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A *C. elegans* homolog of the Cockayne syndrome complementation group A gene

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Abstract

Cockayne syndrome (CS) is a debilitating and complex disorder that result from inherited mutations in the CS complementation genes A and B, *CSA* and *CSB*. The links between the molecular functions of the CS genes and the complex pathophysiology of CS are as of yet poorly understood and are the subject of intense debate. While mouse models reflect the complexity of CS, studies on simpler genet models might shed new light on the consequences of CS mutations. Here we describe a functional homolog of the human *CSA* gene in *Caenorhabditis elegans*. Similar to its human counterpart, mutations in the nematode *csa-1* gene lead to developmental growth defects as a consequence of DNA lesions.

Keywords

Nucleotide excision repair; *C. elegans*; Cockayne syndrome

1. Introduction

Congenital defects in DNA repair pathways lead to highly complex disease syndromes that are characterized by various degrees of developmental failure, segmental premature ageing, and tissue-specific cancer susceptibility. These DNA repair deficiencies have suggested a causal role of DNA damage in human pathology[1]. However, the link between specific unrepaired lesions and mechanistic roles of DNA repair molecules to the disease manifestations is incompletely understood. Nucleotide excision repair (NER) deficiencies provide a particular intriguing example of the complexity of human disorders caused by mutations in DNA repair genes. NER removes helix-distorting lesions such as UV-induced 6-4 photoproducts (6-4PPs) and cyclobutane pyrimidine dimers (CPDs) [2]. These lesions

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are recognized by two distinct detection systems: Global-genome (GG)-NER scans the entire genome, while transcription-coupled (TC)-NER recognizes lesions at sites of RNA polymerase (RNAP) II stalling. Congenital defects that primarily inactivate GG-NER lead to Xeroderma pigmentosum (XP) and an increased risk of skin cancer. On the other hand, mutations in *CSA* and *CSB*, which inactivate TC-NER, cause Cockayne syndrome (CS) [3]. Classical CS cases exhibit postnatal growth retardation, followed by cachexia, neuronal degeneration, loss of retinal cells, and premature death between the ages of 12 and 16. Mutations in *CSA* or *CSB* can also cause mild UV hypersensitivity syndrome (UVSS), as well as the more severe type II CS and Cerebro-oculo-facio-skeletal syndrome (COFS) [4]. The causal relationship between mutations in *CSA* and *CSB* and these disorders remains obscure, especially in light of the variations in pathology and severity. *S. cerevisiae* budding yeast strains with certain mutations in Rad28 and Rad26 (homologs of *CSA* and *CSB*) display only mild increases in UV sensitivity [5]. In contrast, mutations in the *S. pombe CSA* homolog *ckn1+* do not confer UV-sensitivity in an otherwise repair-proficient background [6]. Mice that are defective in *Csa* or *Csb* develop mild symptoms that mirror CS and show elevated UV-induced skin cancer susceptibility [7,8]. Only upon additional inactivation of the GG-NER factor *Xpc* or the general NER factor *Xpa* do the mice develop severe growth retardation and degenerative phenotypes reminiscent of the severe CS pathology in humans [9]. While the murine studies have been important for understanding physiological alterations caused by NER defects, the disease complexity in mammals makes it very challenging to gain a basic mechanistic understanding of the outcomes of transcription-blocking DNA lesions and TC-NER deficiencies in multicellular organisms. Therefore, it is critical to establish simple metazoan systems to study TC-NER functions and the consequences of transcription-blocking DNA lesions. The utility of such model systems depends on the identification of functional homologs of TC-NER genes. While *CSB* homologs are clearly conserved throughout evolution, a nematode homolog for *CSA* has remained elusive. The CSA protein is comprised of WD40 domains spanning nearly the entire protein sequence [10]. WD40 domains occur in a large variety of proteins and make the unequivocal assignment of homologous genes in the absence of other functional domains difficult [11]. We have identified the open reading frame D2013.3, which we propose to name *csa-1*, as the closest homolog of *CSA* in the nematode worm *C. elegans*. A functional homology has been confirmed by demonstrating that mutations in *csa-1* sensitize animals to UV irradiation during developmental growth. Moreover, we show that *csa-1* forms an epistasis group with *csb-1*, and, similar to *Csa* mutations in yeast and mice, loss of functional *csa-1* enhances the UV sensitivity of GG-NER defective nematode strains. Our results form the basis of further analysis of the roles of the CSA protein in the context of a simple metazoan model system.

