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The Snail-like CES-1 protein of *C. elegans* can block the expression of the *BH3-only* cell-death activator gene *egl-1* by antagonizing the function of bHLH proteins

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SUMMARY

The NSM cells of the nematode *Caenorhabditis elegans* differentiate into serotonergic neurons, while their sisters, the NSM sister cells, undergo programmed cell death during embryogenesis. The programmed death of the NSM sister cells is dependent on the cell-death activator EGL-1, a BH3-only protein required for programmed cell death in *C. elegans*, and can be prevented by a gain-of-function (gf) mutation in the cell-death specification gene *ces-1*, which encodes a Snail-like DNA-binding protein. Here, we show that the genes *hlh-2* and *hlh-3*, which encode a Daughterless-like and an Achaete-scute-like bHLH protein, respectively, are required to kill the NSM sister cells. A heterodimer composed of HLH-2 and HLH-3, HLH-2/HLH-3, binds to Snail-binding sites/E-boxes in a cis-regulatory region of the *egl-1* locus in vitro that is required for the death of the NSM sister cells in vivo. Hence, we propose that HLH-2/HLH-3 is a direct, cell-type specific activator of *egl-1* transcription. Furthermore, the Snail-like CES-1 protein can block the death of the NSM sister cells by acting through the same Snail-binding sites/E-boxes in the *egl-1* locus. In *ces-1*(gf) animals, CES-1 might therefore prevent the death of the NSM sister cells by successfully competing with HLH-2/HLH-3 for binding to the *egl-1* locus.

Key words: Apoptosis, *C. elegans*, *egl-1*, Snail-like transcription factor, bHLH proteins

INTRODUCTION

The elimination of unwanted cells by programmed cell death or apoptosis is a fundamental feature of animal development and the mechanisms of programmed cell death have been conserved through evolution (reviewed by Ellis et al., 1991; Strasser et al., 2000). For example, more than 50% of all neurons formed by neurogenesis in vertebrates are removed by programmed cell death before adulthood (reviewed by Oppenheim, 1991). By removing neurons that are superfluous or that have not made appropriate connections with their targets, programmed cell death plays an important role in the formation of a functional vertebrate nervous system. What triggers neurons to undergo programmed cell death in the developing nervous system is largely unknown. One inducer of neuronal death appears to be the removal of trophic factors, such as nerve growth factor (NGF). NGF deprivation has been shown to induce programmed cell death in a cell non-autonomous manner in a number of models of neuronal death, such as the death of sympathetic neurons (reviewed by Deshmukh and Johnson, 1997). The death of these neurons is not only dependent on NGF deprivation but on the synthesis of macromolecules, i.e. on an active transcription and translation machinery. The programmed death of neurons by trophic factor removal therefore also depends on intrinsic, cell-autonomous programs and/or the fate of the cell.

Programmed cell death is also a fundamental feature of neurogenesis in invertebrates, including the nematode *Caenorhabditis elegans*. The nervous system of a wild-type *C. elegans* hermaphrodite is composed of 302 neurons of at least 118 different types (Sulston and Horvitz, 1977; Sulston et al., 1983). During the development of a *C. elegans* hermaphrodite, 131 somatic cells die by programmed cell death, a process that is determined by the essentially invariant *C. elegans* cell lineage. Most of these 131 cells are sisters of cells that differentiate into neurons and, when prevented from undergoing programmed cell death, often adopt a neuronal fate themselves (Ellis and Horvitz, 1986; Avery and Horvitz, 1987). Hence, more than a quarter of the cells that are initially generated and that have the potential to form neurons are eliminated by programmed cell death during the development of the *C. elegans* nervous system.

Genetic analyses of programmed cell death in *C. elegans* have been instrumental for our current understanding of the conserved, molecular mechanisms of this essential process (reviewed by Horvitz, 1999). Four genes, *egl-1* (*egl*, egg-laying

defective), *ced-9* (*ced*, cell-death defective), *ced-4* and *ced-3*, have been identified, which, when mutated, can block programmed cell death during development. These four genes act in a simple genetic pathway, in which *egl-1* negatively regulates *ced-9*, *ced-9* negatively regulates *ced-4* and *ced-4* positively regulates *ced-3*, the activity of which is required for programmed cell death. The *ced-3* gene encodes a pro-caspase, *ced-4* an Apaf1-like adaptor, *ced-9* a Bcl-2-like cell-death inhibitor and *egl-1* a pro-apoptotic member of the Bcl-2 family, a BH3-only protein. Genetic and cell biological observations indicate that CED-9, CED-4 and proCED-3 proteins are present in most if not all cells during *C. elegans* embryogenesis. It has been proposed that in cells that live, CED-9 blocks the activity of CED-4 and, hence, the CED-4-dependent activation of proCED-3. Conversely, in cells destined to die, EGL-1 negatively regulates CED-9 thereby allowing the activation of proCED-3 (Chen et al., 2000).

Little is known thus far about how the central cell-death pathway and, in particular, the activity of its most upstream component, the BH3-only protein EGL-1, is regulated to specifically cause the death of the 131 cells that are destined to die during *C. elegans* development. EGL-1 is likely to be regulated by cell-specific factors because genes have been identified that act upstream of *egl-1* and that, when mutated, block specific cell deaths. For example, mutations in the genes *ces-2* and *ces-1* (*ces*, cell-death specification) block specifically the death of the NSM sister cells, and of the NSM sister cells and the I2 sister cells respectively (Ellis and Horvitz, 1991). Furthermore, a mutation in the gene *tra-1* (*tra*, transformer) prevents the death of the hermaphrodite-specific neurons (HSNs) in males (Conradt and Horvitz, 1999). In addition to being involved in the specification of the male-specific death of the HSNs, the *tra-1* gene has a more general role during *C. elegans* development. *tra-1* functions as the terminal regulator of all somatic sexual fates in *C. elegans* (reviewed by Goodwin and Ellis, 2002). The cell-specific pathways that regulate EGL-1 activity therefore may not be cell-death specific pathways but rather pathways that play additional, important roles during development. *tra-1* encodes a zinc-finger DNA-binding protein, TRA-1A, which directly represses the transcription of *egl-1* in the HSNs of hermaphrodites but not in the HSNs of males. At least in the HSNs, in which the life-versus-death decision is determined by somatic sex, EGL-1 activity is therefore regulated at the transcriptional level (Conradt and Horvitz, 1999).

In contrast to the male-specific death of the HSNs, the death of the NSM sister cells appears to be determined solely by lineage. Whether the signal that triggers this particular death also does so by regulating *egl-1* transcription has not yet been determined. As described above, two genes, *ces-1* and *ces-2*, have been identified that, when mutated, can block the death of the NSM sister cells and therefore cause a cell-death specification or Ces phenotype (Ellis and Horvitz, 1991). A loss-of-function (lf) mutation in the gene *ces-2* blocks the death of the NSM sister cells, indicating that *ces-2* is required for their programmed death. The death of the NSM sister cells is also blocked by a gain-of-function (gf) mutation in *ces-1*, which suggests that *ces-1* has cell-death protective activity and that it can function to prevent the death of the NSM sister cells. A *ces-1*(lf) mutation causes no obvious phenotype; however, it suppresses the ability of the *ces-2*(lf) mutation to block the death of the NSM sister cells, suggesting that *ces-2* causes the NSM sister cells to die by negatively regulating the cell-death protective activity of *ces-1*. *ces-2* encodes a DNA-binding protein most closely related to the proline- and acid-rich (PAR) subfamily of basic leucine-zipper (bZIP) transcription factors of vertebrates (Metzstein et al., 1996). *ces-1* encodes a DNA-binding protein most similar to members of the Snail family of zinc-finger transcription factors (Metzstein and Horvitz, 1999). *cis*-regulatory regions upstream of the *ces-1* transcription unit include a potential CES-2 binding site, which suggests that CES-2 might be a direct, negative regulator of *ces-1* transcription. The *ces-1*(gf) mutation is located adjacent to this potential CES-2 binding site, which suggests that this mutation results in NSM sister cell survival by causing overexpression of *ces-1* in the NSM sister cells. This hypothesis is supported by the observation that overexpression of *ces-1* from extra-chromosomal arrays carrying the wild-type *ces-1* locus causes NSM sister cell survival (Metzstein and Horvitz, 1999).

Members of the Snail family of zinc-finger DNA-binding proteins act predominantly as repressors of transcription (reviewed by Hemavathy et al., 2000). It has therefore been proposed that in *ces-1*(gf) animals, CES-1 blocks the death of the NSM sister cells by blocking the transcription of a pro-apoptotic gene (Metzstein and Horvitz, 1999). As *ces-1* acts upstream of the cell-death activator gene *egl-1*, *egl-1* is a candidate target of *ces-1* (Conradt and Horvitz, 1998). In this paper we present data indicating that the basic helix-loop-helix (bHLH) proteins HLH-2 and HLH-3 are at least partially required for the death of the NSM sister cells and that a heterodimer composed of HLH-2 and HLH-3, HLH-2/HLH-3, acts as a direct activator of *egl-1* transcription. Furthermore, we describe studies that suggest that in *ces-1*(gf) animals, CES-1 acts as a direct repressor of *egl-1* transcription by antagonizing the function of HLH-2/HLH-3.

MATERIALS AND METHODS

General methods and strains

C. elegans was cultured and maintained as described by Brenner on NGM medium at 20°C unless otherwise noted (Brenner, 1974). The Bristol strain N2 was used as the standard wild-type strain. Mutations used in this study are listed below and are described by Riddle et al. (Riddle et al., 1997), except where noted otherwise. LGI: *unc-87*(*e1216*), *ces-1*(*n703*), *ces-1*(*n703 n1434*), *ces-2*(*n732*ts), *hlh-2*(*bx108*) (Portman and Emmons, 2000). LGIII: *cnd-1*(*ju29*) (Hallam et al., 2000), *bcIs1*($P_{egl\text{-}1}$*gfp*; this study). LGIV: *ced-3*(*n717*), *bcIs25* ($P_{tph\text{-}1}$*gfp*; this study). LGV: *egl-1*(*n1084 n3082*) (Conradt and Horvitz, 1998), *unc-76*(*e911*), *bcIs37* ($P_{egl\text{-}1}$*his24-gfp*; this study). LGX: *lin-15*(*n765*), *bcIs24* ($P_{tph\text{-}1}$*gfp*; this study), *bcIs30* ($P_{tph\text{-}1}$*gfp*; this study).

