Development, Growth & Differentiation
The Japanese Society of Developmental Biologists

Develop. Growth Differ. (2015) **57**, 430–443 doi: 10.1111/dgd.12223

Original Article

Nse1 and Nse4, subunits of the Smc5–Smc6 complex, are involved in *Dictyostelium* development upon starvation

Hideo Taniura,* Naoya Tanabe, Yumi Bando and Natsumi Arai

Laboratory of Neurochemistry, College of Pharmaceutical Sciences, Ritsumeikan University, 1-1-1 Nojihigashi, Kusatsu, Shiga 525-8577, Japan

The Smc5–Smc6 complex contains a heterodimeric core of two SMC proteins and non-Smc elements (Nse1–6), and plays an important role in DNA repair. We investigated the functional roles of Nse4 and Nse1 in *Dictyostelium discoideum*. Nse4 and Nse3 expressed as Flag-tagged fusion proteins were highly enriched in nuclei, while Nse1 was localized in whole cells. Using yeast two-hybrid assays, only the interaction between Nse3 and Nse1 was detected among the combinations. However, all of the interactions among these three proteins were recognized by co-immunoprecipitation assay using cell lysates prepared from the cells expressing green fluorescent protein (GFP)- or Flag-tagged fusion proteins. GFP-tagged Nse1, which localized in whole cells, was translocated to nuclei when co-expressed with Flag-tagged Nse3 or Nse4. RNAi-mediated Nse1 and Nse4 knockdown cells (Nse1 KD and Nse4 KD cells) were generated and found to be more sensitive to UV-induced cell death than control cells. Upon starvation, Nse1 and Nse4 KD cells had increases in the number of smaller fruiting bodies that formed on non-nutrient agar plates or aggregates that formed under submerged culture. We found a reduction in the mRNA level of *pdsA*, in vegetative and 8 h-starved Nse4 KD cells, and pdsA knockdown cells displayed effects similar to Nse4 KD cells. Our results suggest that Nse4 and Nse1 are involved in not only the cellular DNA damage response but also cellular development in *D. discoideum*.

Key words: Nse1, Nse3, Nse4, Smc5–Smc6 complex, pdsA, cellular development, *Dictyostelium discoideum*.

Introduction

The structural maintenance of chromosome (SMC) proteins play important roles in sister chromosome cohesion, chromosome condensation and DNA repair (De Piccoli *et al.* 2009). Three separate SMC protein complexes are conserved in all eukaryotes: cohesin, condensin and the Smc5–Smc6 complex. Each complex contains a heterodimeric core of two SMC proteins, together with 2–6 non-SMC proteins. Smc1 and Smc3 form the core of the cohesin complex, Smc2 and Smc4 are components of the condensin complex, and Smc5 and Smc6 form a third complex known as the Smc5–Smc6 complex. Cohesin and condensin play roles in sister chromatid cohesion and chromosome condensation, respectively. In yeasts, at least six additional subunits are associated with Smc5 and Smc6; these subunits are termed non-Smc elements (Nse1–6). Nse1, Nse3 and Nse4 form a subcomplex and bridge the head domains of the Smc5–Smc6 heterodimer (Hudson *et al.* 2011). Mutation of any subunit of the Smc5–Smc6 complex induces hypersensitivity to DNA damage agents including ionizing radiation, alkylating agents and UV light in yeast. The mutants are defective in DNA repair and recovery after treatment with genotoxic agents, which results in cell death (De Piccoli *et al.* 2009).

While the Smc5–Smc6 complex has been studied extensively regarding the DNA damage response, Nse3 and Nse4 may be associated with cellular differentiation. Nse3 is related to the MAGE (melanoma-associated antigen) family of proteins (De Piccoli *et al.* 2009; Hudson *et al.* 2011). While only one MAGE

*Author to whom all correspondence should be addressed.
Email: hideo@fc.ritsumei.ac.jp
Received 10 June 2014; revised 6 April 2015; accepted 22 April 2015.

gene (the Nse3 gene) exists in the genomes of plants, nematodes, insects and even non-mammalian vertebrates, mammalian cells carry 55 different MAGE genes (De Piccoli *et al.* 2009). Although the functions of most MAGE proteins in mammals are relatively poorly understood, Necdin, a MAGE protein, may be important in the development of the brain and in the differentiation of multiple cell types. Necdin has been reported to associate with the homeodomain transcription factor Dlx via MAGE-D1 to promote the differentiation of GABAergic neurons in mouse embryonic forebrain; mutant mice lacking the necdin gene showed significant reductions in forebrain GABAergic neurons differentiation *in vivo* and *in vitro* (Kuwajima *et al.* 2006). Nse4 is related to the EID (E1A-like inhibitor of differentiation) family of transcriptional repressors (Palecek *et al.* 2006; Hudson *et al.* 2011). The adenoviral transforming protein E1A is a viral oncoprotein that targets both CBP and p300, which serve as transcriptional co-activators. Similar to the adenoviral protein E1A, EID1, a mammalian EID, binds the A-B pocket of Rb, impairs transactivation by the muscle determination protein, MyoD, and binds to p300, inhibiting its histone acetyltransferase activity. EID1 overexpression in skeletal muscle inhibits tissue-specific transcription (MacLellan *et al.* 2000). Nse1 contains a RING finger motif (a zinc finger-like motif) that resembles those found in ubiquitin ligases (McDonald *et al.* 2003; De Piccoli *et al.* 2009). The RING finger motif is essential for ubiquitin ligase activity. No information has been published regarding a role for Nse1 in cellular differentiation.

The social amoeba *Dictyostelium discoideum* is one of the eukaryotic model organisms suitable for cellular differentiation and development research. However, the Smc5–Smc6 complex has not yet been described in *Dictyostelium*. In this study, we identified and characterized Nse1, Nse3 and Nse4 derived from *Dictyostelium discoideum*, including their cellular distribution and binding activities. A group of single *Dictyostelium* cells develops a multicellular fruiting body upon starvation. Streams of cells connect to each other and form a multicellular aggregate (Jang & Gomer 2008). Then, the aggregate undergoes a program of cell differentiation to produce an intermediate slug and finally a fruiting body that contains a supporting stalk with spores. To investigate the functional role of Nse proteins of the Smc5–Smc6 complex in *Dictyostelium* development, we isolated Nse4 and Nse1 knockdown transformants by RNA interference (RNAi), and found that these transformants showed altered development upon starvation. Our findings suggest that Nse proteins of the Smc5–Smc6 complex play a role in the cellular development of *D. discoideum.*