2. Materials and methods

2.1. Growth conditions

C. elegans strains were cultured according to standard conditions at 20°C on nematode growth medium (NGM) plates with *E. coli* strain OP50 [12]. Strains used were: N2 (Bristol; wild type), *csb-1* (*ok2335*), *xpa-1* (*ok698*), *xpc-1* (*tm3886*), *csa-1* (*tm4539*), *csa-1* (*tm5232*), *csa-1* (*tm4916*).). *csa-1* mutant strains were obtained from National Bioresource Project;

other strains were provided from the *Caenorhabditis* Genetics Center. All strains were backcrossed a minimum of four times.

2.2. Somatic UV-sensitivity assay

Eggs were obtained from gravid adult worms by hypochlorite treatment and were allowed to hatch over night in M9 buffer. The following day, synchronized L1 larvae were transferred to NGM agar plates with OP50 and 4 hours later, UV-irradiated (310nm) with the indicated doses using a UV6 lamp (Philips) in a Waldmann UV236B device. A minimum of 40 worms was used for each UV-dose, and each treatment was conducted in triplicate. Post-UV, worms were allowed to develop for 65 hours at 20°C. Afterwards, the percentage of different larval stages in each treatment was counted and quantified to assess the UV-sensitivity.

2.3. Germline UV-sensitivity assay

Staged young adults were treated with indicated UV-doses and allowed to recover for 16 hours. Following recovery, timed egg laying was conducted by transferring three worms per UV-dose to new NGM plates with OP50 for 3 hours. Each treatment was conducted in triplicate. The total number of eggs laid was counted and, 48 hours later, the number of viable eggs was determined. To assess UV-sensitivity, the percentage of eggs that were laid after UV-treatment was compared to $0J/m^2$ and the percentage of viable eggs was determined after hatching.

2.4. Illudin-M sensitivity assay

Synchronized L1 larvae were obtained by hypochlorite treatment and treated with the indicated concentrations of illudin-M in liquid S-basal medium with OP50 for 24 hours at 20°C on a shaking platform. Worms were then transferred to NGM plates with OP50 and allowed to grow for another 40 hours. A minimum of 40 worms was used for each illudin-M dose and each treatment was conducted in triplicate. Illudin-M sensitivity was measured by determining the percentage of different larval stages in each treatment. Illudin-M was a kind gift from Rainer Schobert.

3. Results and discussion

3.1. Identification of the C. elegans CSA homolog

While the CSB protein has a clearly identifiable homolog in *C. elegans*, no homolog for CSA has been uncovered thus far. CSA is largely comprised of WD40-repeat domains that are found in a large number of sequence-related proteins. For the bioinformatic identification of a *C. elegans* CSA homolog, we constructed a generalized sequence profile from a multiple sequence alignment of established CSA sequences. This profile was used to screen a database of *C. elegans* protein sequences (current version of Wormbase [13]). The procedure identified 55 different *C. elegans* gene products with significant similarity to the profile, most containing no biological relationship to CSA except for the presence of WD40-repeats. A dendrogram using the neighbor-joining method [14] was calculated from a multiple alignment [15] of the *C. elegans* proteins and representative CSA sequences (Figure 1A, Supplementary Figure 1). As shown in Figure 1A, the uncharacterized *C.*

elegans ORF D2013.3 clusters with the CSA proteins and was thus selected for further experimental characterization. D2013.3 is 1968 nucleotides long and is organized into seven exons. The coding sequence is 1083 nucleotides long and encodes a 360 amino acid protein. We obtained three deletion alleles for D2013.3 from National BioResource Project [16]. The *tm4539* and *tm5232* alleles contain 150 bp and 212 bp deletions, which delete parts of intron 4 and exon 5 (respectively). Both alleles are expected to encode null mutations as they introduce frameshifts that should be polar on the downstream parts of the open reading frames. The *tm4916* allele encodes a 171 nucleotide deletion in intron 4 (Figure 1B).