Molecular analysis and PCR mutagenesis

Standard molecular biology protocols were used unless otherwise noted. Primers used throughout this study were based on sequences determined by the *C. elegans* Sequencing Consortium (The *C. elegans* Sequencing Consortium, 1998). Plasmid pBC105 was generated by amplifying *his24-gfp* from plasmid pJH2.19 (M. Dunn and G. Seydoux, personal communication), using the polymerase chain reaction (PCR) and appropriate primers. The PCR product was digested with *Sma*I and *Nco*I and used to replace the *Sma*I-*Nco*I fragment of plasmid pBC99 (Conradt and Horvitz, 1999). Plasmid pBC08 (Conradt and Horvitz, 1998) was used to generate plasmids

pBC119, pBC13, pBC11, and pBC149. Plasmid pBC148, which is based on pBluescript and contains a 2.9 kb *Pst*I-*Xho*I fragment of pBC08, including Region B, was used to mutagenize the four Snail-binding sites/E-boxes in Region B. PCR-mediated mutagenesis was performed. The sequence of the resulting plasmids carrying four Snail$^-$/E-box$^-$ sites (5′-CATATA-3′) (pBC170) or four Snail$^-$/E-box$^+$ sites (5′-CATATA-3′) (pBC180) was confirmed by sequence analysis using an automated ABI sequencer (Applied Biosystems). A 1.4 kb *Hpa*I-*Pst*I fragment, which includes the four Snail-binding sites/E-boxes, of pBC170 and pBC180 was used to replace the *Hpa*I-*Pst*I fragment of pBC08 and to generate plasmids pBC181 (Snail$^-$/E-box$^-$) and pBC182 (Snail$^-$/E-box$^+$). The plasmid for the expression in *E. coli* of dsRNA of *hlh-2* (pBC132A) was constructed by subcloning the corresponding cDNA from plasmid pKM1199 into the *Bam*HI site of vector L4440 (Krause et al., 1997; Timmons et al., 2001). The plasmid for the expression of dsRNA of *hlh-3* in vitro was obtained by subcloning the corresponding cDNA from pKM1195 into the *Bam*HI site of pBluescript to generate pBC226. Plasmids for the expression of *hlh-4*, *hlh-6*, *hlh-12* or *hlh-14* in *E. coli* were obtained by PCR-amplifying coding regions of the genes from genomic DNA using the following primers: 5′-gaa ggg atc ctg ttc tga aac aac atc ttcc aac g-3′ and 5′-gaa ggg atc cgc agt tga tgg ttg ata gaa ata tg-3′ for *hlh-4*, 5′-gaa ggg atc caa ttc cac att cca act tcc-3′ and 5′-gaa ggg atc ccc aaa ctg atg agc tga aaa tt-3′ for *hlh-6*, 5′-gaa ggg atc cag cca cct ctt aca taa ttc-3′ and 5′-gaa ggg atc cat ata aac att ggt ttg ggg-3′ for *hlh-12*, 5′-gaa ggg atc cct gag ctc aga ttt tca g-3′ and 5′-gaa ggg atc ctg cgt tct ctc tca ttt ctg-3′ for *hlh-14*. PCR fragments were digested with *Bam*HI and ligated into pBluescript to generate plasmids pBC227 (*hlh-4*), pBC228 (*hlh-6*), pBC229 (*hlh-12*) and pBC230 (*hlh-14*).

Transgenic animals

Germline transformation was performed as described by Mello and Fire (Mello and Fire, 1995). For transformation with the P$_{egl-1}$*his24-gfp* reporter, *ced-3*(*n717*); *lin-15*(*n765*) animals were injected with plasmid pBC105 (P$_{egl-1}$*his24-gfp*) (2.5 ng/μl) and the co-injection marker pL15-EK (50 ng/μl), which rescues the *lin-15*(*n765*) multivulva or Muv phenotype (Clark et al., 1994). Injected animals were shifted to 25°C, and non-Muv F1 animals were picked to establish transgenic lines. The line carrying the extrachromosomal array *bcEx78* was used to integrate the array into the genome. *ced-3*(*n717*); *lin-15*(*n765*); *bcEx78* animals were mutagenized using ethyl methanesulfonate (EMS) (Brenner, 1974) and transgenic F2 animals were selected that gave rise to 100% non-Muv progeny at 25°C. The strain carrying the integration *bcIs37* V was backcrossed five times to *ced-3*(*n717*); *lin-15*(*n765*). For transformation with the P$_{tph-1}$*gfp* reporter, we injected *lin-15*(*n765*) animals with plasmid pBY668 (P$_{tph-1}$*gfp*) (Rohrig et al., 2000) (50 ng/μl) and pL15-EK (50 ng/μl). Transgenic lines were selected and maintained at 25°C. The line carrying the array *bcEx113* was used for integration. The strains carrying the integrations *bcIs24*, *bcIs25* and *bcIs30* were backcrossed four times to N2. For NSM sister cell death rescue, *egl-1*(*n1084 n3082*) *unc-76*(*e911*); *lin-15*(*n765*) *bcIs24* animals were injected with the *egl-1* rescuing plasmids pBC08 (2.0 ng/μl), pBC119 (1.5 ng/μl), pBC13 (1.1 ng/μl), pBC11 (1.0 ng/μl), or pBC149 (1.1 ng/μl) and the co-injection marker p76-16B (75 ng/μl), which rescues the uncoordinated or Unc phenotype of *unc-76*(*e911*) animals (Bloom and Horvitz, 1997). (*egl-1* rescuing fragments are toxic and were therefore injected at such low concentrations.) Non-Unc F1 animals were selected to establish transgenic lines. *ces-2*(*n732*)*; bcIs25; egl-1*(*n1084 n3082*) *unc-76*(*e911*) animals were injected with the *egl-1* rescuing plasmids pBC08 (2 ng/μl), pBC181 (2 ng/μl) or pBC182 (2 ng/μl) and p76-16B (75 ng/μl). *bcIs1* is an integrated array of plasmid pBC99 (P$_{egl-1}$*gfp*) (2.0 ng/μl) (Conradt and Horvitz, 1999) and the co-injection marker pL15-EK (50 ng/μl).

Electrophoretic mobility shift assays and protein production

For electrophoretic mobility shift assays, probes were generated by PCR amplification in the presence of 10 μCi [^{32}P]-dATP using the primers 5′-aac tca tcc acg tca cca aa-3′ and 5′-ttg tcc act cgt tta cca ca-3′ and plasmids pBC08 (wild-type), pBC181 (Snail$^-$/E-box$^-$) or pBC182 (Snail$^-$/E-box$^+$) as templates. The labeled PCR products were purified on a 6% acrylamide/TBE gel. A GST-CES-1 zinc-finger fusion protein construct, expressing GST fused to amino acids 117-270 of CES-1 (referred to as GST-CES-1), was made as described by Metzstein et al. (Metzstein et al., 1999). GST-CES-1 was produced and purified from *E. coli* as described by Ip et al. (Ip et al., 1992) (4.25 ng represents 1×10^{-13} moles). Expression plasmids for His$_6$-HLH-2 (pKM1199) and His$_6$-HLH-3 (pKM1195) fusion proteins were provided by M. Krause (Krause et al., 1997). His$_6$-HLH-2 and His$_6$-HLH-3 fusion proteins were produced in *E. coli* strain BL21-CodonPlus(DE3)-RIL (Stratagene) and purified as described (Portman and Emmons, 2000). 5.0 ng and 3.2 ng of His$_6$-HLH-2 and His$_6$-HLH-3 fusion protein, respectively, represents 1×10^{-13} mol. The purity and concentration of the fusion proteins were assessed by SDS-PAGE and the BioRad protein assay (BioRad). EMSAs were performed as described for HLH-2 and HLH-3, and CES-1 (Krause et al., 1997; Metzstein and Horvitz, 1999). Binding was quantified using a phosphoimager (Fujifilm BAS-2500) and appropriate software (Aida Image Analyzer V. 3.21).

RNAi experiments

For RNAi experiments using feeding as the method of delivering dsRNA, plasmids pBC132A, pBC124A, pBC188, pBC189, pBC190 and pBC191 were transformed into *E. coli* strain HT115 (Timmons et al., 2001). NGM plates containing 6 mM IPTG, 50 μg/ml ampicillin and 12.5 μg/ml tetracycline were inoculated with transformed HT115 bacteria. The expression of dsRNA was induced overnight at room temperature. The plates were subsequently inoculated with L4 larvae. Animals were cultured at 15°C and their progeny was analysed. For RNAi experiments using injection as the method of delivering dsRNA, plasmids pBC226, pBC227, pBC228, pBC229 and pBC230 were used to PCR-amplify their inserts flanked by the *T3* and *T7* promoter using appropriate primers. PCR products were used as templates for in vitro transcription using the *T3* and *T7* polymerases (Promega). Reactions contained 100 U *T3* or *T7* polymerase, 2 mM each rATP, rCTP, rGTP, rUTP, 10 mM DTT in a final volume of 100 μl in DEPC-H_2O buffered with transcription buffer. After incubating for 2 hours at 37°C, five units DNase was added and the reaction incubated for another 20 minutes at 37°C. The RNA was purified by phenol/chloroform extraction and resuspended in 20 μl DEPC-H_2O. Single stranded sense RNA was annealed with an equal amount of the corresponding antisense RNA at 37°C for 20 minutes, centrifuged at 4°C for 10 minutes and injected into the gonad of young adult worms, which were subsequently incubated at 25°C. The progeny of injected animals was analysed.

Analysis of P$_{egl-1}$ *his24-gfp* expression and NSM sister cell survival

For the analysis of P$_{egl-1}$ *his24-gfp* expression in the NSMs and NSM sister cells, the NSMs and NSM sister cells were identified in L1 larvae using Nomarski microscopy as described (Ellis and Horvitz, 1991) and analysed for *his24-gfp* expression using epifluorescence. Percent NSM sister cell survival was determined in L3 or L4 larvae carrying the P$_{tph-1}$*gfp* reporter using a combination of Nomarski microscopy and epifluorescence. To obtain the number of surviving NSM sister cells per animal, the total number of GFP-positive cells in the anterior pharynx of an animal was determined and subtracted by two. Percent NSM sister cell survival represents the percent of NSM sister cells that survived and n the maximum number of NSM sister cells that could have survived in the number of animals analysed.

Immunohistochemistry

Embryos were prepared in 10 μl H_2O on poly L-Lysine coated slides

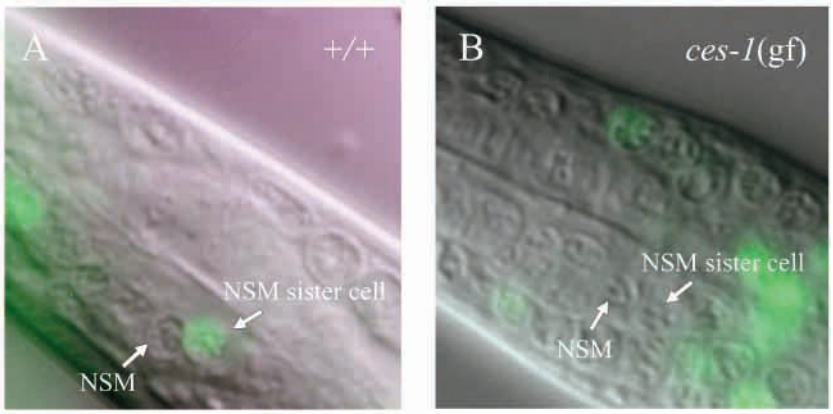

Fig. 1. *egl-1* is transcriptionally active in the NSM sister cells but not in the NSMs or undead NSM sister cells. (A) Merged Nomarski and epifluorescence image of the anterior bulb of the pharynx of a *ced-3*(*n717*) L1 larva carrying an integrated $P_{egl\text{-}1}$*his24-gfp* reporter construct (*bcIs37*). $P_{egl\text{-}1}$*his24-gfp* is expressed in the NSM sister cell but not in the NSM. The complete genotype of the animals scored was *ced-3*(*n717*); *bcIs37*. (B) Merged Nomarski and epifluorescence image of the anterior bulb of the pharynx of a *ces-1*(*703*gf); *ced-3*(*n717*) L1 larva carrying an integrated $P_{egl\text{-}1}$*his24-gfp* reporter construct (*bcIs37*). $P_{egl\text{-}1}$*his24-gfp* is not expressed in the undead NSM sister cell or the NSM. The complete genotype of the animals scored was *unc-87*(*e1216*) *ces-1*(*n703*gf); *ced-3*(*n717*); *bcIs37*.

and allowed to develop until the 1.5-fold stage in a moist chamber. They were fixed with 5% paraformaldehyde and stained as described (Krause et al., 1990; Krause et al., 1997). HLH-2 was detected using a polyclonal anti-HLH-2 antibody raised in rabbits (provided by Mike Krause) and GFP was detected using a monoclonal anti-GFP antibody (Clontech). Immunofluorescence was viewed using a Leica TCS NT confocal microscope.