Materials and methods

Growth and development of Dictyostelium

Dictyostelium discoideum Ax-2 cells were provided by the NBRP (National BioResources Project; cellular slime molds) of Japan. Ax-2 cells were grown axenically in HL-5 medium at 21°C. All the transformed cells were grown in HL-5 medium supplemented with 10 μg/mL G418. For development on agar plates, the cells were washed twice in 10 mmol/L Na-K phosphate buffer (10 mmol/L Na-K phosphate buffer, 20 mmol/L $MgSO_4$ and 2 mmol/L $CaCl_2$, pH 6.1), and 100 μL of cells (5×10^6 cells/mL) was plated on each 1.5% agar plates in KK2 (16.5 mmol/L KH_2PO_4, 3.8 mmol/L K_2HPO_4). For development under submerged culture, the cells were washed twice in 10 mmol/L Na-K phosphate buffer, and 500 μL of cells (2×10^6 cells/mL) was added to 10 mmol/L Na-K phosphate buffer in four-well dishes (1.9 cm^2/well; Thermo Fisher Scientific, MA, USA).

cDNA cloning

The entire coding sequence of Nse4 (DDB_G0281991) was amplified from cDNA and cloned into pBluescript SK+ (Agilent Technologies, CA, USA) using BamHI and XhoI sites. The following primers were used for polymerase chain reaction (PCR) amplification: 5′-TGC GGA TCC ATG AGT AGA TCA CAA CCA CA-3′ and 5′-GCG CTC GAG TTA AAA ATC CAT TCT TGT CGA-3′. The Nse4 sequence was matched completely to the sequence in the Dictybase. Nse1 (DDB_G0279231) entire coding sequence was amplified from cDNA and cloned into pBluescript SK+ using BamHI and XhoI sites. The following primers were used for PCR amplification: 5′-TGC GGA TCC ATG AGT AGA AGA TCA CAA CCA CA-3′ and 5′-GCG CTC GAG TTA AAA ATC CAT TCT TGT CGA-3′. The Nse1 sequence was matched completely to the Dictybase. The entire coding sequence of Nse3 (DDB_G0284679) was amplified from cDNA and cloned into pBluescript SK+ using BamHI and XhoI sites. The following primers were used for PCR amplification: 5′-TGC GGA ATG TCA AGA AGA TCA CAA CAA TC-3′ and 5′-GCG CTC GAG TTA TCT TCT TGT ACT TCT TTG AG-3′. The Nse3 sequence showed one nucleotide substitution at position +580 (+1 at the beginning of the ATG start codon) (T to C, no amino acid substitution). The entire coding sequence of pdsA (DDB_G0285995) was amplified from cDNA and cloned into pBluescript SK+ (Stratagene) using BamHI and XhoI sites. The following primers were used for PCR amplification: 5′-TGC GGA TCC ATG GCA TTA AAT AAA AAA T-3′ and 5′-GCG CTC

GAG TTA AAT ACA AAT TGG ATC ACC-3′. The pdsA sequence was matched completely to the Dictybase.

Expression of Flag-tagged Nse4, Nse1 and Nse3, and of GFP-tagged Nse1 for immunocytochemistry

The pTX expression vector was kindly provided by Dr Thomas T. Egelhoff at Case Western Reserve School of Medicine (Levi *et al.* 2000). Flag tags were fused at the N termini of Nse4, Nse1 and Nse3. The constructs were introduced into cells by electroporation, and stable transformants were selected with G418. A GFP tag fused at the N terminus of Nse1 was used to determine altered cellular distribution. For fluorescence immunocytochemistry, cells were fixed in 4% formaldehyde in phosphate buffered saline (PBS) at 4°C for 20 min, permeabilized with methanol at room temperature for 20 min and incubated with the anti-Flag M2 antibody (Sigma-Aldrich, MO, USA) or anti-GFP rabbit monoclonal antibody (Life Technologies). For 4′6′-diamidino-2-phenylindole dihydrochloride (DAPI) (Life Technologies) staining, the fixed cells were treated with 300 nmol/L DAPI in PBS for 5 min at room temperature and observed using a fluorescence microscope.

Yeast two-hybrid assay

cDNAs for Nse4, Nse1, and Nse3 were directionally inserted into the Gal4 DNA-binding domain vector (pGBT9) (Clontech Laboratories) and/or Gal4 activation domain vector (pGAD424) (Clontech Laboratories), and introduced into *Saccharomyces cerevisiae* AH109 cells. The transformants were grown on leucine-, tryptophan- and histidine-deficient synthetic dropout medium plates at 30°C for 3 days. If the DNA-binding domain and activation domain derived from the GAL4 transcription factor can be brought into close physical proximity to the promoter region, then the transcriptional activation function will be restored. The AH109 strain contains two reporter genes (*HIS3* and *lacZ*) under the control of a Gal4-responsive element. When expressing the *HIS3* gene, the yeast can grow without histidine.

Immunoprecipitation

Green fluorescent protein (GFP) tags fused at the N termini of Nse4 and Nse1 were used for co-immunoprecipitation. Combinations of expression vectors for GFP, GFP-Nse1, GFP-Nse4 and Flag-Nse3 were transfected into Ax-2 cells, and the transformants were selected with G418. In another experiment, combinations of expression vectors for GFP-Nse4 with Flag-Nse1, Flag-Nse3 or both of Flag-Nse1 and Flag-Nse3 were used. Cell lysates from each transformant were prepared in a buffer containing 10 mmol/L PIPES (pH 7.0), 100 mmol/L NaCl, 300 mmol/L sucrose, 3 mmol/L $MgCl_2$, 1 mmol/L EGTA, 0.5% Triton X-100 and complete protease inhibitors (Roche, Switzerland). Aliquots (400 μg of protein) of extracts were incubated for 2 h at 4°C with anti-GFP rabbit monoclonal antibody (Life Technologies). The antibody–protein complexes were pelleted with protein A-Sepharose (GE Healthcare, WI), separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and analyzed by immunoblotting with anti-Flag M2 monoclonal antibody (Sigma-Aldrich).

RNAi knockdown mutants of Nse4, Nse1 and pdsA

To knockdown the expression of Nse1, Nse4 and pdsA, we constructed expression vector containing stem-loop RNAs directed against Nse1, Nse4 and pdsA mRNA, respectively. For Nse1, one fragment containing nucleotides +48 to +406 of the coding region of Nse1 (+1 at the beginning of the ATG start codon; 359 bp) was cloned in sense orientation into pTX BamHI/XhoI sites. Another fragment containing nucleotides +48 to +777 of the coding region (+1 at the beginning of the ATG start codon; 730 bp) was fused in antisense orientation into the above pTX XhoI/XbaI sites. For Nse4, one fragment containing nucleotides +106 to +616 of the coding region of Nse4 (+1 at the beginning of the ATG start codon; 511 bp) was cloned in sense orientation into pTX BamHI/XhoI sites. Another fragment containing nucleotides +106 to +1072 of the coding region (+1 at the beginning of the ATG start codon; 967 bp) was fused in antisense orientation into the above pTX XhoI/XbaI sites. For pdsA, one fragment containing nucleotides +78 to +682 of the coding region of pdsA (+1 at the beginning of the ATG start codon; 604 bp) was cloned in sense orientation into pTX BamHI/XhoI sites. Another fragment containing nucleotides +78 to +1196 of coding region (+1 at the beginning of the ATG start codon; 1119 bp) was fused in antisense orientation into the above pTX XhoI/XbaI sites. The Nse1-RNAi, Nse4-RNAi and pdsA-RNAi vectors were introduced into Ax-2 cells and stable transformants were selected with G418. RT–PCR was performed to quantify the levels of Nse1, Nse4 and pdsA mRNA expression. Quantification of the results was performed using ImageJ software, and each gene product was normalized against both the *DdmgluPR* or *actin15* RNA level as internal controls (Taniura *et al.* 2006). Statistical significance was tested using Student's *t*-test. We also performed RT–PCR to analyze the mRNA levels of *pdsA*, *ctnA* and *gca* in Nse4 KD cells. Total RNA was prepared from vegetative and