3.2. D2013.3 mutants are UV-hypersensitive

The *C. elegans* life cycle consists of four larval stages: L1, L2, L3, and L4, which precede adulthood. In *C. elegans*, the two branches of NER lead to UV hypersensitivity at distinct developmental stages [17,18]. Maintenance of the germline of adult animals and the embryos requires predominantly GG-NER, while late embryos and L1 larvae mostly rely on TC-NER. Most developmental cell divisions occur during embryogenesis [19] and the adult germline contains populations of mitotic and meiotic cells (in contrast, the adult soma is post-mitotic); thus the requirement of GG-NER in these contexts corresponds with their high proliferative activity. Consequently, the germ cells of GG-NER deficient *xpc-1* mutant strains are highly UV sensitive. TC-NER, in contrast, is required during late embryonic and early larval development when cells are expected to be highly transcriptionally active. So far, the only TC-NER specific mutation in *C. elegans* is a deletion in the *CSB* homolog, *csb-1* [17].To investigate whether D2013.3 is involved in TC-NER, we subjected L1 larvae carrying one of the three deletion alleles to different UV doses and followed their development. 65 hours post-UV, the untreated worms reached adulthood, whereas worms carrying the *tm4539* and *tm5232* alleles displayed UV-dependent development arrest after a dose of 60 mJ/cm^2, similar to a *csb-1* mutant strain (Figure 2A). In contrast to the other two alleles, the *tm4916* allele displayed no UV-hypersensitivity and developed similar to wild-type animals. This observation could be explained by the fact that the deletion in tm4916 only affects intronic sequence, while the exons remain intact. The UV-hypersensitivity of D2013.3 supports our bioinformatic identification and we now refer to D2013.3 as *csa-1*. The *csa-1*(*tm4539*) mutant allele was used for further experiments.

3.3. csa-1 is dispensable for GG-NER

To validate the specificity for *csa-1* in TC-NER, we next examined the UV sensitivity of germ cells, which mainly rely on GG-NER. UV-treatment of GG-NER-deficient *xpc-1* mutant animals leads to a reduction in fertility [17]. To examine the role of *csa-1* in GG-NER, young adult worms were UV-treated, allowed to recover for 16 hours and their egg-laying activity was assessed over the course of three hours. The *xpc-1* mutant animals displayed a dose-dependent reduction in the number of eggs laid and the percentage of eggs hatched. In contrast, UV irradiation had no significant effect on the germline activity of *csa-1* mutant worms, as there was no significant reduction in the number of eggs laid after UV or the percentage of eggs hatched (Figure 2B). Hence, *csa-1* is dispensable for GG-NER.

3.4. csa-1 mutants are sensitive to Illudin-M

To further elucidate the role of *csa-1* in NER, we tested the sensitivity of *csa-1* mutant animals to illudins: sesquiterpenoid compounds isolated from the mushroom *Omphalotus illudens* with tumor-therapeutic properties [20]. Though the nature of the DNA lesions induced by illudins is not clearly understood, previous studies have demonstrated that illudin-induced lesions interfere with transcription and require TC-NER for their removal [21]. We hypothesized that the illudin treatment would induce a developmental arrest in TC-NER deficient worms similar to UV-induced DNA lesions. 40μM illudin-M induced a developmental delay in *csa-1* and *csb-1* mutant worms and a higher dose (40μM) caused developmental arrest (Figure 3). As expected, the development of the GG-NER deficient *xpc-1* mutant strain was only very mildly affected. These results further corroborate a TC-NER-specific function of *C. elegans csa-1*.

3.5. csa-1 is transcription-coupled NER specific

In order to integrate *csa-1* into the genetics of NER, we next tested the genetic interactions of *csa-1* with TC-NER-deficient *csb-1*, GG-NER deficient *xpc-1* and total NER deficient *xpa-1* mutant strains (Figure 4). As expected, *csa-1*;*csb-1* double mutants displayed similar UV-sensitivity as the corresponding single mutants, indicating that *csa-1* and *csb-1* are epistatic. Likewise, UV-sensitivity of *csa-1*;*xpa-1* was similar to *xpa-1*, suggesting that *csa-1* acts in the same pathway as *csb-1* and upstream of *xpa-1*. UV-sensitivity of *csa-1*;*xpc-1* double mutants was enhanced compared to both single mutants, reminiscent of the UV sensitivity of *csb-1*;*xpc-1* double mutants. This relationship suggests that in *csa-1*;*xpc-1* double mutants, both the NER sub-pathways are compromised, as they are in *csb-1*;*xpc-1* double mutants. Hence, *csa-1* is TC-NER-specific and, along with *csb-1*, acts in parallel to *xpc-1* to repair UV-induced DNA lesions.