RESULTS

egl-1 is regulated at the transcriptional level in the NSMs and NSM sister cells

About 400 minutes after the first cleavage of a *C. elegans* embryo, two bilaterally symmetric cells, ABaraapapaa and ABaraapppaa, each divide. The ventral daughters of these cells survive and differentiate into serotonergic, neurosecretory motoneurons, called NSMs, which are located in the anterior pharynx. Their dorsal sisters, the NSM sister cells, however, die by programmed cell death shortly after they are born (at about 420-430 minutes) (Sulston et al., 1983). The programmed death of the NSM sister cells is blocked by lf mutations in the *C. elegans* cell-death activator genes *egl-1*, *ced-3* and *ced-4*, and by a gf mutation in the *C. elegans* cell-death inhibitor gene *ced-9*, indicating that these specific cell deaths are dependent on the activation of the central cell-death pathway (Horvitz, 1999).

In the case of another specific cell death, the death of the HSNs in males, it has been shown that the decision between life and death is specified by *egl-1* expression (Conradt and Horvitz, 1999). To determine whether *egl-1* expression also specifies the cell-death fate of the NSM sister cells, we monitored the expression of *egl-1* in these cells, using an integrated $P_{egl\text{-}1}$*his24-gfp* transgene. $P_{egl\text{-}1}$*his24-gfp* expresses a nuclearly localized fusion of the His24 protein and the green fluorescent protein (GFP) under the control of the cis-regulatory regions of the *egl-1* gene. The NSM sister cells normally die during a stage called the 1.5-fold stage of embryogenesis. At this stage of development it is difficult to identify cells on the basis of their position within the embryo. We therefore analysed the expression of $P_{egl\text{-}1}$*his24-gfp* in the background of the *ced-3* lf mutation *n717*. *ced-3*(*n717*) blocks programmed cell death, including the death of the NSM sister cells (Ellis and Horvitz, 1986). However, because *ced-3* acts downstream of *egl-1* genetically, *ced-3*(*n717*) should not interfere with the expression of the $P_{egl\text{-}1}$*his24-gfp* transgene. This experimental design enabled us to analyse the expression of $P_{egl\text{-}1}$*his24-gfp* in the NSMs and in surviving 'undead' NSM sister cells in animals of the first larval stage of development (L1 larvae), in which these cells are identifiable by position in the anterior pharynx using Nomarski differential interference contrast (Nomarski microscopy) (Ellis and Horvitz, 1991). In *ced-3(n717)*; $P_{egl\text{-}1}$*his24-gfp* larvae, we observed GFP in 88% of the NSM sister cells, which normally die, and in 0% of the NSMs, which normally survive (n=51) (Fig. 1A). This indicates that the activity of EGL-1 is regulated at the transcriptional level in the NSMs and NSM sister cells and, hence, that the cell-death fate of these cells is specified by *egl-1* expression.

ces-1 acts upstream of *egl-1* and a gf mutation of *ces-1*, *n703*, specifically blocks the death of the NSM sister cells. To determine whether *ces-1*(*n703*gf) affects *egl-1* expression in the NSM sister cells, we analysed the expression of $P_{egl\text{-}1}$*his24-gfp* in *ces-1*(*n703*gf); *ced-3*(*n717*) animals. In this background, we detected GFP in 2% of the NSM sister cells and in 0% of the NSMs (n=51) (Fig. 1B). The *ces-1*(gf) mutation therefore prevents the death of the NSM sister cells by blocking the transcription of *egl-1* in these cells.

Region B of the *egl-1* locus is required for the death of the NSM sister cells in vivo

The *egl-1* lf mutation *n1084 n3082* blocks programmed cell death, including the death of the NSM sister cells. A 7660 bp genomic fragment of cosmid C01G9, pBC08, which includes the *egl-1* transcription unit, 1036 bp of its upstream region and 5575 bp of its downstream region, when introduced as an extra-chromosomal array, can rescue the cell-death defect of *egl-1*(*n1084 n3082*) animals, including the death of the NSM sister cells (Fig. 2) (Conradt and Horvitz, 1998). We therefore conclude that pBC08 contains the *cis*-regulatory region or regions required for the expression of *egl-1* in the NSM sister cells. To determine which region or regions of pBC08 are specifically required for the expression of *egl-1* in these cells, we analysed subclones of pBC08 for their ability to rescue the death of the NSM sister cells in *egl-1*(*n1084 n3082*) animals (Fig. 2A).

Undead NSM sister cells are located close to and posterior to their sisters, the NSMs, in the anterior pharynx and can therefore be identified due to their position using Nomarski microscopy (Ellis and Horvitz, 1991). In addition, like their

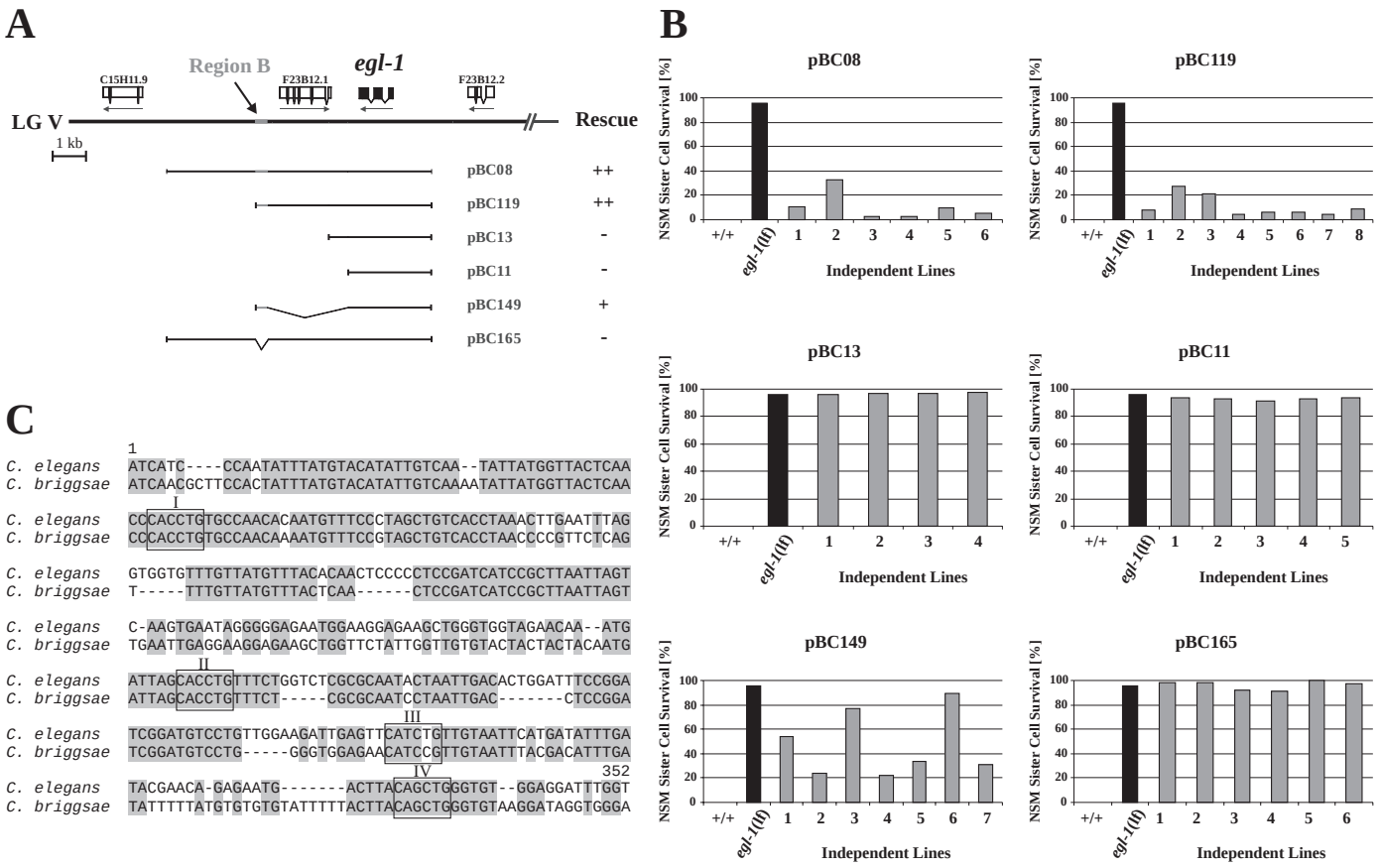

Fig. 2. Region B of the *egl-1* locus is required for the specification of the NSM sister cell death. (A) Schematic representation of the *egl-1* locus on linkage group V. pBC119, pBC13, pBC11, pBC149 and pBC165 represent subclones of the *egl-1* rescuing fragment pBC08. The abilities of the fragments to rescue NSM sister cell death (see below) are summarized under 'Rescue'. The arrow indicates Region B. (B) pBC08, pBC119 and pBC149 can rescue the death of the NSM sister cells in *egl-1*(lf) animals. Plasmids containing the fragments indicated were introduced by germline transformation into hermaphrodites of genotype *egl-1*(*n1084 n3082*) *unc-76*(*e911*)*; lin-15*(*n765*) carrying an integrated $P_{tph\text{-}1}gfp$ reporter construct (*bcIs24*) as described in the Materials and Methods. Rescue of NSM sister cell death was analysed as described in the Materials and Methods (n=100-240). For every plasmid, several independent transgenic lines were generated and characterized (n=4-7). (C) Region B of the *egl-1* locus is highly conserved. Alignment of the sequence of *C. elegans* Region B (*C. elegans* sequence VF23B12L bp 2659-2792 and *C. elegans* cosmid F23B12 bp 1-218) with the sequence of the corresponding region of *C. briggsae* (*C. briggsae* cosmid G12D19.2 bp 10590-10248). Identical nucleotides are shaded. The four conserved Snail-binding sites are indicated by boxes.

sisters, undead NSM sister cells differentiate into serotonin-expressing neurons. The NSMs are the only serotonin-expressing neurons in the anterior pharynx of *C. elegans*. Hence, in wild-type animals, two serotonin-expressing cells can be detected in this part of the pharynx, the two NSMs, and in animals, in which the NSM sister cells survive, four serotonin-expressing cells, the two NSMs and the two undead NSM sister cells (Ellis and Horvitz, 1991). The $P_{tph\text{-}1}gfp$ (*tph*, tryptophan hydroxylase) transgene expresses *gfp* under the control of the *cis*-regulatory regions of the *tph-1* gene, which encodes an enzyme required for serotonin biosynthesis and which is expressed in serotonergic neurons, including the NSMs (Sze et al., 2000). To test whether the $P_{tph\text{-}1}gfp$ transgene is also expressed in undead NSM sister cells (and therefore can be used to conveniently identify undead NSM sister cells), we analysed the expression of this reporter in wild-type animals, in which the NSM sister cells die, and in animals homozygous for *egl-1*(*n1084 n3082*), in which the NSM sister cells survive. In wild-type animals, we observed GFP in only two cells located in the anterior part of the pharynx, the NSMs (0% NSM sister cell survival, n=414). In most *egl-1*(*n1084 n3082*) animals, however, we observed GFP in the two NSMs and in two additional cells located more posterior, the undead NSM sister cells (96% NSM sister cell survival, n=120). Throughout our studies, NSM sister cell survival was therefore analysed using a combination of Nomarski microscopy and $P_{tph\text{-}1}gfp$/epifluorescence.