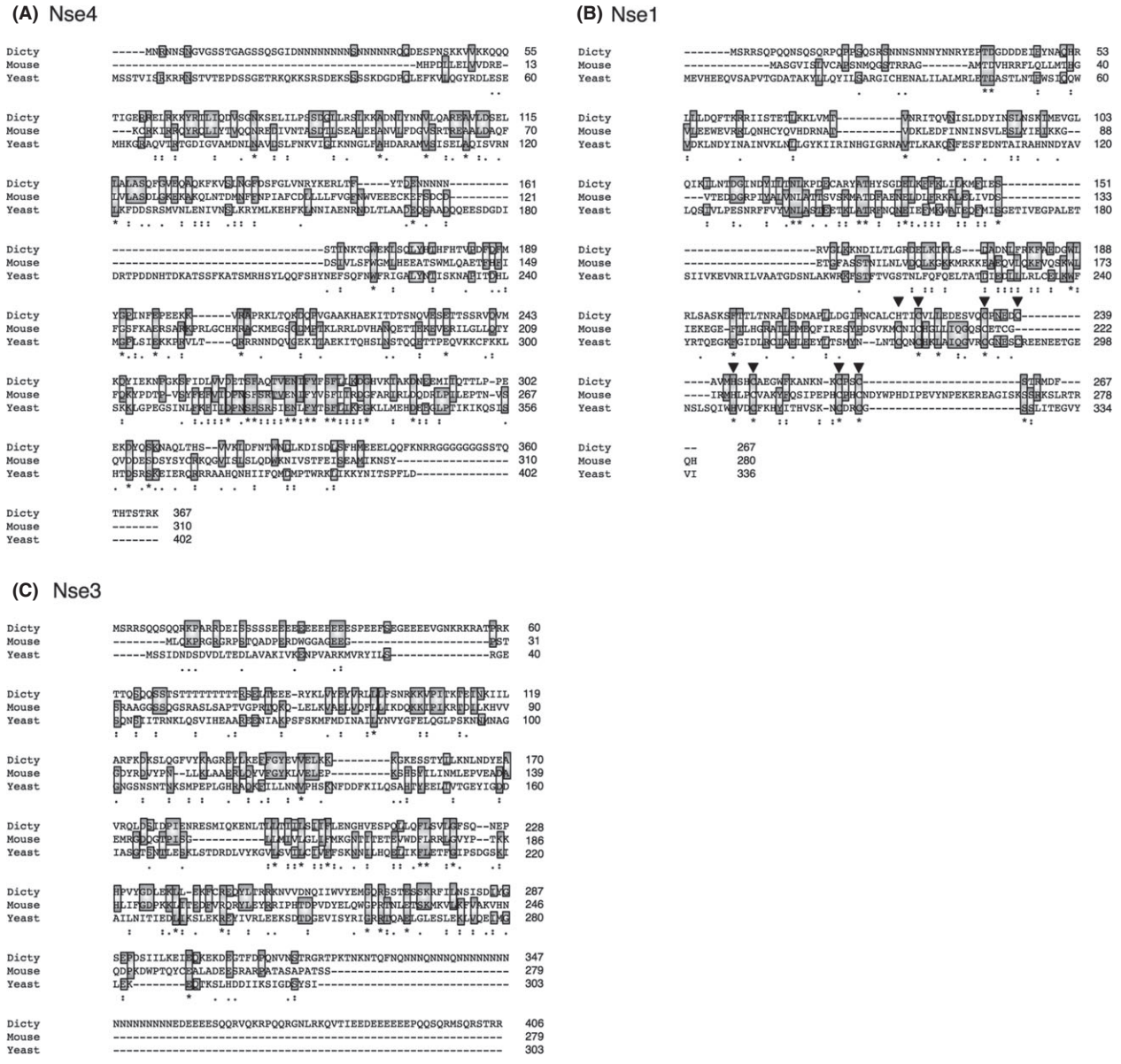


Fig. 1. Amino acid sequence alignment of Nse4, Nse1 and Nse3 derived from *Dictyostelium discoideum* (Dicty), murine (mouse) and *Saccharomyces cerevisiae* (yeast). (A) Amino acid sequences of Nse4 from *D. discoideum* (Dictybase DDB_G0281991), murine (NCBI BC099545) and *S. cerevisiae* (NCBI NM001180164) were aligned using ClustalW. Identical residues between sequences are boxed. (*), conserved residues; (:), strongly similar residues; (.), weakly similar residues. (B) Amino acid sequences of Nse1 from *D. discoideum* (Dictybase DDB_G0279231), murine (NCBI NM026330) and *S. cerevisiae* (NCBI NM001181894) were aligned using ClustalW. Identical residues between sequences are boxed. (*), conserved residues; (:), strongly similar residues; (.), weakly similar residues. Arrowheads (▼) are placed at predicted Nse1 RING finger domains. (C) Amino acid sequences of Nse3 from *D. discoideum* (Dictybase DDB_G0284679), murine MAGE-G1 (NCBI NM023239) and *S. cerevisiae* (NCBI NM001180596) were aligned using ClustalW. Identical residues between sequences are boxed. (*), conserved residues; (:), strongly similar residues; (.), weakly similar residues. Amino acid residue numbers are shown at the end of each line.

8 h-starved Nse4 KD and control cells under submerged culture. *Actin15* RNA levels were used as internal controls. The following primers were used: *actin15*: 5′-TAA ATC CAA AAG CCA ACA GAG-3′ and 5′-TTG GAA AGT TGA GAG TGA AGC-3′; *pdsA*: 5′-GAG TTAT TTC ATT GGT CAC AG-3′ and 5′-TTA GTC ATT GGT GGA GAG GTT-3′; *ctnA*: 5′-GCG ACT TAT TAT GCG ATT ATG-3′ and 5′-AAG AAC CAG CAC CAG ACA TTG-3′; *gca*: 5′-AAA CAA GCA TAC CAT AGT CGT-3′ and 5′-TTT CTG CCT CTT CCCA TCT AC-3′.