3.6. Discussion

The remarkable complexity of pathologies caused by *CSA* mutations necessitates the establishment of experimentally tractable multicellular systems for studying NER mutations. Here, we report the identification of a functional homolog of the Cockayne Syndrome gene, *CSA*, in the metazoan model *C. elegans*. We demonstrated that *csa-1* clusters most closely with budding yeast *Rad28*. In yeast TC-NER is not required to resistance to UV irradiation [5]; however, TC-NER mutations further aggravate the UV-hypersensitivity of GG-NER mutants. Similarly, TC-NER mutations in mice only give rise to mild CS-like phenotypes, while mutations in Xpc strongly enhance the consequences of *Csa* and *Csb* loss [9]. In contrast, most human patients carrying mutations in *CSA* or *CSB* develop classical type I CS with severe growth defects and mental retardation and do not survive beyond the second decade of life [4]. Similar to yeast, proliferating cells in *C. elegans* mostly rely on GG-NER to repair UV-induced lesions. In contrast to unicellular yeast, however, post-mitotic tissues in *C. elegans* become primarily reliant on TC-NER to withstand UV irradiation. Hence, developing larvae that have completed most of their cell divisions during embryogenesis require *csa-1* and *csb-1* when challenged with UV. Nonetheless, *xpc-1* mutations strongly enhance UV sensitivity of TC-NER mutants during development, arguing for a backup function of GG-NER, similar to the genetic observations in mice. The *C. elegans* system

provides a clear developmental distinction between the utilization of the two NER branches. The availability of *csa-1* mutant strains will help to further define the function of TC-NER components during developmental growth and genome integrity in differentiated tissues.

4. Conclusions

NER genes are highly conserved from nematodes to mammals. Intriguingly, the distinct functions of the two NER branches: TC-NER during development and GG-NER in proliferating cell types, is phenotypically consistent in *C. elegans*. This conservation reflects the distinct pathologies of developmental impairments in CS patients and the genome instability that leads to UV-induced skin cancer development in XP patients. The identification of a functional homolog of *csa-1* in *C. elegans* further establishes the nematode model for investigating the function of TC-NER during development and ageing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

bp	Base pairs
6-4 PP	6-4 photoproduct
cm	Centimetre
CPD	Cyclobutane pyrimidine dimer
CS	Cockayne syndrome
COFS	Cerebro-oculo-facio-skeletal-syndrome
GG-NER	Global-genome nucleotide excision repair
mJ	Milli-Joule
NER	Nucleotide excision repair
NGM	Nematode growth medium
nt	Nucleotide
ORF	Open reading frame
RNAP II	RNA polymerase II

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TC-NER	Transcription-coupled nucleotide excision repair
UTR	Un-translated region
UV	Ultra-violet
UVSS	UV-hypersensitivity syndrome
XP	Xeroderma pigmentosum

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Highlights

- *C. elegans* possesses an orthologue for the Cockayne syndrome complementation group A (*CSA*) gene
- *C. elegans csa-1* mutants are sensitive the UV-induced DNA damage during development
- Genetically *csa-1* is epistatic to *csb-1* and mutant *csa-1* enhances UV-sensitivity of global-genome NER defective *xpc-1* mutants