As mentioned above, in wild-type animals, 100% of the NSM sister cells die; however, 96% of them survive in *egl-1*(*n1084 n3082*) animals. In transgenic *egl-1*(*n1084 n3082*) animals carrying extra-chromosomal arrays of the *egl-1* rescuing fragment pBC08, between 35% and 5% of the NSM sister cells survived (6/6 lines), indicating that pBC08 rescues the death of the NSM sister cells (Fig. 2B). (Rescue was defined as 40% or less NSM sister cell survival.) Similarly, subclone pBC119, which includes 3447 bp of the region downstream of the *egl-1* transcription unit, rescued the cell-death defect of *egl-1*(*n1084 n3082*) animals (8/8 lines). By

contrast, subclones pBC13 (806 bp of downstream region) and pBC11 (377 bp of downstream region) failed to rescue (0/4 lines and 0/5 lines). pBC13 lacks 2641 bp of pBC119 that includes a 352 bp region, called Region B, which is conserved between the *egl-1* locus of *C. elegans* and *C. briggsae* (Fig. 2A,C) (see below). As sequence conservation between these two *Caenorhabditis* species suggests functional relevance (Heschl and Baillie, 1990), we tested whether addition of Region B alone can restore the rescuing activity of pBC11 (pBC149). We found that pBC149 rescued NSM sister cell death in four out of seven lines (4/7). Conversely, pBC08 lacking only Region B (pBC165) failed to rescue (0/6 lines). These results indicate that Region B of the *egl-1* locus is required to rescue the death of the NSM sister cells in *egl-1*(*n1084 n3082*) animals. As the death of the NSM sister cells is dependent on the expression of *egl-1* in the NSM sister cells, Region B most probably contains the cis-regulatory regions necessary for the transcription of *egl-1* in these cells.

Region B contains four conserved Snail-binding sites to which a GST-CES-1 fusion protein can bind in vitro

An alignment of the sequence of Region B of the *C. elegans egl-1* locus with the sequence of the corresponding region of the *C. briggsae egl-1* locus, revealed extensive sequence conservation at the nucleotide level. Over their entire length, the sequences are 76% identical (Fig. 2C). Sequence inspection revealed that Region B contains four closely spaced Snail-binding sites, DNA-binding sites for members of the Snail family of zinc-finger transcription factors (Hemavathy et al., 2000). The core motif of three of these binding sites is completely conserved in *C. briggsae* (binding sites I, II and IV; 6/6 bases identical), and one of them has one base change in *C. briggsae* (binding site III; 5/6 bases identical). The sequence of binding sites I and II of *C. elegans* is a perfect match to the sequence of the core motif of a consensus Snail-binding site (5′-CACCTG-3′) whereas the sequences of binding sites III and IV have one mismatch to the consensus sequence (5′-CATCTG-3′ and 5′-CAGCTG-3′, respectively).

CES-1 is a member of the Snail family of DNA-binding proteins and has previously been shown to bind to Snail-binding sites in vitro (Metzstein and Horvitz, 1999). In addition, in *ces-1*(gf) animals, CES-1 prevents the death of the NSM sister cells by blocking *egl-1* expression. To determine whether in *ces-1*(gf) animals, CES-1 might block the transcription of *egl-1* directly by binding to the four Snail-binding sites in Region B, we tested whether CES-1 can bind to these sites in vitro, using electrophoretic mobility shift assays (EMSAs). A bacterially produced, affinity-purified CES-1 fusion protein consisting of the C-terminal half of CES-1 (which includes all five zinc fingers of CES-1) fused to glutathione S-transferase (GST) could bind and shift a radioactively labeled 390 bp DNA fragment consisting of wild-type Region B, including the four Snail-binding sites (Fig. 3, lanes 1-6). Long exposures and the use of various fragments of Region B indicate that CES-1 can bind to at least three of the four Snail-binding sites in vitro (data not shown). Binding of CES-1 to Region B was severely reduced after the introduction of point mutations that destroy the core motif of the four Snail-binding sites (5′-CACCTG-3′ to 5′-CATATA-3′) (Fig. 3, lanes 7-12). Using an amount of CES-1 that binds about 50% of the wild-type probe (set to 100% binding), CES-1 binding was reduced to on average 6% (*n*=2) (Fig. 3, compare lanes 4 and 10). Intact Snail-binding sites are therefore required for the ability of CES-1 to bind to Region B in vitro. The specificity of the observed binding was furthermore confirmed by competition experiments, using the wild-type Region B as a probe and a wild-type or mutant Snail-binding site as competitor (data not shown).

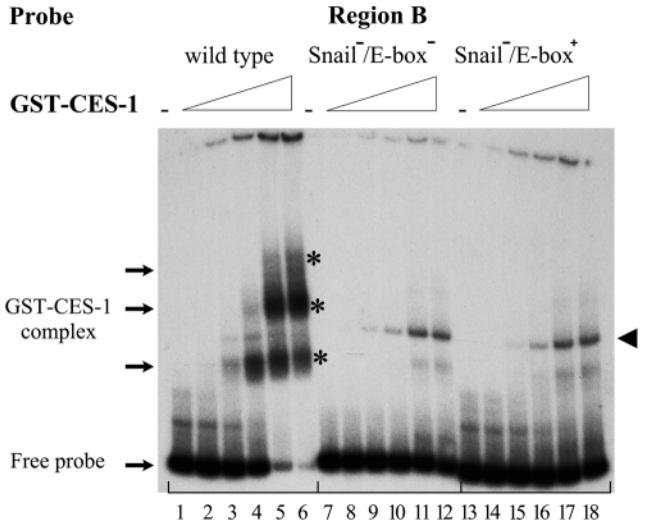

Fig. 3. CES-1 binds to the Snail-binding sites/E-boxes in Region B of the *egl-1* locus in vitro. A GST-CES-1 fusion protein binds to wild-type but not mutant Region B in vitro. Increasing amounts of bacterially expressed, affinity-purified GST-CES-1 fusion protein [0 mol (lanes 1, 7 and 13), 8×10^{-13} mol (lanes 2, 8 and 14), 2×10^{-12} mol (lanes 3, 9 and 15), 4×10^{-12} mol (lanes 4, 10 and 16), 6×10^{-12} mol (lanes 5, 11 and 17), 8×10^{-12} mol (lanes 6, 12 and 18) were incubated with 7 ng of radioactively labeled wild-type Region B with four intact Snail-binding sites (lanes 1-6), or mutant Region B with all four Snail-binding sites mutated to Snail^{-}/E-box^{-} sites (lanes 7-12) or to Snail^{-}/E-box^{+} sites (lanes 13-18). Electrophoretic mobility shift assays were performed as described in the Materials and Methods. Asterisks indicate protein-DNA complexes with one, two or three CES-1 molecules bound to Region B. Triangle indicates a protein-DNA complex that is most likely a bacterial contaminant bound to Region B.

These results suggest that in *ces-1*(gf) animals, in which the NSM sister cells survive, CES-1 might bind to the Snail-binding sites in Region B of the *egl-1* locus thereby directly repressing *egl-1* transcription in the NSM sister cells.

The *C. elegans* bHLH genes *hlh-2* and *hlh-3* are required for the programmed death of the NSM sister cells

Region B of the *egl-1* locus is required to rescue the death of the NSM sister cells in *egl-1*(lf) mutants and is therefore most likely to be necessary for the expression of *egl-1* in these cells. We therefore sought to identify transcriptional activators that are required for the death of the NSM sister cells and that act through Region B. The core motif of a Snail-binding site (5′-CACCTG-3′) also represents an E-box motif (5′-CANNTG-3′), the DNA-binding site for members of the family of bHLH DNA-binding proteins, many of which function as transcriptional activators (reviewed by Massari and Murre,

2000). Indeed, it has been suggested that members of the Snail family of transcription factors can functionally antagonise bHLH proteins by competing for binding to Snail-binding sites/E-boxes (Fuse et al., 1994; Kataoka et al., 2000; Nakayama et al., 1998). We therefore set out to test whether *C. elegans* bHLH proteins are involved in the specification of the NSM sister cell death in vivo. We were particularly interested in the *C. elegans* homologues of neuronal bHLH proteins, which can be divided into two families: the Achaete-scute complex-related proteins and the Atonal-related proteins. The Atonal-related proteins are further subdivided into three groups: the Neurogenin group, the NeuroD group and the ATO group (reviewed by Hassan and Bellen, 2000; Lee, 1997). As tissue-specific bHLH proteins form DNA-binding heterodimers with ubiquitously expressed E proteins or Daughterless-like proteins, we also analysed *C. elegans* homologues of this class of bHLH proteins. The *C. elegans* genome contains at least 35 *bHLH* genes, including one *daughterless*-like, five *achaete-scute*-like, one *atonal*-like and one *NeuroD*-like gene (reviewed by Ledent and Vervoort, 2001). Using existing mutants and RNA-mediated interference (RNAi) (Fire et al., 1998), we tested whether these genes are involved in specifying the death of the NSM sister cells.

The only *C. elegans daughterless*-like gene, *hlh-2*, has so far only been defined by weak lf mutations, such as *bx108*, which were identified in a screen for enhancers of the phenotype caused by a weak lf mutation of *lin-32*, the only *C. elegans atonal*-like gene (Portman and Emmons, 2000). *bx108* is a missense mutation in the helix-loop-helix dimerization domain of HLH-2 and is predicted to affect the ability of the protein to form heterodimers with other bHLH proteins such as LIN-32 (Portman and Emmons, 2000). However, *bx108* has so far not been shown to cause a phenotype in an otherwise wild-type background. The inactivation of *hlh-2* by RNAi results in embryonic lethality, indicating that *hlh-2* is essential for development (Krause et al., 1997). To determine whether reducing *hlh-2* function has an effect on the survival of the NSM sister cells, we analysed *hlh-2*(*bx108*) animals and 'escapers' of *hlh-2*(*RNAi*) for the survival of NSM sister cells using the $P_{tph\text{-}1}gfp$ reporter. We found that *hlh-2*(*bx108*) leads to the survival of up to 5% of the NSM sister cells, an effect that is temperature sensitive (Table 1A). Furthermore, we found that 15% of the NSM sister cells survived in *hlh-2*(*RNAi*) embryos that escaped early developmental arrest and developed to a stage, at which the $P_{tph\text{-}1}gfp$ reporter is expressed (Table 1A).

To confirm that the additional GFP positive cells observed represent undead NSM sister cells and not other pharyngeal cells that have acquired a serotonergic fate, we analysed *ces-1*(*n703*gf); *hlh-2*(*RNAi*); $P_{tph\text{-}1}gfp$ animals. In *ces-1*(*n703*gf); $P_{tph\text{-}1}gfp$ animals, the NSM sister cells survive and therefore four GFP positive cells can be observed in the anterior pharynx, the NSMs and the undead NSM sister cells. If the additional GFP positive cells observed in *hlh-2*(*RNAi*); $P_{tph\text{-}1}gfp$ embryos are not undead NSM sister cells, we would expect to detect more than four GFP positive cells in *ces-1*(*n703*gf); *hlh-2*(*RNAi*); $P_{tph\text{-}1}gfp$ embryos. We found that 34% of the *hlh-2*(*RNAi*); $P_{tph\text{-}1}gfp$ animals analysed had more than two GFP positive cells (n=45) and that only 2% of the *ces-1*(*n703*gf); *hlh-2*(*RNAi*); $P_{tph\text{-}1}gfp$ animals (n=42) had more than four GFP positive cells. This indicates that the majority of additional GFP positive cells in *hlh-2*(*RNAi*); $P_{tph\text{-}1}gfp$ embryos represent undead NSM sister cells.