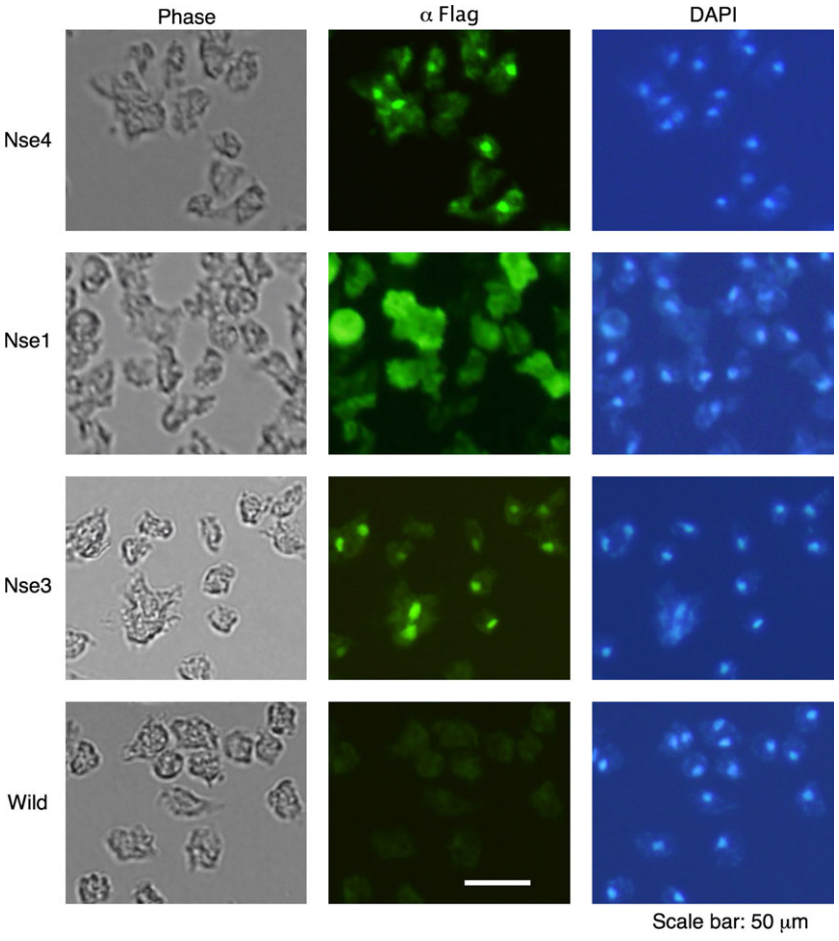


Fig. 2. Localization of Nse4, Nse1 and Nse3. Localization of Flag-tagged Nse4, Nse1 and Nse3 in axenically grown Ax-2 cells. Expression of Flag-tagged Nse4, Nse1 or Nse3 was detected using anti-Flag M2 antibody directed against the Flag epitope under a fluorescence microscope (middle panel, α-Flag). The same cells were also stained with 4′6′-diamidino-2-phenylindole dihydrochloride (DAPI) to show the nuclei (right panel, DAPI). The left panel shows the phase contrast image of the same field of cells (phase). Scale bar: 50 μm.

UV treatment

Empty vector-transfected control cells and RNAi-mediated Nse1 and Nse4 knockdown cells (Nse1 KD cells and Nse4 KD cells) were plated into four-well dish at 2×10^6 cells/mL in HL5 medium. Two hours after the cells were plated, they were subjected to UV-irradiation with different intensities at 250 and 500×100 μJ/cm^2 (Stratalinker 1800UV crosslinker; Agilent Technologies) immediately after the supernatant medium was removed. After the cells were irradiated, fresh medium was added, and the cells were allowed to recover for 2 h. To detect cell death, the cells were incubated with 2 μmol/L EthD-1 (Life Technologies) in PBS at room temperature for 30 min. EthD-1-positive cells were counted and expressed as a percentage of cell death. Statistical significance was tested using Student's *t*-test.

Development of Nse1, Nse4 and pdsA KD cells upon starvation

Nse1, Nse4 and pdsA KD cells were developed on both non-nutrient agar plates and under submerged culture conditions upon starvation. The number of fruiting bodies that formed from 100 μL of 5×10^6 cells/mL on non-nutrient agar plates was counted at 24 h after starvation. The number of aggregates that formed from 500 μL of 2×10^6 cells/mL under submerged culture conditions was counted at 24 h after starvation. Statistical significance was tested using Student's *t*-test. Cell streaming of Nse4 and Nse1 KD cells was observed at 8 and 11 h after starvation under submerged culture conditions.

Results

Nse1, Nse4 and Nse3 sequences

We obtained the Nse4, Nse1 and Nse3 gene sequences from a bioinformatics database. Sequence similarities showed 20% identity between *Dictyostelium* and mouse Nse4 and 12% identity between *Dictyostelium* and yeast Nse4 (Fig. 1A). Sequence similarities showed 19% identity between *Dictyostelium* and mouse Nse1 and 14% identity between *Dictyostelium* and yeast Nse1 (Fig. 1B). Nse1 contains a RING finger-like domain also found in

Nse1 homologs from different species (McDonald *et al.* 2003). The predicted *Dictyostelium* Nse1 RING finger is conserved as shown in Figure 1B. Nse3 cDNA sequence showed one nucleotide substitution at position +580 (+1 at the beginning of the ATG start codon) (T to C, no amino acid substitution). Sequence similarities showed 16.7% identity between *Dictyostelium* and mouse Nse3 and 8.6% identity between *Dictyostelium* and yeast Nse3 (Fig. 1C).

Cellular localization of Nse4, Nse1 and Nse3

To examine the subcellular localization of Nse4, Nse1 and Nse3 proteins, we expressed Nse4, Nse1 and Nse3 proteins carrying Flag tags at their N termini. The Flag-tagged Nse4 and Nse3 immunoreactivities were highly enriched in nuclei (Fig. 2), whereas, Nse1 immunoreactivity was enriched in whole cells.