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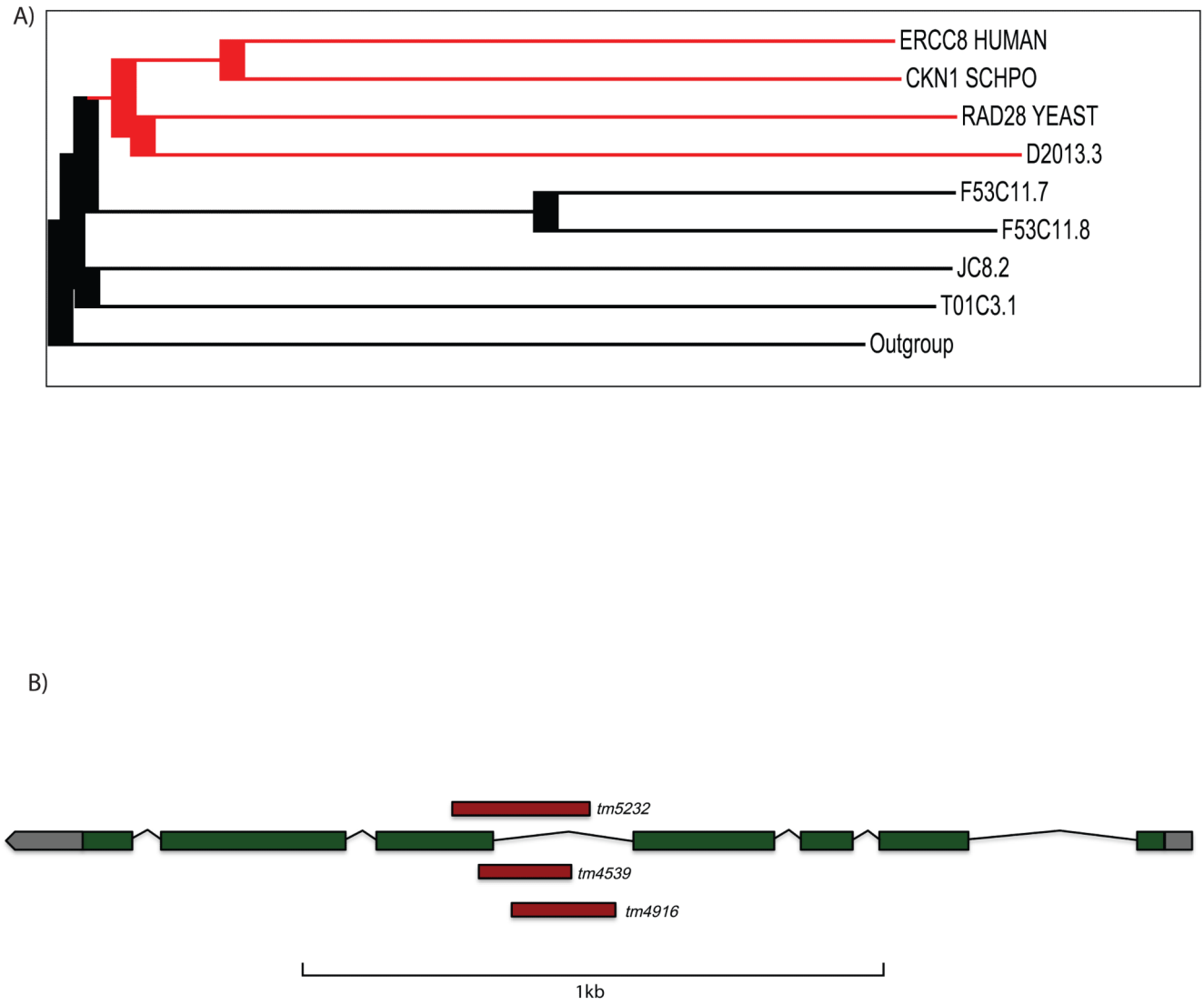

Figure 1. Bioinfomatical identification of D2101.3 as a CSA homolog

(A) A dendrogram showing the alignment of *C. elegans* proteins and known CSA sequences, calculated using the neighbour-joining method. The uncharacterized *C. elegans* ORF D2013.3 clusters with the CSA sequences. (B) Representation of the genomic architecture of D2013.3. Green boxes represent exons, black lines represent introns, and untranslated regions are in gray. The region deleted in the corresponding alleles is represented in red.