Table 1. Reducing the activity of *hlh-2* and *hlh-3* causes the NSM sister cells to survive

A

	% NSM sister cell survival (n) at:		
Genotype	15°C	20°C	25°C
+/+	0 (416)	0 (414)	0 (408)
hlh-2(*bx108*)	1 (350)	4 (400)	5 (410)
hlh-6(*RNAi*)	0 (116)		
hlh-2(*RNAi*)	15 (130)		

B

Genotype	% NSM sister cell survival (n) at 25°C
hlh-6(*RNAi*)	0±0 (136)
hlh-2(*bx108*)	5 (410)
hlh-2(*bx108*);*hlh-6*(*RNAi*)	4±1 (176)
hlh-3(*RNAi*)	7±3 (178)
hlh-2(*bx108*);*hlh-3*(*RNAi*)	30±11 (140)

(A) Reducing the activity of *hlh-2* causes NSM sister cell survival. The presence of NSM sister cells was scored as described in the Materials and Methods using the $P_{tph\text{-}1}gfp$ reporter. dsRNA for RNAi was delivered by feeding. The complete genotype of the animals scored was as follows: *bcIs25, hlh-2(bx108)*; *bcIs25, hlh-6*(RNAi); *bcIs25, hlh-2*(RNAi); *bcIs25*.

(B) Reducing the activity of *hlh-3* causes NSM sister cell survival and enhances *hlh-2*(*bx108*). The presence of NSM sister cells was scored as described in the Materials and Methods using the $P_{tph\text{-}1}gfp$ reporter. dsRNA for RNAi was delivered by injections. The complete genotype of the animals scored were as follows: *hlh-6*(RNAi); *bcIs25, hlh-2*(*bx108*); *bcIs25, hlh-2*(*bx108*); *hlh-6*(RNAi); *bcIs25, hlh-3*(RNAi); *bcIs25, hlh-2*(*bx108*); *hlh-3*(RNAi); *bcIs25*. Values are average percent survival obtained for the progeny of different injected animals±s.d.

The *C. elegans atonal* and *NeuroD*-like genes *lin-32* (*lin*, lineage abnormal) and *cnd-1* (*cnd*, *C. elegans* NeuroD), respectively, have been defined by non-lethal, strong lf mutations (Zhao and Emmons, 1995; Hallam et al., 2000). To determine whether these two genes are involved in specifying the NSM sister cell death, we analysed NSM sister cell survival in animals carrying lf mutations in either gene. In *cnd-1*(*ju29*) animals, 0% of the NSM sister cells survived (n=60), indicating that the *C. elegans NeuroD* homologue is probably not required for the NSM sister cell death. Similarly, lf mutations of *lin-32* did not affect NSM sister cell survival in an otherwise wild-type background or in a *hlh-2*(*bx108*) background (data not shown).

The five *C. elegans achaete-scute*-like genes (*hlh-3*, *hlh-4*, *hlh-6*, *hlh-12* and *hlh-14*) have so far not been defined by mutations. For this reason, we used RNAi to analyse their potential role in the specification of the NSM sister cell death. No effect on NSM sister cell survival was observed in *hlh-4*(*RNAi*), *hlh-6*(*RNAi*), *hlh-12*(*RNAi*) or *hlh-14*(*RNAi*) animals (Table 1B and data not shown). *hlh-3*(*RNAi*), however, caused 7% of the NSM sister cells to survive (n=178), indicating that *hlh-3* is at least partially required for the death of the NSM sister cells (Table 1B). Moreover, *hlh-3*(*RNAi*) [but not *hlh-4*(*RNAi*), *hlh-6*(*RNAi*), *hlh-12*(*RNAi*) or *hlh-14*(*RNAi*)] increased NSM sister cell survival in *hlh-2*(*bx108*) animals from 4% to 30%. *hlh-2* and *hlh-3* therefore might act together to cause the death of the NSM sister cells.

To determine whether reducing *hlh-2* and *hlh-3* function

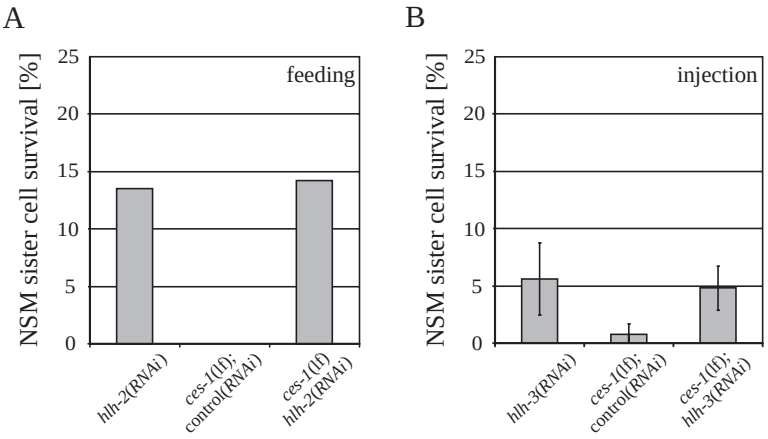

Fig. 4. A *ces-1*(lf) mutation is unable to suppress the NSM sister cell survival caused by *hlh-2*(*RNAi*) or *hlh-3*(*RNAi*). (A) A *ces*-1(lf) mutation is not able to suppress the NSM sister cell survival induced by *hlh-2*(*RNAi*). NSM sister cell survival was analysed as described in the Materials and Methods (*n*=96-142). dsRNA was delivered by feeding. *hlh-6* dsRNA was used for control RNAi. The complete genotype of the animals analysed was as follows *hlh-2*(*RNAi*)*; bcIs25, ces-1*(*n703 n1434*lf); *hlh-6*(*RNAi*); *bcIs25, ces-1*(*n703 n1434*lf); *hlh-2*(*RNAi*); *bcIs25*. (B) A *ces*-1(lf) mutation is not able to suppress the NSM sister cell survival induced by *hlh-3*(*RNAi*). NSM sister cell survival was analysed as described in the Materials and Methods (*n*=174-218). dsRNA was delivered by injection. *hlh-6* dsRNA was used for control RNAi. The complete genotype of the animals analysed was as follows: *hlh-3*(*RNAi*); *bcIs25, ces-1*(*n703 n1434*lf); *hlh-6*(*RNAi*); *bcIs25, ces-1*(*n703 n1434*lf); *hlh-3*(*RNAi*); *bcIs25.* Error bars represent the standard deviation of the average percent survival obtained for the progeny of different injected animals.

causes a general block in programmed cell death or specifically results in the survival of the NSM sister cells, we analysed the survival of other cells that normally die in *hlh-2*(*bx108*); *hlh-3*(*RNAi*) animals using Nomarski microscopy. During the development of the anterior pharynx, 16 cells undergo programmed cell death and mutations that block programmed cell death in general, such as *egl-1*(*n1084 n3082*), block many of these cell deaths. *egl-1*(*n1084 n3082*) animals therefore have on average about 12 extra cells in this part of the pharynx (Conradt and Horvitz, 1998). We found that *hlh-2*(*bx108*); *hlh-3*(*RNAi*) animals have on average 1.3 extra cells (*n*=16). 57% of these extra cells were undead NSM sister cells, as confirmed by the position of their nuclei and by $P_{tph\text{-}1}$*gfp* expression, and 23% possibly were undead m2 sister cells, as determined by the position of their nuclei. We were unable to determine the identity of 20% of the extra cells. Therefore, the majority of the surviving cells are NSM sister cells. Reducing the activity of *hlh-2* and *hlh-3*, hence, results in the survival predominantly of the NSM sister cells.

hlh-2 and *hlh-3* act downstream of or in parallel to *ces-1* to kill the NSM sister cells

The ability of the *ces-2* lf mutation *n732* to cause NSM sister cell survival depends on a functional *ces-1* gene, which suggests that *ces-1* acts downstream of *ces-2*. To determine whether the ability of *hlh-2*(*RNAi*) and *hlh-3*(*RNAi*) to cause NSM sister cell survival similarly depends on a functional *ces-1* gene, we tested whether the *ces-1* lf mutation *n703 n1434* can block the NSM sister cell survival observed in *hlh-2*(*RNAi*) and *hlh-3*(*RNAi*) animals. In *hlh-2*(*RNAi*) animals, 14% of the NSM sister cells survived and in *ces-1*(*n703 n1434*lf) animals treated with control RNA, 0% survived (dsRNA delivered by feeding) (Timmons et al., 2001) (Fig. 4A). In *ces-1*(*n703 n1434*lf); *hlh-2*(*RNAi*) animals, 14% of the NSM sister cells survived. Similarly, in *hlh-3*(*RNAi*) animals, 6% of the NSM sister cells survived and in *ces-1*(*n703 n1434*lf) animals treated with control RNA, 1% survived (dsRNA delivered by injection) (Fire et al., 1998). In *ces-1*(*n703 n1434*lf); *hlh-3*(*RNAi*) animals, 5% of the NSM sister cells survived (Fig. 4B). These data indicate that a functional *ces-1* gene is not required for the ability of *hlh-2*(*RNAi*) or *hlh-3*(*RNAi*) to cause NSM sister cell survival, which suggests that *ces-1* is not acting downstream of *hlh-2* and *hlh-3*. *hlh-2* and *hlh-3* therefore act downstream of or in parallel to *ces-1* to kill the NSM sister cells.

A HLH-2/HLH-3 heterodimer can bind to the Snail-binding sites/E-boxes in Region B of the *egl-1* locus in vitro

To determine whether HLH-2 and HLH-3 can bind to the Snail-binding sites/E-boxes in Region B of the *egl-1* locus, we performed EMSAs, using bacterially produced, affinity-purified His-tagged HLH-2 and HLH-3 fusion proteins. Using the 390 bp DNA fragment consisting of wild-type Region B, including all four Snail-binding sites/E-boxes as a probe, we found that homodimers of HLH-2 and heterodimers of HLH-2 and HLH-3 but not homodimers of HLH-3 could bind and shift the probe (Fig. 5A). Upon longer exposures and the use of various fragments of Region B, we could determine that HLH-2/HLH-3 heterodimers can bind to at least three of the four Snail-binding sites/E-boxes in vitro (data not shown). The amount of protein required to detect binding to Region B and to bind 50% of the probe was 10-fold and fivefold higher for CES-1 than for HLH-2/HLH-3, respectively. This suggests that at least in vitro, the binding affinity of HLH-2/HLH-3 for these Snail-binding sites/E-boxes is higher than the binding affinity of CES-1 [compare Fig. 3 (lane 3) with Fig. 5A (lane 13), and Fig. 3 (lane 4) with Fig. 5A (lane 15)]. The binding of HLH-2/HLH-3 heterodimers was much reduced when a DNA fragment consisting of mutant Region B, in which the four Snail-binding sites/E-boxes had been mutated (5′-CACCTG-3′ to 5′-CATATA-3′) was used as a probe (Fig. 5B, lanes 7-12). Using amounts of HLH-2/HLH-3 that are sufficient to bind 50% of the wild-type probe (100% binding), binding to mutant Region B was reduced to on average 4% (*n*=3) (compare Fig. 5B, lanes 4 and 10). HLH-2/HLH-3 binding to Region B in vitro therefore is dependent on functional Snail-binding sites/E-boxes. We confirmed the specificity of the binding observed by competition experiments (data not shown). These results suggest that HLH-2 and HLH-3 might cause the NSM sister cells to die by acting as a direct activator of *egl-1* transcription in the NSM sister cells.

