Fig. 5. Increased UV-induced apoptosis in the germ cells of *Cecsb*(RNA*i*) *C. elegans*. Wild type and F1 *Cecsb*(RNA*i*) worms were irradiated with UV (100 J/m$^2$) at the young adult stage. A: At every 12 h after the UV irradiation, the worms were stained with SYTO12 dye and observed under a fluorescence microscope with DIC optics. The SYTO12-positive cells were scored for both gonad arms in a worm and over 20 worms were examined for each data point. Circles, wild type; triangles, *Cecsb*(RNA*i*) *C. elegans*. B: Time lapse microscopy of apoptotic germ cells in *Cecsb*(RNA*i*) *C. elegans* after UV irradiation. Apoptotic germ cells near the gonad loop, marked as a–d, showed progressive changes in fluorescence intensity.

cells [19]. As shown in Fig. 5A, the number of SYTO12-positive germ cells increased four-fold in *Cecsb*(RNA*i*) worms, whereas wild type worms showed a less than two-fold increase in number. Cell death progression was observed under Nomarski optics as in Fig. 5B, where pachytene stage cells near the gonad loop region are shown. The morphological changes of the 'a' and 'b' cells clearly showed disappearance of the cells with time lapse and the intensities of the SYTO12 stain weakened, coinciding with the morphological changes. In the 'd' cell, the cell corpse appeared together with the SYTO12 stain, and the 'c' cell, showing a progressively weakening SYTO12 stain, fused with a neighboring cell and then almost disappeared. DNA damage-induced germ cell deaths have been demonstrated in *C. elegans* gonads by Gartner et al. [20] using γ-rays, and the cell death frequency was much higher in their work than in ours, suggesting the effectiveness of double-stranded DNA breaks relative to adductive DNA modification. As previously observed in the work by Gartner et al. [20], mitotic cells at the wild type gonad tip showed enlargement of nuclear volume (marked with arrowheads in Fig. 6) and sparse packing of the cells, indicating cell proliferation arrest. In the *Cecsb*(RNA*i*) gonad, the nuclear enlargement appeared much earlier (at 6 h and 12 h after UV irradiation in Fig. 6) and led to much larger increases in cell volume and aggravated abnormalities in cellular morphology later on. These more severe phenotypes of morphological change and proliferation arrest in the mitotic germ cells of the *Cecsb*(RNA*i*) gonad, relative to the wild type in Fig. 6, again demonstrated that the CSB protein functions against DNA damages induced by UV radiation. The increased apoptosis at the pachytene stage of the *Cecsb*(RNA*i*) gonad, shown in Fig. 5, was probably due to ineffective NER in the absence of CSB and the resulting accumulation of DNA damages. When P0 and F1 *csb*(RNA*i*) worms were placed in NGM plates containing methyl methanesulfonate, no increased sensitivity compared with the N2 wild type strain was observed, verifying that the RNA*i* effects were specific to NER, and not to other DNA damage responses.

Although no clear phenotypes were observed following RNA*i* of the *csb* gene, marked effects on germ cell development and proliferation (Figs. 5 and 6), and also embryogenesis (Fig. 4), were observed upon UV irradiation, as expected from the well-known function of human CSB in NER. The relatively stronger *Cecsb::gfp* expression in dividing cells and cells that play physiologically important roles coincided with the role of CSB in transcription-coupled NER. To further explore the possibility of using the *csb*(RNA*i*) *C. elegans* as a model organism for CS, physiological properties such as defects in neurons or life span should be examined in detail and correlated with the phenotypes of human CS.

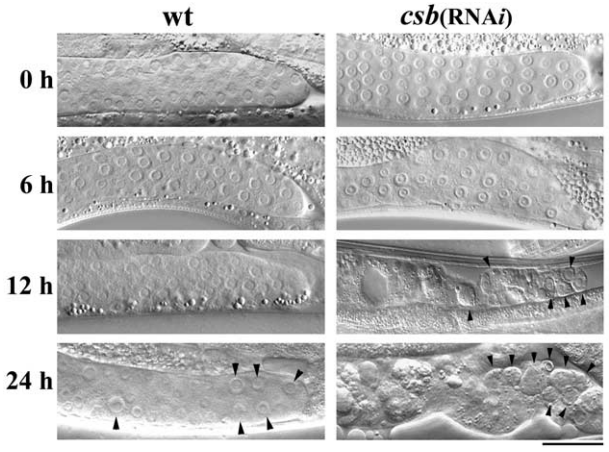


Fig. 6. Morphological abnormalities in mitotic germ cells of the *Cecsb*(RNA*i*) *C. elegans* following UV irradiation. Wild type and F1 *Cecsb*(RNA*i*) worms were irradiated with UV (100 J/m$^2$) at the young adult stage. At the indicated hours after UV irradiation, the gonad tip region of the worms was observed under DIC optics. Nuclear expansion (marked with arrowheads) was apparent at 12 h in *Cecsb*(RNA*i*) worms, while the wild type showed later and milder nuclear expansion. Scale bar is 20 μm.

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