Interactions among Nse4, Nse1, and Nse3

Nse1, Nse3, and Nse4 form a subcomplex of the yeast Smc5–Smc6 complex (De Piccoli *et al.* 2009). We tested interactions among Nse1, Nse3, and Nse4 derived from *D. discoideum* by yeast two-hybrid assays (Fig. 3). Nse3 and Nse4 cDNAs were fused to the Gal4 binding and activation domains, respectively. Nse1 cDNA fused to either domain was used depending on the partner tested. The interaction-dependent transcriptional activation of the *HIS3* gene was assayed on histidine-deficient synthetic dropout plates. We detected only the interaction between Nse3 and Nse1 among the combinations. Then, we tested the interaction of Nse1 or Nse4 with Nse3 by co-immunoprecipitation assay (Fig. 4A). GFP-tagged Nse1 or Nse4 was co-expressed with Flag-tagged Nse3. All the fusion proteins expressed the predicted size products (Fig. 4A, left and middle); however, many degradation products were observed in the lysates from cells that expressed GFP-Nse4 (Fig. 4A, left). Flag-Nse3 was co-precipitated with GFP-Nse1 and GFP-Nse4 by anti-GFP antibody, but not with GFP alone (Fig. 4A, right), also suggesting an interaction between Nse4 and Nse3. We investigated whether Nse1 alters its intracellular distribution when it interacts with Nse3. Ax-2 cells were transfected with expression vectors for GFP-tagged Nse1 and Flag-tagged Nse3. GFP-Nse1 was distributed in whole cells, and Nse3 expression translocated GFP-Nse1 to nuclei (Fig. 4B). These results suggest that Nse1 alters its subcellular distribution under the influence of its binding partner. We also tested the interactions of GFP-Nse4 with Flag-Nse1 or Flag-Nse3 (Fig. 4C). All the expressed fusion proteins are shown in Figure 4C (left). Flag-Nse1 or Flag-Nse3 was co-precipitated with GFP-Nse4 by anti-GFP antibody (Fig. 4C, right), also suggesting an interaction between Nse4 and Nse1 as well. When both Flag-tagged Nse1 and Nse3 were co-expressed with GFP-tagged Nse4, both Flag-tagged proteins were co-precipitated. Nse4 expression also translocated GFP-Nse1 to nuclei (Fig. 4C, bottom).

RNAi-mediated knockdown of Nse4 or Nse1 expression

To determine the functional importance of Nse4 and Nse1, we isolated Nse4 or Nse1 knockdown transformants by RNAi. RNAi-mediated gene silencing has been established in *Dictyostelium* (Martens *et al.* 2002). To knockdown the expression of Nse4 or Nse1, we constructed a plasmid to express a stem-loop RNA directed against Nse4 or Nse1 mRNA under the control of the actin 15 promoter (Fig. 5A). The expression levels of Nse4 and Nse1 mRNAs in the RNAi expression vector-transfected cells were reduced to approximately 20% and 35.6%, respec-

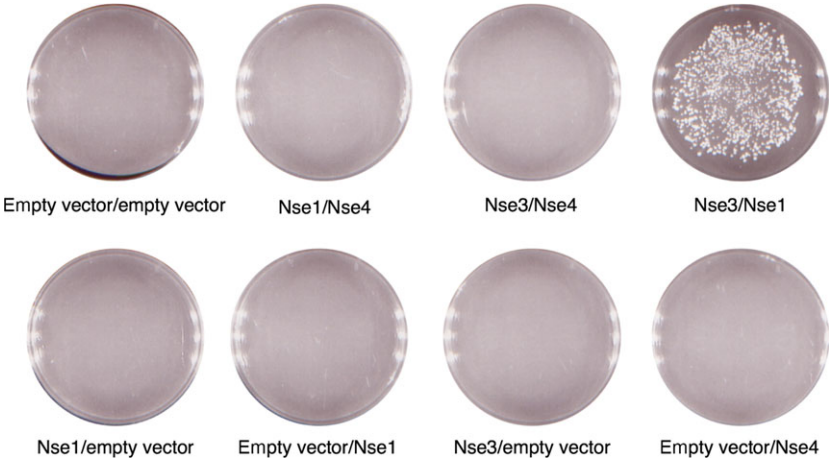


Fig. 3. Yeast two-hybrid assays. Yeast two-hybrid plasmids expressing Nse4 fused to the Gal4 activation domain and Nse3 or Nse1 fused to the Gal4 DNA-binding domain were co-expressed in yeast cells. Yeast two-hybrid plasmids expressing Nse1 fused to the Gal4 activation domain and Nse3 fused to the Gal4 DNA-binding domain were co-expressed in yeast cells. All the plasmids expressing Nse1, Nse3 or Nse4 were also tested with empty vector as indicated. Transformants were grown on leucine-, tryptophan- and histidine-deficient synthetic dropout medium plates at 30°C for 3 days.