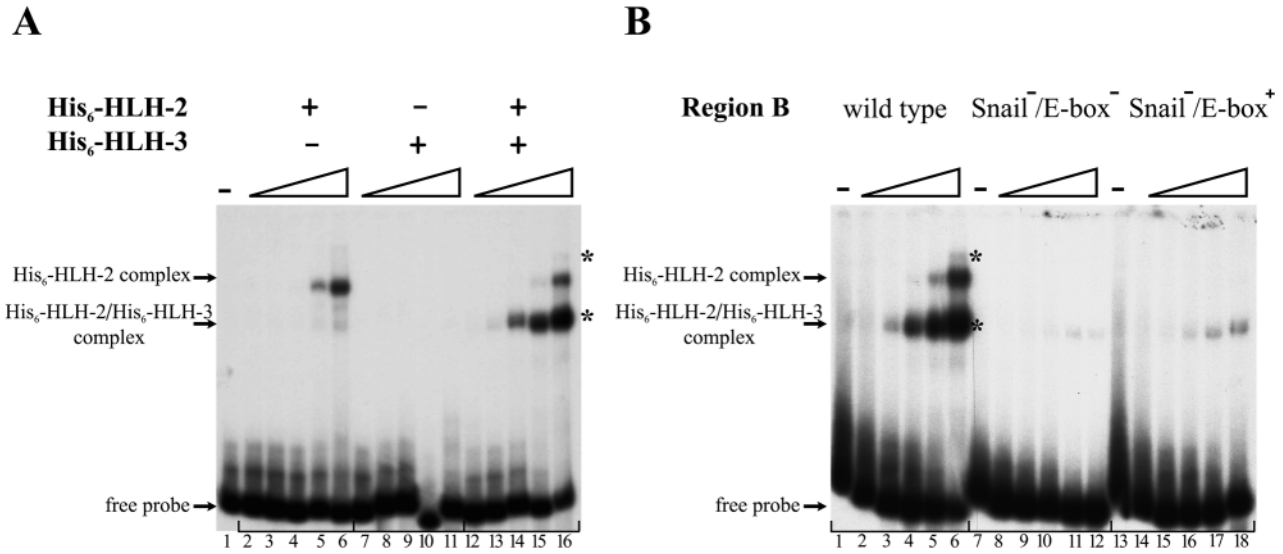

Fig. 5. HLH-2/HLH-3 binds to the Snail-binding sites/E-boxes in Region B of the *egl-1* locus in vitro. (A) A HLH-2 homodimer and HLH-2/HLH-3 heterodimer bind to wild-type Region B in vitro. Increasing amounts of bacterially expressed, affinity-purified His_6-tagged HLH-2 (lanes 2-6), HLH-3 (lanes 7-11) or both HLH-2 and HLH-3 (lanes 12-16) fusion proteins [0 mol (lane 1), 8×10^{-14} mol (lanes 2, 7 and 12), 2×10^{-13} mol (lanes 3, 8 and 13), 4×10^{-13} mol (lanes 4, 9 and 14), 8×10^{-13} mol (lanes 5, 10 and 15), 2×10^{-12} mol (lanes 6, 11 and 16)] were incubated with 7 ng of radioactively labeled wild-type Region B. Electrophoretic mobility shift assays were performed as described in the Materials and Methods. Asterisks indicate a DNA-protein complex with one or two heterodimers bound to Region B. (B) A HLH-2/HLH-3 heterodimer still binds to Snail$^-$/E-box$^+$ binding sites in Region B. Increasing amounts of both His_6-tagged HLH-2 and HLH-3 [0 mol (lane 1, 7 and 13), 8×10^{-14} mol (lanes 2, 8 and 14), 2×10^{-13} mol (lanes 3, 9 and 15), 4×10^{-13} mol (lanes 4, 10 and 16), 8×10^{-13} mol (lanes 5, 11 and 17), 2×10^{-12} mol (lanes 6, 12 and 18)] were incubated with 7 ng of radioactively labeled wild-type Region B with four intact Snail-binding sites (lanes 1-6), or mutant Region B with all four Snail-binding sites mutated to Snail$^-$/E-box$^-$ sites (lanes 7-12) or to Snail$^-$/E-box$^+$ sites (lanes 13-18). Electrophoretic mobility shift assays were performed as described in Materials and Methods. Asterisks indicate a DNA-protein complex with one or two heterodimers bound to Region B.

HLH-2 is most probably present in the NSM sister cells at the time their cell death fate is determined

If HLH-2 acts as a direct activator of *egl-1* transcription, HLH-2 should be present in the NSM sister cells at the time their cell-death fate is specified, i.e. in the NSM sister cells of embryos at the 1.5-fold stage of development (about 400-430 minutes after the first cleavage). HLH-2 has been shown to be broadly distributed throughout the embryo during the proliferative phase of embryogenesis (until about 350 minutes). During later stages of embryogenesis, the distribution of HLH-2 becomes progressively restricted (Krause et al., 1997). Using an antibody specific for HLH-2 (Krause et al., 1997), we stained 1.5-fold stage *ced-3*(*n717*) embryos and found that at that stage of development HLH-2 is still present in a number of nuclei predominantly in the head and tail region (Fig. 6). However, owing to the lack of a marker for the early NSM lineage, we were unable to determine whether the HLH-2-positive cells include the NSM sister cells. We therefore stained *ced-3*(*n717*) embryos carrying an integrated $P_{egl\text{-}1}$*gfp* transgene [$P_{egl\text{-}1}$*gfp; ced-3*(*n717*)], which expresses the *gfp* gene under the control of the *cis*-regulatory regions of *egl-1*. *egl-1* appears to be specifically expressed in cells destined to die during embryogenesis (R. Schnabel and B.C., unpublished), including, as shown above, the NSM sister cells. In a wild-type background, about 98 cells have undergone programmed cell death by the time an embryo reaches the 1.5-fold stage (Sulston et al., 1983). In a *ced-3*(*n717*) background, most if not all of these 98 cells survive (Ellis and Horvitz, 1986). As *egl-1* acts upstream of *ced-3*, *ced-3*(*n717*) does not interfere with the expression of the $P_{egl\text{-}1}$*gfp* transgene. In a $P_{egl\text{-}1}$*gfp; ced-3*(*n717*) embryo at the 1.5-fold stage, a large number of undead, GFP-positive cells can therefore be detected (Fig. 6). When staining these embryos for HLH-2, we found that a total of four cells expressed $P_{egl\text{-}1}$*gfp* and were positive for HLH-2 (Fig. 6). All four cells are located in the head region where the developing pharynx is found. To determine whether two of these four cells may represent the NSM sister cells, we stained embryos of genotype *ces-1*(*n703*gf); $P_{egl\text{-}1}$*gfp; ced-3*(*n717*). As shown above, *ces-1*(*n703*gf) prevents the deaths of the NSM sister cells by blocking the expression of *egl-1* in these cells. In a *ces-1*(*n703*gf); *ced-3*(*n717*) background, the distribution of HLH-2 and the expression of $P_{egl\text{-}1}$*gfp* in 1.5-fold stage embryos was overall unchanged; however, a total of only two cells expressed $P_{egl\text{-}1}$*gfp* and were positive for HLH-2 (Fig. 6). The two cells that are still positive for HLH-2 but no longer express $P_{egl\text{-}1}$*gfp* may well be the NSM sister cells. This result suggests that HLH-2 is most probably present in the NSM sister cells at the time their cell-death fate is determined.

Uncoupling bHLH binding to the four Snail-binding sites/E-boxes in Region B from CES-1 binding

Our results suggest that in the NSM sister cells, a repressor of *egl-1* transcription, CES-1, as well as an activator of *egl-1* transcription, HLH-2/HLH-3, act on *egl-1* transcription through the same DNA-binding sites in Region B of the *egl-1*

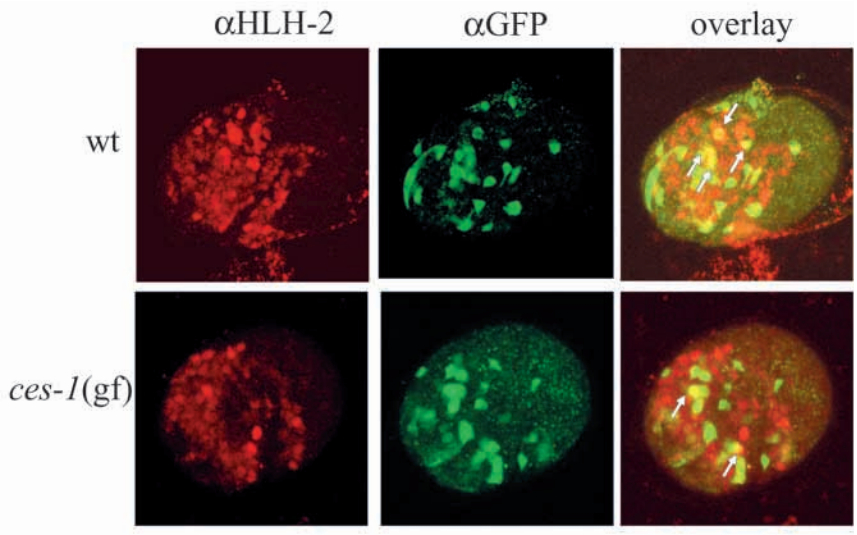

Fig. 6. HLH-2 is most probably present in the NSM sister cells. (Upper panel) Anti-HLH-2 and anti-GFP immunofluorescence staining of a 1.5-fold embryo of genotype *bcIs1* ($P_{egl\text{-}1}gfp$); *ced-3*(*n717*), and overlay (from left to right). The images presented are stacks of a confocal series through an entire embryo. The four double positive cells found in embryos of this genetic background are indicated by arrows. (Lower panel) Anti-HLH-2 and anti-GFP immunofluorescence staining of a 1.5-fold embryo of genotype *unc-87*(*e1216*) *ces-1*(*n703*gf); *bcIs1* ($P_{egl\text{-}1}gfp$); *ced-3*(*n717*), and overlay. The images presented are stacks of a confocal series through an entire embryo. The two double positive cells found in embryos of this genetic background are indicated by arrows.

locus. To test this hypothesis in vivo, we sought experimental approaches that would allow us to analyse the effect of bHLH binding to Region B in the absence of CES-1 binding.

As shown above, CES-1 and HLH-2/HLH-3 bind to wild-type Region B with the four intact Snail-binding sites/E-boxes (referred to as 'wild-type' sites) in vitro but fail to efficiently bind to mutant Region B with the four Snail-binding sites/E-boxes mutated to 5′-CATATA-3′ (referred to as 'Snail$^-$/E-box$^-$' sites) (Fig. 3, Fig. 5B). In contrast to the consensus sequence for Snail binding (5′-CACCTG-3′), bases 3 and 4 of the consensus sequence for bHLH binding can be variable (5′-CANNTG-3′) (Massari and Murre, 2000). We therefore tested whether mutating the four Snail-binding sites/E-boxes in Region B to 5′-CATATG-3′ (referred to as 'Snail$^-$/E-box$^+$' sites) would disrupt CES-1 binding but still allow the binding of bHLH proteins such as HLH-2/HLH-3 in vitro. As shown in Fig. 3, CES-1 binding to a probe consisting of Region B, in which the four Snail-binding sites/E-boxes have been mutated to Snail$^-$/E-box$^+$, is severely reduced when compared with CES-1 binding to the wild-type probe (Fig. 3, compare lanes 1-6 with lanes 13-18). Using an amount of CES-1 protein that is sufficient to bind about 50% of the wild-type probe (100% binding), the introduction of the Snail$^-$/E-box$^+$ mutation reduced binding to on average 6% (n=2) (compare Fig. 3, lanes 4 and 16). On the contrary, using an amount of HLH-2/HLH-3 that was sufficient to bind about 50% of the wild-type probe (100% binding), the introduction of the Snail$^-$/E-box$^+$ mutation only reduced binding to on average 17% (n=3) (Fig. 5B; compare lanes 4 and 16). At least in vitro, the introduction into Region B of Snail$^-$/E-box$^+$ mutations therefore affects the binding of CES-1 more dramatically than the binding of HLH-2/HLH-3.