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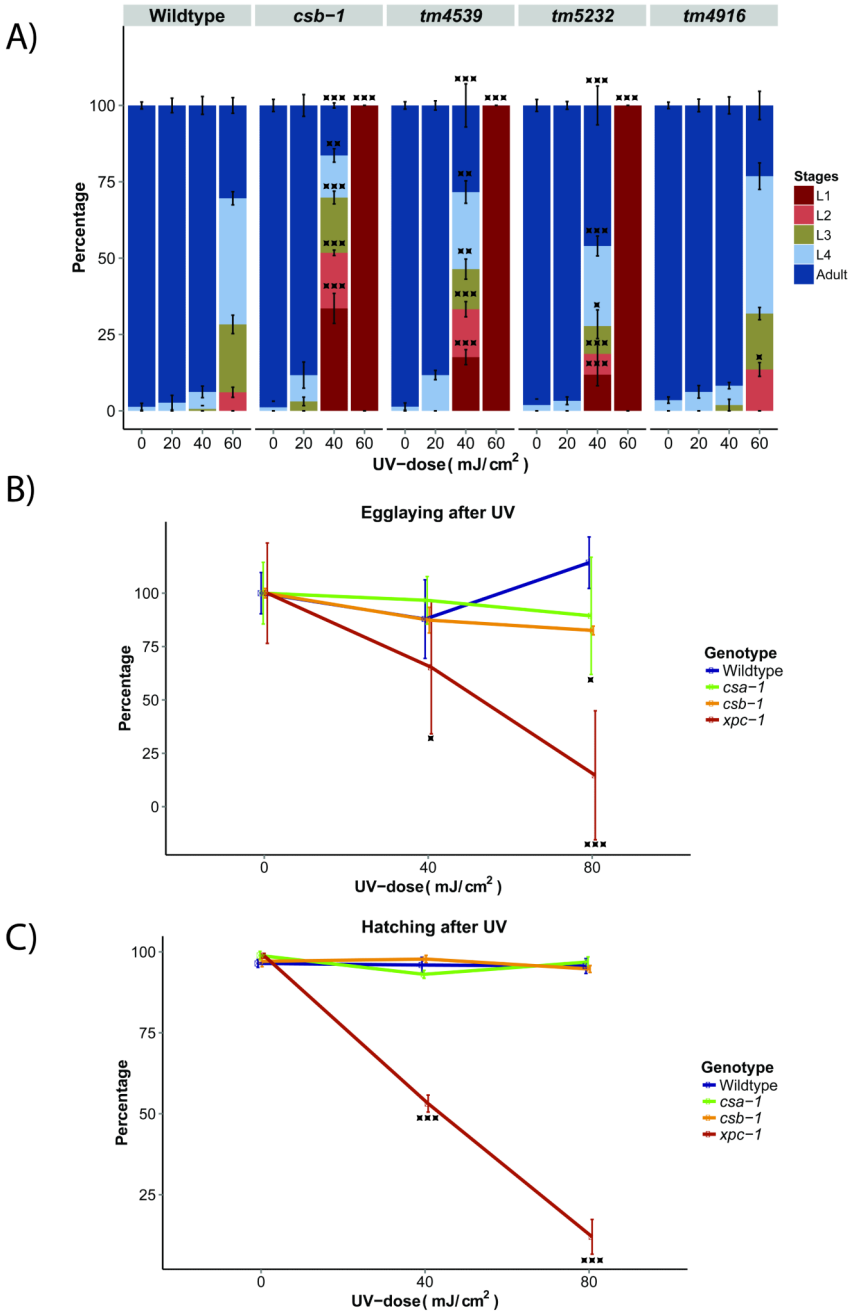

Figure 2. D2013.3 is required for TC-NER but dispensable for GG-NER

(A) The percentage of larval stages 65 hours after UV-treatment administered at the L1 larval stage. The *tm4539* and *tm5232* alleles display UV-sensitivity similar to the TC-NER-defective *csb-1* mutant strain. A minimum of 40 worms were used for each UV-dose, and each treatment was conducted in triplicate. Error bars represent SD. *=p<0.05, **=p<0.01, ***=p<0.001, two-tailed t-test, compared to wildtype. (B) Egg-laying activity of the indicated mutants 16 hours after UV-treatment at young adult stage. Timed egg-laying was conducted with three worms per UV-dose for 3 hours. Each treatment was conducted in

triplicate. (C) Percentage of hatched eggs after UV-treatment. Egg-laying and hatching of *csa-1* mutant worms are unaffected, unlike GG-NER deficient *xpc-1* mutant worms. B,C-Error bars represent SD. Statistical significance was calculated using two-tailed t-test, comparing treatments to 0mJ/cm^2.