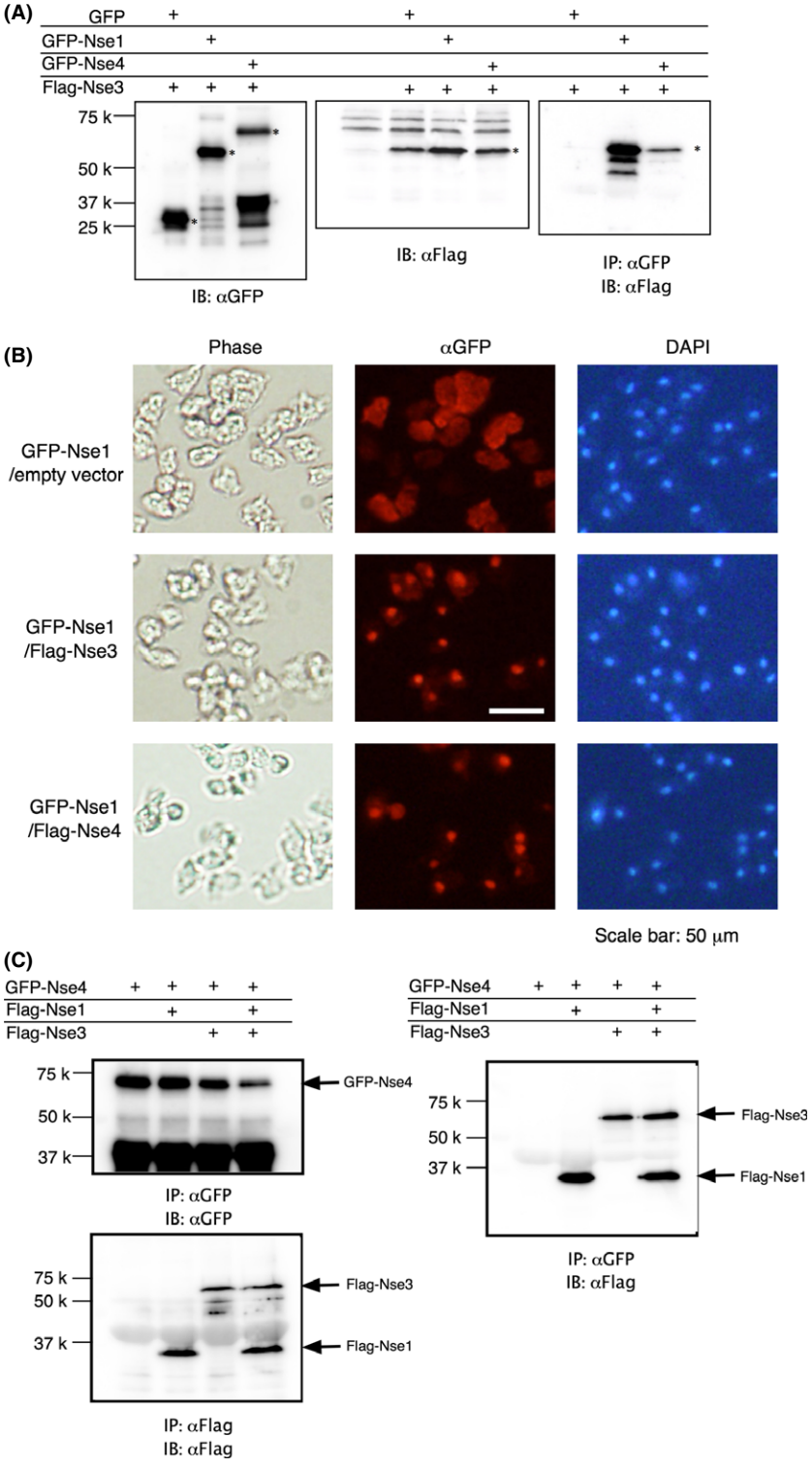


Fig. 4. Interactions among Nse4, Nse1, and Nse3. (A) Immunoprecipitation cell lysates were prepared from Ax-2 cells co-expressing green fluorescent protein (GFP), GFP-Nse1 or GFP-Nse4 with Flag-Nse3. Expression levels of these proteins are shown in the left and middle panels as detected by anti-GFP antibody (left panel) or anti-Flag antibody (middle panel). Cell lysates were immunoprecipitated with anti-GFP antibody, and Flag-Nse3 was detected by anti-Flag antibody (right panel). IB, immunoblot; IP, immunoprecipitation; α GFP, anti-GFP antibody; α Flag, anti-Flag antibody. Asterisks indicates the fusion protein products. (B) Translocation of Nse1 with co-expressed Nse3 or Nse4. GFP-tagged Nse1 was expressed alone (GFP-Nse1/empty vector) or co-expressed with Flag-tagged Nse3 (GFP-Nse1/Flag-Nse3) or Nse4 (GFP-Nse1/Flag-Nse4). GFP-tagged Nse1 was detected using anti-GFP antibody (middle panel, α-GFP). The same cells were also stained with DAPI to show the nuclei (right panel, DAPI). The left panel shows the phase contrast image of the same field of cells (phase). Scale bar: 50 μm. (C) Immunoprecipitation cell lysates were prepared from Ax-2 cells co-expressing Flag, Flag-Nse1 or Flag-Nse3 with GFP-Nse4. Expression levels of these proteins are shown in the left 2 panels as detected by anti-GFP antibody (left upper panel) or anti-Flag antibody (left lower panel). Cell lysates were immunoprecipitated with anti-GFP antibody, and Flag-Nse1 and/or Flag-Nse3 was detected by anti-Flag antibody (right panel). IB, immunoblot; IP, immunoprecipitation; α GFP, anti-GFP antibody; α Flag, anti-Flag antibody. Arrows indicate the fusion protein products.

tively, compared to those in the empty vector-transfected control cells (Fig. 5B). Nse4 and Nse1 KD cells grew normally but slight slower at low cell density compared with the control cells (Fig. 6A). Because Nse4 and Nse1 yeast mutants are hypersensitive to DNA damage (Harvey *et al.* 2004; Hu *et al.* 2005), we tested the sensitivity of Nse4 and Nse1 KD cells to the lethal effects of UV irradiation. Cell death was assessed using EthD-1 (ethidium homodimer), a fluorescent dye that is excluded by viable cells. Almost