Functional E-boxes in Region B are required to kill the NSM sister cells and functional Snail-binding sites for the ability of CES-1 to cause their survival

To determine the effect of bHLH binding and CES-1 binding to the four Snail-binding sites/E-boxes in Region B in vivo, we introduced the Snail$^-$/E-box$^-$ and Snail$^-$/E-box$^+$ mutations into Region B of the rescuing fragment pBC08 and analysed the ability of the resulting fragments to rescue NSM sister cell death in *ces-2*(*n732*ts); *egl-1*(*n1084 n3082*) animals. *n732*ts is a temperature sensitive lf mutation of *ces-2*: 13% of the NSM sister cells survive in *ces-2*(*n732*ts) animals raised at 15°C, the permissive temperature; and 73% survive in animals raised at 25°C, the non-permissive temperature (Fig. 7) (Ellis and Horvitz, 1991). *ces-2*(*n732*ts) has been proposed to cause NSM sister cell survival as a result of *ces-1* overexpression in the NSM sister cells (Metzstein and Horvitz, 1999). Culturing transgenic *ces-2*(*n732*ts); *egl-1*(*n1084 n3082*) animals at 15°C and 25°C therefore allowed us to analyse the ability of the fragments to rescue the NSM sister cell death defect caused by *egl-1*(*n1084 n3082*) in the presence of slightly or strongly elevated levels of CES-1 protein in the NSM sister cells, respectively.

Using $P_{tph\text{-}1}gfp$ to analyse NSM sister cell survival, we found that the wild-type fragment pBC08 rescued the death of the NSM sister cells in *ces-2*(*n732*ts); *egl-1*(*n1084 n3082*) animals grown at 15°C (5/5 independent lines) but not at 25°C (0/5 independent lines) (Fig. 7A). This suggests that an activator of *egl-1* transcription, which is required to activate *egl-1* transcription in the NSM sister cells, can function through cis-regulatory regions contained in pBC08, and that high levels of CES-1 in the NSM sister cells can block this activator. The ability of this activator to activate *egl-1* transcription is dependent on functional Snail-binding sites/E-boxes in Region B of the *egl-1* locus, as the Snail$^-$/E-box$^-$ fragment (pBC181) failed to rescue NSM sister cell death in animals grown at 15°C (0/3 independent lines) or at 25°C (0/3 independent lines) (Fig. 6B). Finally, the Snail$^-$/E-box$^+$ fragment (pBC182) rescued the NSM sister cell death in animals grown at 15°C (6/6 independent lines) and also in animals grown at 25°C (6/6 independent lines) (Fig. 7C). The ability of the activator to activate *egl-1* transcription is therefore dependent on functional E-boxes but not on functional Snail-binding sites in Region B of the *egl-1* locus. This indicates that the activator of *egl-1* transcription in the NSM sister cells is most likely an E-box binding bHLH protein. In light of our in vivo data demonstrating a requirement for the genes *hlh-2* and *hlh-3* for NSM sister cell death and our in vitro data demonstrating binding of a HLH-2/HLH-3 heterodimer to the Snail-binding sites/E-boxes in Region B, we propose that a heterodimer of

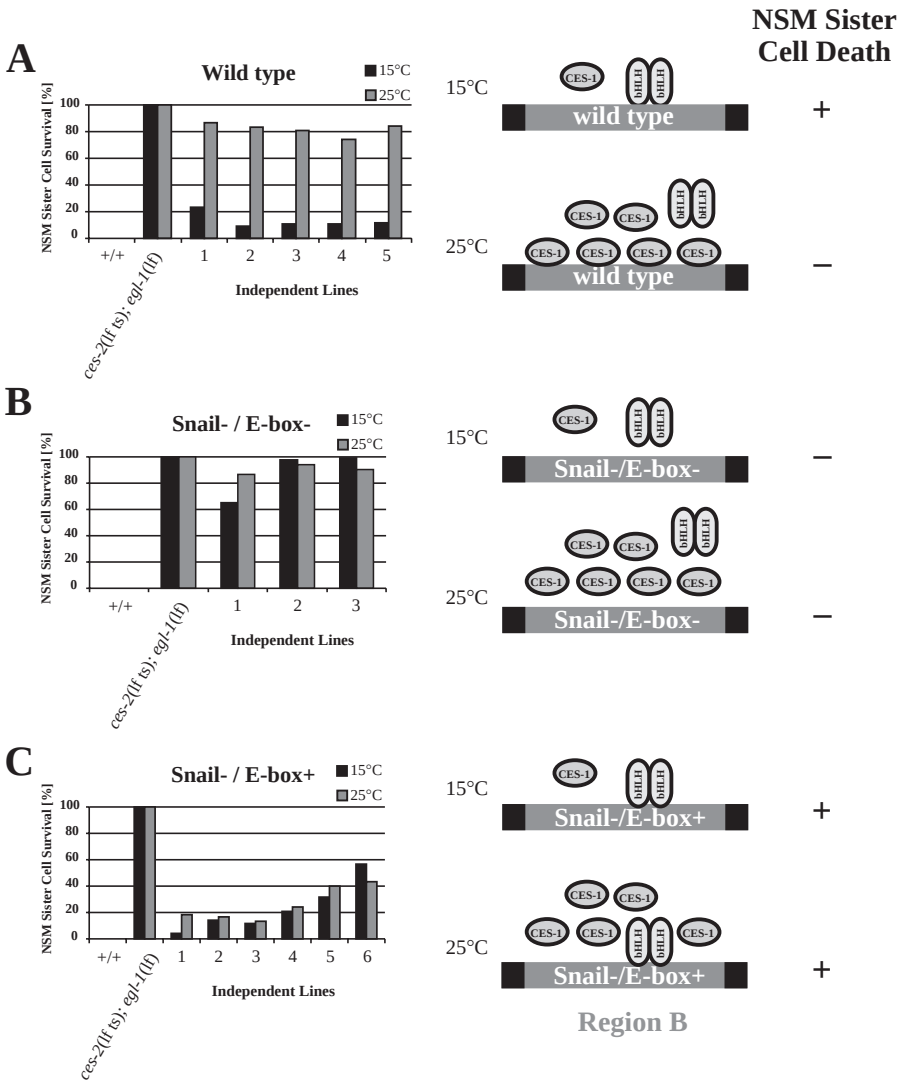

Fig. 7. CES-1 and an activator of *egl-1* expression function through the Snail-binding sites/E-boxes in Region B in vivo. Plasmids pBC08 (wild-type Region B) (A), pBC181 (Region B with Snail$^-$/E-box$^-$ sites) (B) or pBC182 (Region B with Snail$^-$/E-box$^+$ sites) (C) were introduced by germline transformation into hermaphrodites of genotype *ces-2*(*n732*ts)*; bcIs25; egl-1*(*n1084 n3082*) *unc-76*(*e911*) as described in the Materials and Methods. Transgenic lines were cultured at 15°C and 25°C. Rescue of the NSM sister cell death was analysed as described in the Materials and Methods (n=60-222). For every plasmid, several independent transgenic lines were generated and analysed (n=3-6).

HLH-2 and HLH-3 activates *egl-1* transcription in the NSM sister cells to cause these cells to undergo programmed cell death.

The results obtained with the Snail$^-$/E-box$^+$ fragment furthermore indicate that the ability of high levels of CES-1 to block the activator of *egl-1* transcription is dependent on functional Snail-binding sites in Region B of the *egl-1* locus. As CES-1 and HLH-2/HLH-3 therefore act through overlapping DNA-binding sites, the NSM sister cells might survive in *ces-2*(*n732*ts) animals grown at 25°C because high levels of CES-1 protein successfully compete with HLH-2/HLH-3 for binding to Region B in the *egl-1* locus.

DISCUSSION

hlh-2 and *hlh-3* are at least partially required for the death of the NSM sister cells and might encode a direct activator of *egl-1* transcription

We found that reducing the activity of *hlh-2* or *hlh-3* by RNAi results in the survival of 15% and 7% of the NSM sister cells, respectively. Reducing the activity of *hlh-2* and *hlh-3* simultaneously by a weak hypomorphic mutation and by RNAi leads to the survival of on average 30% of the NSM sister cells. *hlh-2* and *hlh-3* are therefore at least partially required for the death of the NSM sister cells. Furthermore, a heterodimer composed of HLH-2 and HLH-3, HLH-2/HLH-3, can bind to Snail-binding sites/E-boxes in the *egl-1* locus in vitro, which are required to kill the NSM sister cells in vivo, and HLH-2 appears to be present in the NSM sister cells at the time their cell-death fate is determined. Hence, we propose that HLH-2/HLH-3 most probably acts as a direct activator of *egl-1* transcription in the NSM sister cells.

hlh-2 and *hlh-3* have so far not been defined by null mutations. It is therefore unclear whether the low penetrance of the Ces phenotype observed is due to additional factors that can kill the NSM sister cells in the absence of *hlh-2* and *hlh-3* or residual *hlh-2* and *hlh-3* activity in the NSM sister cells. It has been shown that *C. elegans* neurons are more resistant to the inactivation of gene function by RNAi than other cell types (Timmons et al., 2001). As this also appears to be the case for the NSMs and NSM sister cells (J.H. and B.C., unpublished), we favour the possibility that the low penetrance of the Ces phenotype observed is the result of the incomplete inactivation of *hlh-2* and *hlh-3* in the NSM sister cells. The inactivation by mutation of the Snail-binding sites/E-boxes in the *egl-1* locus, through which we propose HLH-2/HLH-3 activates *egl-1* transcription in the NSM sister cells, results in the complete failure to kill NSM sister cells. This observation suggests that, if additional factors exist that can kill the NSM sister cells in the absence of *hlh-2* and *hlh-3*, they are most likely to be additional E-box binding proteins, i.e. additional bHLH proteins.