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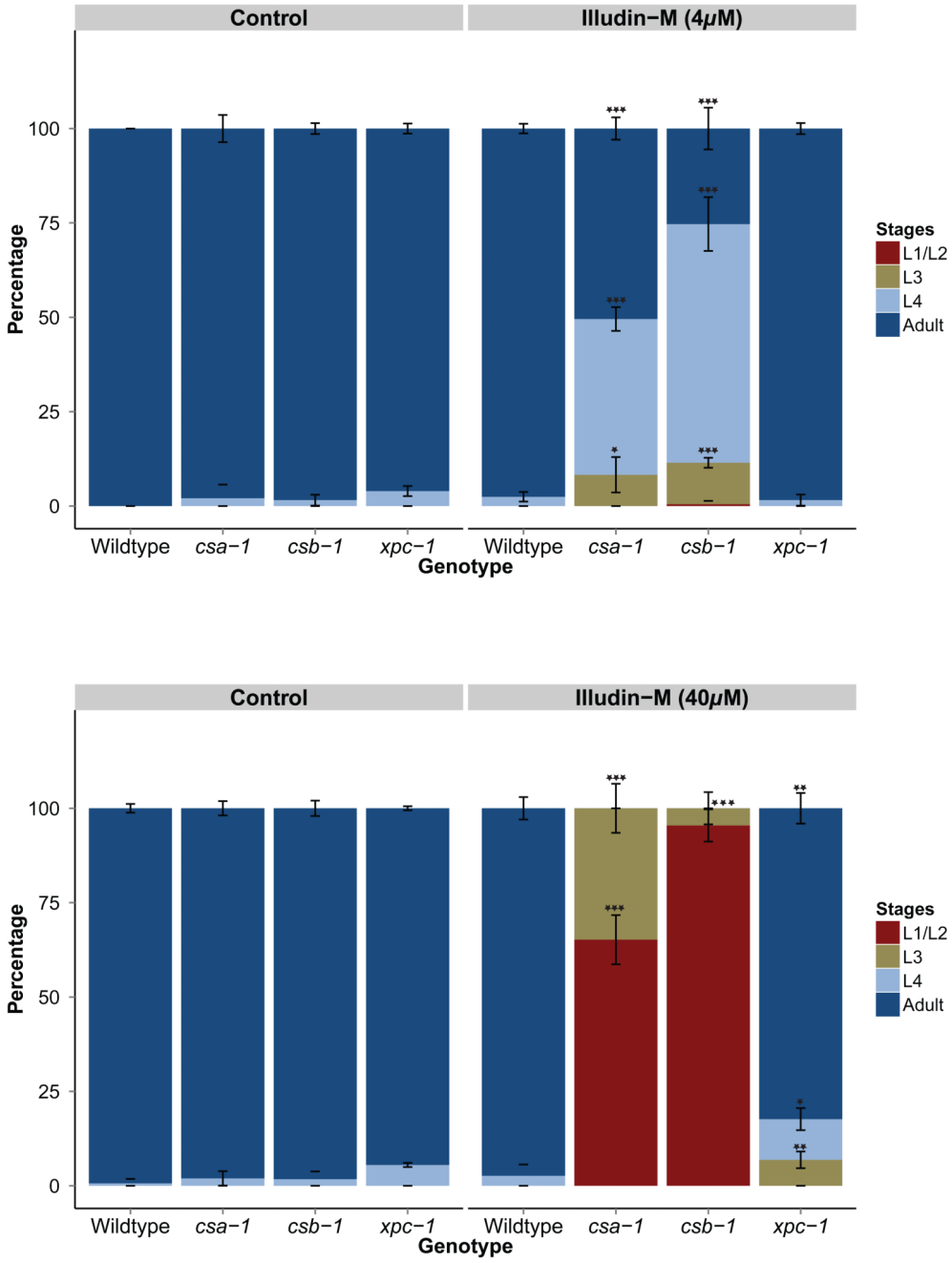

Figure 3. *csa-1* mutants are illudin-M sensitive

Percentage of larval stages 65 hours after 4μM or 40μM illudin-M treatment. Illudin-M induces a developmental delay at 4μM and a developmental arrest at 40μM in *csa-1* and *csb-1* mutants. A minimum of 40 worms was used for each illudin-M dose and each treatment was conducted in triplicate. Error bars represent SD. *=p<0.05, **=p<0.01, ***=p<0.001, two-tailed t-test, compared to wildtype.