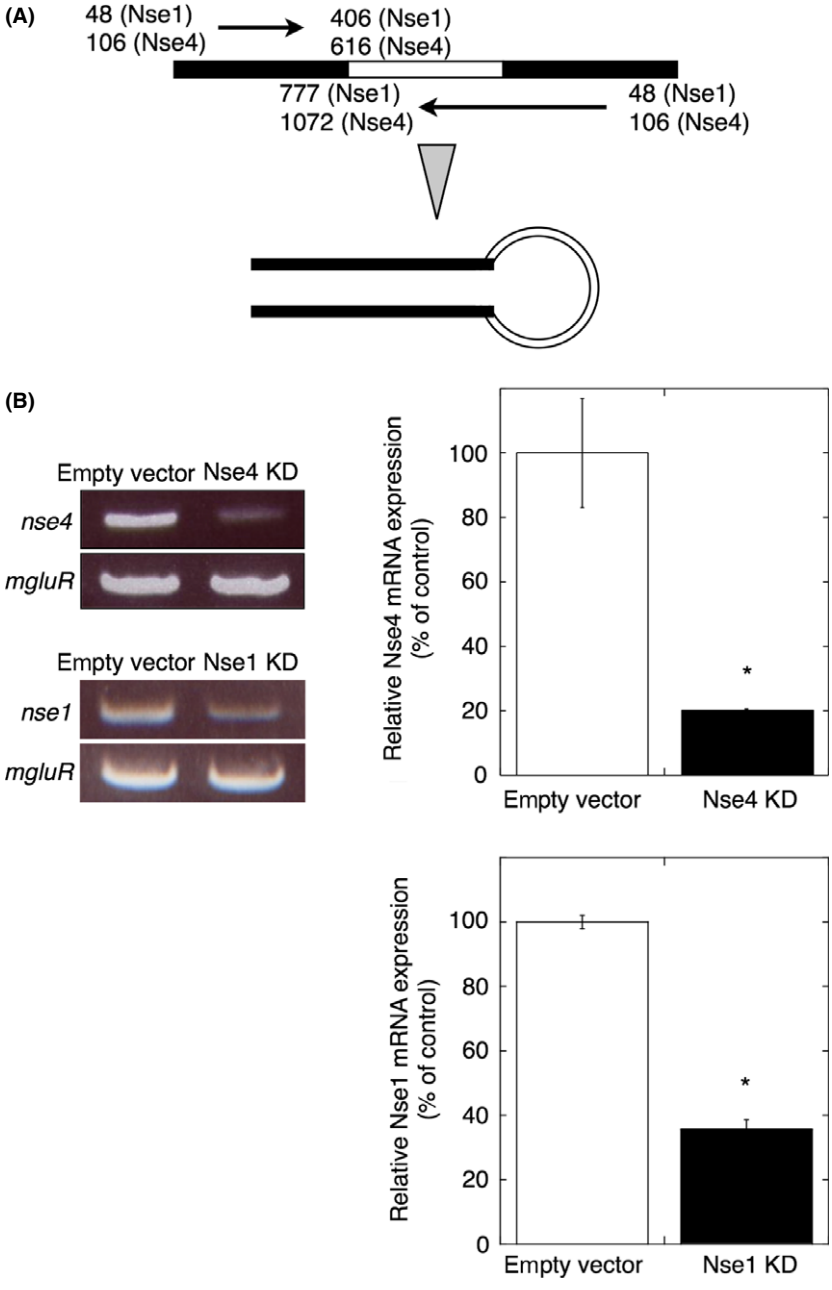


Fig. 5. RNAi-mediated knockdown of Nse4 or Nse1 expression. (A) Construction of stem-loop RNAs directed against Nse4 and Nse1. One fragment containing nucleotides +106 to +616 of the coding region of Nse4 (+1 at the beginning of the ATG start codon; 511 bp), and another fragment containing nucleotides +106 to +1072 of the coding region (967 bp) were fused in reverse orientation and cloned into the pTX expression vector under the control of the actin 15 promoter. One fragment containing nucleotides +48 to +406 of the coding region of Nse1 (+1 at the beginning of the ATG start codon; 359 bp) and another fragment containing nucleotides +48 to +777 of the coding region (730 bp) were fused in reverse orientation and cloned into the pTX expression vector under the control of the actin 15 promoter. The Nse4-RNAi or Nse1-RNAi vector was introduced into Ax-2 cells, and stable transformants were selected with G418. Empty vector was introduced into Ax-2 cells used as a control. (B) reverse transcription–polymerase chain reaction (RT–PCR) analysis of Nse4 and Nse1 knockdown cells (Nse4 and Nse1 KD cells). Total RNA extracted from vegetative cells of control (empty vector), and Nse4 and Nse1 KD cells was used for oligo(dT)-primed RT–PCR (left panel). *DdmGluPR* expression in vegetative cells was used as an internal standard. Quantified results were normalized against *DdmgluPR* RNA levels and expressed as % of the control (right panel). Each value represents the mean ± standard error (SE) ($n = 3$). The asterisk indicates a value that significantly differs ($P < 0.005$) from the value of the control.

no cell death was observed without UV irradiation (empty vector, 0.27 ± 0.07%; Nse4 KD, 1.13 ± 0.29%; Nse1 KD, 0.73 ± 0.18%). When Nse4 and Nse1 KD cells and control cells (empty vector) were irradiated with intensities of UV light at 250 and 400 × 100 μJ/cm^2, Nse4 and Nse1 KD cells were more sensitive to UV irradiation-induced death compared to control cells (Fig. 6B,C). The result suggests DNA repair dysfunction in response to UV-induced DNA damage in Nse4 and Nse1 KD cells.

The development of Nse4, Nse1 and pdsA KD cells

Dictyostelium lives as a single-celled amoeba; however, upon starvation, these cells initiate a developmental cycle. This cycle leads to the aggregation of cells and to the formation of a multicellular fruiting body that carries spores at its top. To examine the development of Nse4 and Nse1 KD cells, we observed the starved cells grown on non-nutrient agar and under submerged culture. Nse4 and Nse1 KD cells displayed large increases in the number of smaller fruiting bodies that formed on

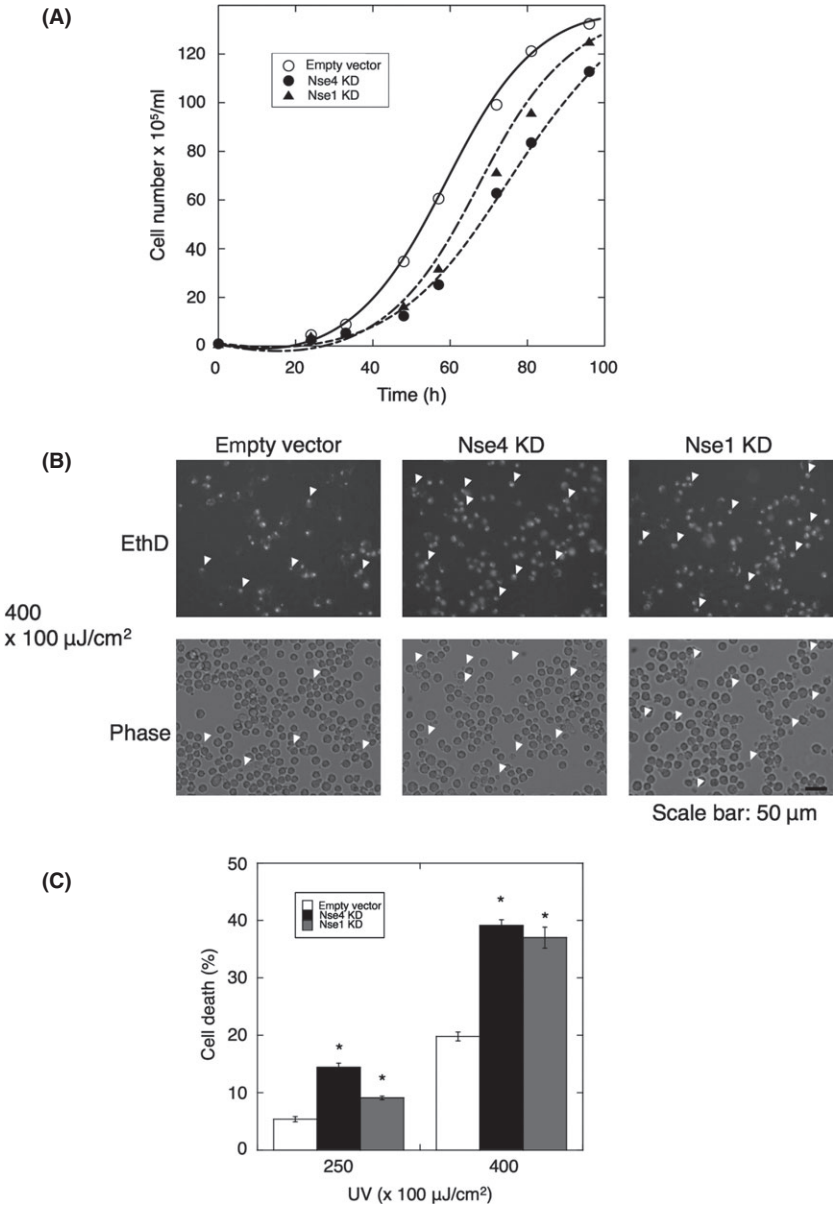


Fig. 6. DNA damage sensitivity of Nse4 and Nse1 KD cells. (A) Growth of control (empty vector), Nse4 KD (Nse4 KD) and Nse1 KD (Nse1 KD) cells in an axenic suspension culture. Cells were diluted to 1×10^5 cells/mL in HL5 medium and cultured at 21°C. (B) Control (empty vector), Nse4 KD (Nse4 KD) cells and Nse1 KD (Nse1 KD) cells were irradiated with UV light intensities of 250 and 400 × 100 μJ/cm^2, and cell death was assessed by using EthD-1, a fluorescent dye that is excluded by viable cells. Cell death of control, Nse4 KD cells and Nse1 KD cells irradiated at 400 × 100 μJ/cm^2 is shown. Arrowheads show the EthD-positive cells. Scale bar: 50 μm. (C) EthD-1-positive cells were counted at each intensity of UV light and expressed as cell death percentages. Each value represents the mean ± SE (n = 3). The asterisk indicates a value that significantly differs ($P < 0.005$) from the value of the control.

agar plates or aggregates that formed under submerged culture at 24 h after starvation (Fig. 7A,B). As shown in Figure 7C, we observed loose cell streaming in control cells (empty vector) at 8 h after starvation, whereas no such structure was observed in Nse4 and Nse1 KD cells. Thereafter, control cells (empty vector) formed continuous streams toward the aggregation center, whereas streams of Nse4 and Nse1 KD cells were highly fragmented at 11 h after starvation. These results suggest that the Nse4 and Nse1 cells have impaired cell streaming during development under starvation conditions.