So far one lf mutation of *ces-2*, three gf mutations of *ces-1* (which were found to carry the identical molecular lesion) and one lf mutation of *ces-3*, a gene that acts upstream of or in parallel to *ces-1* and that remains to be characterized at the molecular level, have been identified in genetic screens for mutants, in which the NSM sister cells survive (Ellis and Horvitz, 1991). However, these screens failed to recover

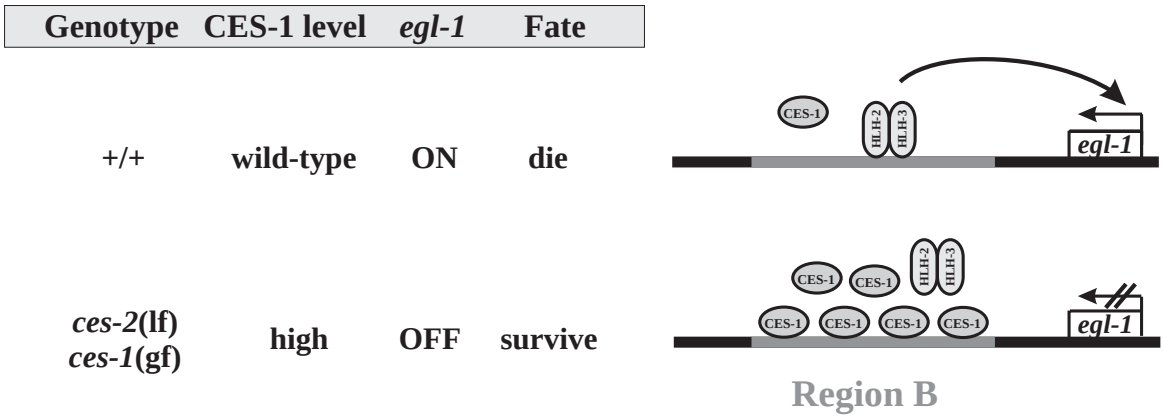

Fig. 8. Competition between CES-1 and HLH-2/HLH-3 for binding to the *egl-1* promoter specifies the cell-death fate of the NSM sister cells. In wild-type animals, the level of CES-1 in the NSM sister cells might not be sufficient to compete with HLH-2/HLH-3 for binding to Region B, resulting in the activation of *egl-1* transcription and NSM sister cell death. In *ces-1*(gf) or *ces-2*(lf) animals, elevated levels of CES-1 in the NSM sister cells might successfully compete with HLH-2/HLH-3 for binding to Region B, resulting in *egl-1* repression and NSM sister cell survival.

mutations in *hlh-2* and *hlh-3*. The failure to identify mutations in these two genes is possibly the result of one or more of the following observations. First, the fact that *ces-2* and *ces-3* have so far only been defined by one lf allele suggests that the screens performed to date were not saturating. Second, *hlh-2*(*RNAi*) results in embryonic lethality (Krause et al., 1997). However, previous screens were performed in a way that did not allow the identification of mutations in essential genes. Finally, it is possible that the complete inactivation of *hlh-2* and *hlh-3* results in a NSM sister cell survival phenotype with low penetrance, decreasing the likelihood of identifying mutations in these genes accordingly.

bHLH proteins play key roles in the specification and execution of cell fates, including neuronal fates, in various organisms (reviewed by Lee, 1997; Massari and Murre, 2000). For example, in *C. elegans*, the *daughterless*-like gene *hlh-2* and the *atonal*-like gene *lin-32* function at multiple steps during the establishment of a specific neuronal sublineage, which gives rise to two neurons and one support cell (Portman and Emmons, 2000). To our knowledge, however, it has not yet been demonstrated that bHLH proteins play a direct role in the activation of programmed cell death. Our results indicate that the *daughterless*-like and *achaete-scute*-like genes *hlh-2* and *hlh-3* of *C. elegans* are at least partially required for the execution of the programmed cell death fate of the NSM sister cells. Furthermore, *hlh-2* and *hlh-3* may not be required for the specification and execution of the neuronal fate of the NSMs, as the $P_{tph\text{-}1}gfp$ reporter continues to be expressed in the NSMs in animals, in which *hlh-2* and/or *hlh-3* have been at least partially inactivated. If *hlh-2* and *hlh-3* function in the specification or execution of the NSM fate as well, the requirement for their function for this process is less stringent than the requirement for their function in promoting the programmed death of the NSM sister cells.

The NSM sister cells might survive in *ces-2*(lf) and *ces-1*(gf) animals, because high levels of CES-1 prevent HLH-2/HLH-3 from binding to the Snail-binding sites/E-boxes in the *egl-1* locus

It has been proposed that the NSM sister cells survive in *ces-2*(lf) or *ces-1*(gf) animals as a result of *ces-1* overexpression in the NSM sister cells (Metzstein and Horvitz, 1999). We have shown that CES-1 can bind to Snail-binding sites/E-boxes in the *egl-1* locus in vitro, which are required for the ability of the *ces-2* lf mutation *n732* to block the death of the NSM sister cells in vivo. This suggests that in *ces-2*(lf) and *ces-1*(gf) animals, CES-1 blocks the death of the NSM sister cells by directly repressing *egl-1* transcription.

Based on our finding that an activator of *egl-1* transcription in the NSM sister cells, most probably HLH-2/HLH-3, acts through the identical Snail-binding sites/E-boxes in the *egl-1* locus, we propose a molecular model for how CES-1 can repress *egl-1* transcription (Fig. 8). We propose that in wild-type animals, in which CES-1 most likely is absent or present at low levels in the NSM sister cells, HLH-2/HLH-3 binds to the Snail-binding sites/E-boxes in the *egl-1* locus thereby activating *egl-1* transcription in the NSM sister cells, which results in the death of these cells. In *ces-2*(lf) or *ces-1*(gf) animals, in which CES-1 levels in the NSM sister cells most likely are elevated, sufficient CES-1 protein is present to successfully bind to the Snail-binding sites/E-boxes thereby preventing HLH-2/HLH-3 from binding to these sites and from activating *egl-1* transcription. This molecular model implies that in wild-type animals, CES-1 might play no role in specifying the cell-death fate of the NSM sister cells. This is supported by the fact that the *ces-1* lf mutation *n703 n1434* does not cause a phenotype in the NSM sister cells. Our model also suggests that in *ces-1*(gf) animals, CES-1 acts by blocking the function of HLH-2/HLH-3, which is supported by our finding that genetically *ces-1* acts upstream of or in parallel to *hlh-2* and *hlh-3*.

What might the function of *ces-1* be in wild-type animals? The *ces-1*-like gene *SLUG* of mammals is required to protect hematopoietic progenitor cells from radiation-induced programmed cell death in vivo (Inoue et al., 2002). One speculative model therefore is that the cell-death protective activity of *ces-1* might be required to protect the NSMs, the fate of which is to survive, from programmed cell death. However, in *ces-1*(lf) animals, the NSMs survive like in wild-type animals, which suggests that either *ces-1* is dispensable

for the survival of the NSMs or that its function is redundant. Hence, the normal function of *ces-1* remains enigmatic.

It has previously been shown that the function of bHLH proteins can be antagonized by Snail-like proteins. For example, the Snail-like protein Escargot of *Drosophila* can repress the E-box-dependent transcriptional activation of a reporter gene by the bHLH proteins Daughterless and Scute in transfection assays (Fuse et al., 1994). In addition, it has been shown that the murine Snail-like protein mSna competes with a homodimer composed of the Daughterless-like protein E47 and with a heterodimer composed of E47 and the Achaete-scute-like protein MASH-2 for binding to E-boxes in vitro and in cultured cells (Nakayama et al., 1998). Finally, the murine Snail-like protein Smuc can block the binding of a heterodimer composed of the Daughterless-like protein E12 and the bHLH protein MyoD to E-boxes in vitro and represses E12/MyoD-dependent activation of a reporter gene in transfection experiments (Kataoka et al., 2000). Our results now demonstrate that by binding to the Snail-binding sites/E-boxes in *cis*-regulatory regions of the *egl-1* locus in vivo, the Snail-like protein CES-1 of *C. elegans* blocks the ability of HLH-2/HLH-3 to activate the cell-death activator gene *egl-1* and to execute the cell-death fate of the NSM sister cells.

Transcriptional activation of the *BH3-only* gene *egl-1* is a common mechanism of cell-death execution

The cell death of at least two types of neurons in *C. elegans* is dependent on the transcriptional activation of the *BH3-only* gene *egl-1*: the male-specific death of the HSN neurons and the death of the NSM sister cells. Transcriptional regulation might therefore be an important mechanism through which the activity of EGL-1 is regulated. The activity of at least four of the 10 mammalian BH3-only proteins identified to date is regulated at the transcriptional level (reviewed by Puthalakath and Strasser, 2002). For example, after DNA damage, the *BH3-only* genes *Noxa* and *Puma/Bbc3* are transcriptionally upregulated in a p53-dependent manner in thymocytes and fibroblasts (Han et al., 2001; Nakano and Vousden, 2001; Oda et al., 2000; Yu et al., 2001). Furthermore, the *BH3-only* genes *Hrk/DP5* and *Bim* have been found to be upregulated in cultured neurons after NGF withdrawal, a process that appears to be dependent on the activation of the c-jun N-terminal kinase (JNK) (Harris and Johnson, 2001; Imaizumi et al., 1999; Putcha et al., 2001; Whitfield et al., 2001). In sympathetic neurons, it could further be shown that the upregulation of *Bim* after NGF-withdrawal is required for their programmed death (Putcha et al., 2001; Whitfield et al., 2001). Hence, the observation that NGF-withdrawal induced death of sympathetic neurons in culture is dependent on the synthesis of macromolecules can be explained by the requirement for *Bim* expression. The transcriptional activation of *BH3-only* genes therefore is crucial for the programmed death of neurons not only in *C. elegans*, in which neuronal death is specified by cell lineage, but also for the programmed death of neurons in mammals, in which neuronal death is predominantly triggered by cell non-autonomous signals.

The cell-death activating function of HLH-2 might be conserved

The cell-death function of *ces-2* and *ces-1* has been conserved through evolution. The homologue of *ces-2* in humans is the proto-oncogene *HLF* (HLF, hepatic leukaemia factor) (Inaba et al., 1996). The oncogenic form of HLF, the E2A-HLF fusion protein, found in patients carrying the t(17; 19) (q22;p13) chromosomal translocation, is composed of the trans-activation domain of the Daughterless-like bHLH protein E2A and the DNA-binding domain of the CES-2-like protein HLF. E2A-HLF has been shown to block the programmed death of pro-B cells thereby allowing their leukaemic transformation and it has been proposed that it does so by inappropriately activating the transcription of a gene with anti-apoptotic function. The *ces-1*-like gene *SLUG* of humans was found to be a target of E2A-HLF and the overexpression of *SLUG* can block the programmed death of leukaemic pro-B cells (Inukai et al., 1999). Furthermore, hematopoietic progenitor cells from mice lacking a functional *SLUG* gene are more sensitive to DNA-damage induced programmed cell death, supporting an anti-apoptotic role for *SLUG* in hematopoietic lineages (Inoue et al., 2002). A mammalian counterpart of the genetic pathway involved in the specification of the NSM sister cell death in *C. elegans* might therefore play an important role in the regulation of the programmed death of pro-B cells in mammals. We have shown that the Snail-like CES-1 protein can prevent the death of the NSM sister cells in *C. elegans* by antagonizing the function of HLH-2 and HLH-3 thereby directly blocking the transcription of the *BH3-only* gene *egl-1* (Fig. 8). It is therefore feasible that homologues of *C. elegans* HLH-2 and HLH-3 in mammals act downstream of or in parallel to SLUG to activate a mammalian *BH3-only* gene in pro-B cells. Indeed, the Daughterless-like bHLH protein E2A, a mammalian homologue of HLH-2, is expressed in B-cell lineages and has been shown to have tumour suppressor activity (Massari and Murre, 2000). Furthermore, a number of *BH3-only* genes have been shown to be expressed in hematopoietic cells in mammals, including *Hrk/DP5* and *Bim* (Puthalakath and Strasser, 2002). Whether *Hrk/DP5* or *Bim* are targets of the conserved, HLF- and SLUG-dependent cell-death pathway in pro-B cells and whether E2A plays a role in their activation remains to be determined.

The *BH3-only* gene *egl-1* of *C. elegans* is required for most if not all of the 131 programmed cell deaths that occur during *C. elegans* development. The studies described resulted in the identification of two new factors, *hlh-2* and *hlh-3*, that are involved in specifying the death of two of these 131 cells, the death of the NSM sister cells. The death of the NSM sister cells is specified by lineage. Like their counterparts in higher organisms, *hlh-2* and *hlh-3*, which encode a Daughterless-like and an Achaete-scute-like bHLH protein, respectively, have been implicated in cell fate determination. The lineage-dependent signal required to kill the NSM sister cells might therefore be transduced by *hlh-2* and *hlh-3*.

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