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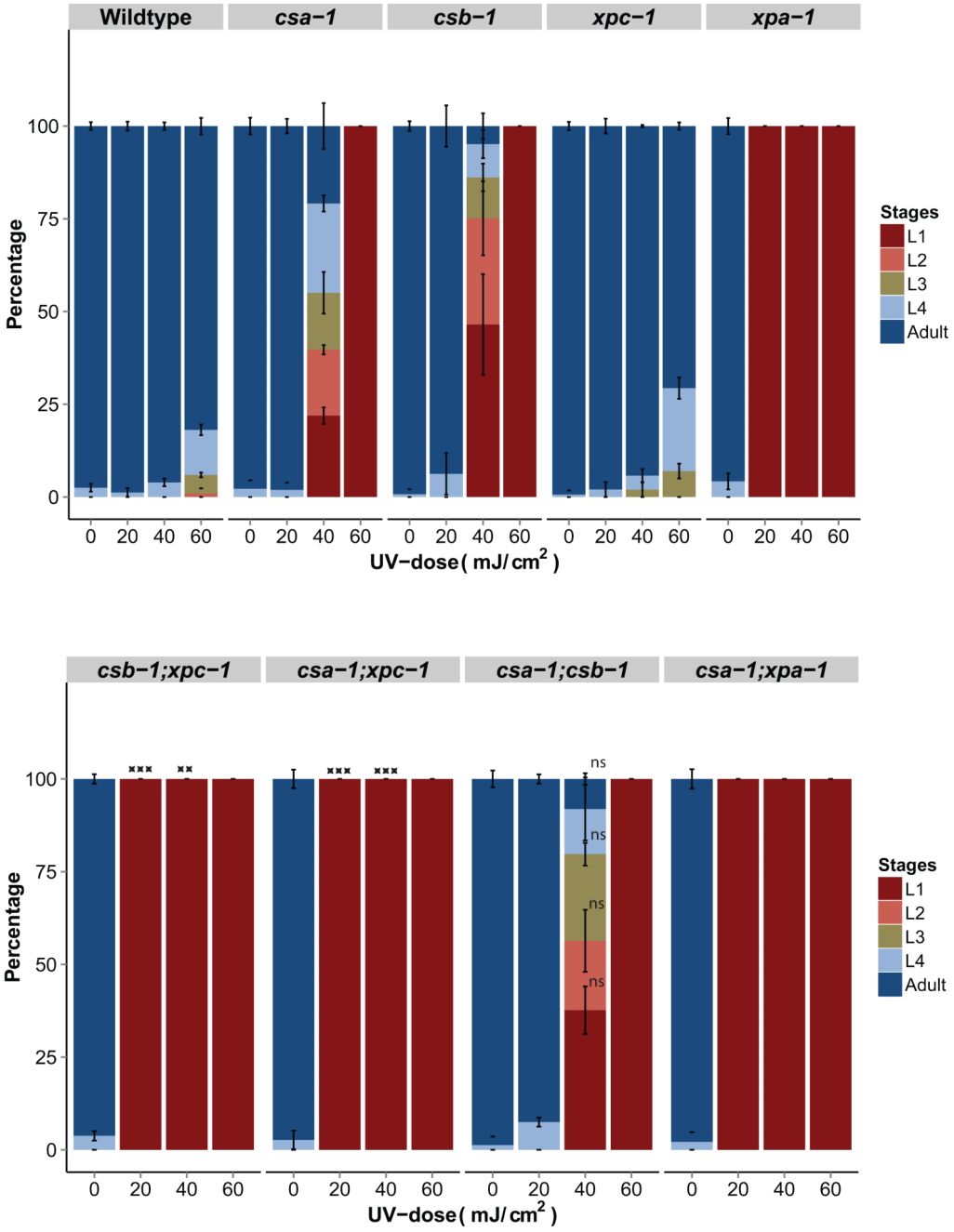

Figure 4. *csa-1* is epistatic to *csb-1* and TC-NER specific

Percentage of larval stages 65 hours after UV-treatment during the L1 larval stage of the indicated genotypes. *csa-1*;*csb-1* double mutants display similar UV-sensitivity as *csa-1* and *csb-1* single mutants. csa1;*xpc-1* double mutants display enhanced UV-sensitivity compared to *csa-1* and *xpc-1* single mutants. A minimum of 40 worms was used for each UV-dose, and each treatment was conducted in triplicate. Error bars represent SD. ns = not significant, two-tailed t-test, compared to *csb-1*.