We analyzed the mRNA levels of *pdsA*, *ctnA* and *gca*, which are genes involved in aggregation size, in Nse4 KD cells under starvation conditions (Faure *et al.* 1988; Brock & Gomer 1999; Veltman & van Haastert 2008). Total RNA was prepared from vegetative and 8 h-starved cells. We found a significant decrease in the mRNA level of *psdA* in Nse4 KD cells compared with control cells (empty vector) derived from both vegetative and 8 h-starved conditions (Fig. 8, 0 and 8 h). The mRNA level of *pdsA* also decreased to 48.4 ± 2.3% and 41.0 ± 3.5% in Nse1 KD cells under vegetative and 8 h-starved, respectively, compared with control cells (data not shown). The *pdsA* gene product is a phosphodiesterase and is involved in the generation of cAMP waves during the development (Faure *et al.* 1988).

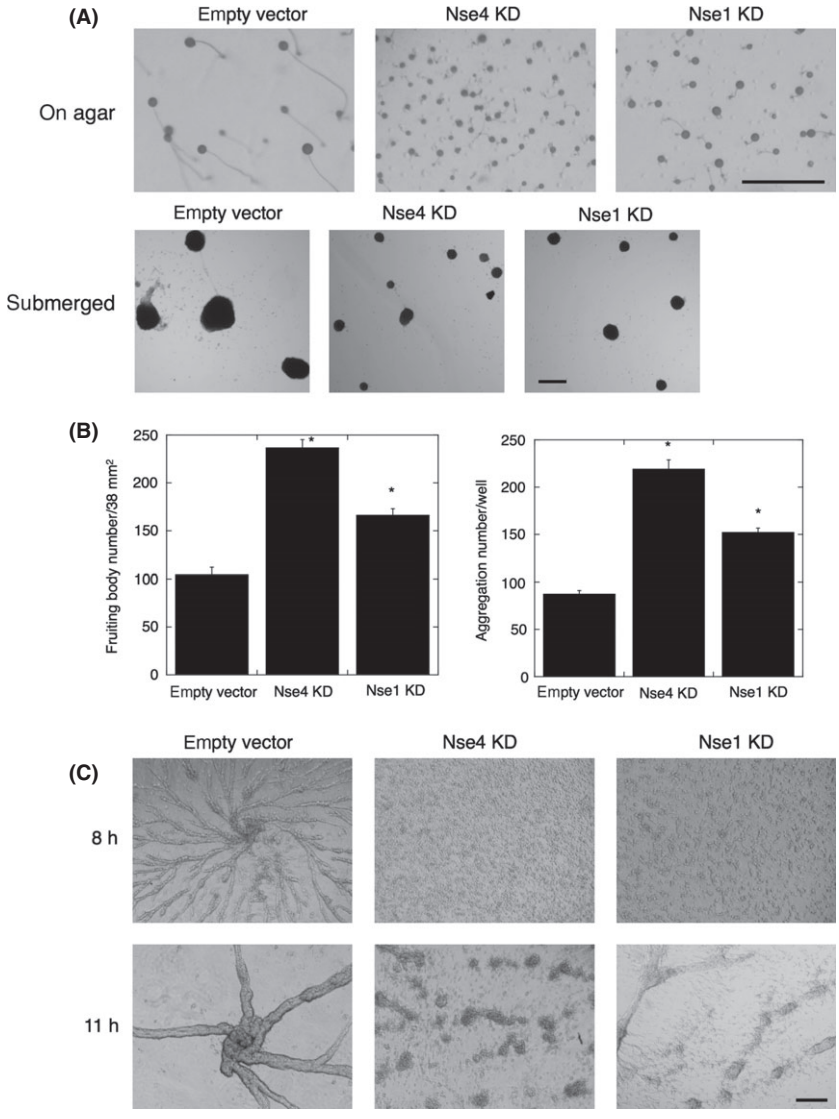


Fig. 7. Nse4 and Nse1 KD cells development upon starvation. (A) (B) Control (empty vector), Nse4 KD (Nse4 KD) cells and Nse1 KD (Nse1 KD) cells were developed on non-nutrient agar and were imaged at 24 h after the onset of starvation. Scale bar: 1 mm (on agar). The number of fruiting bodies that formed on non-nutrient agar plates in a field 38 mm^2 field were counted. Each value represents the mean ± SE (n = 3). The asterisk indicates a value that significantly differs ($P < 0.005$) from the value of the control (empty vector). Control (empty vector), Nse4 KD (Nse4 KD) cells and Nse1 KD (Nse1 KD) cells were developed under submerged culture and were imaged at 24 h after the onset of starvation. Scale bar: 200 μm (submerged). The number of aggregates that formed under submerged culture/well (1.9 cm^2/well) was counted. Each value represents the mean ± SE (n = 8). The asterisk indicates a value that significantly differs ($P < 0.005$) from the value of the control (empty vector). (C) Cell streaming of Nse4 and Nse1 KD cells. Cell streaming of control (empty vector), Nse4 KD (Nse4 KD) cells and Nse1 KD (Nse1 KD) cells were imaged at 8 and 11 h after starvation under submerged culture. Scale bar: 200 μm.

Next, we determined the developmental alterations of pdsA knockdown cells (pdsA KD cells). To knockdown the expression of pdsA, we constructed a plasmid to express a stem-loop RNA directed against pdsA mRNA under the control of the actin 15 promoter (Fig. 9A). We isolated two types of pdsA KD cells, pdsA KD-L (low expression) and pdsA KD-H (high expression) cells. The expression levels of *pdsA* mRNA in pdsA KD-L and KD-H cells were reduced to 10.5% and 29.2%, respectively, compared to that in the control cells that had been transfected with empty vector (Fig. 9B). PdsA KD-L cells showed no cell streaming after 24 h, and a few small aberrant fruiting bodies or a few small aggregates were formed on agar plates or under submerged culture, respectively, by 48 h (Fig. 8C). PdsA KD-H cells also exhibited no cell streaming at 8 h, but displayed a large increase in the number of smaller fruiting bodies that formed on agar plates or aggregates that formed under submerged culture at 24 h after starvation (Fig. 9C,D). PdsA KD exerted effects similar to those of Nse4 KD.

Discussion

In this study, we identified and examined the cellular distribution and binding abilities of Nse1, Nse3 and Nse4 proteins derived from *D. discoideum*. We also have isolated RNAi-mediated Nse4 and Nse1 KD cells and examined their UV-induced cell death sensitivity and developmental alterations upon starvation. We found that Nse3 and Nse4 were enriched in nuclei and that Nse1 was distributed in whole cells. In contrast, all the Smc5–Smc6 complex components including

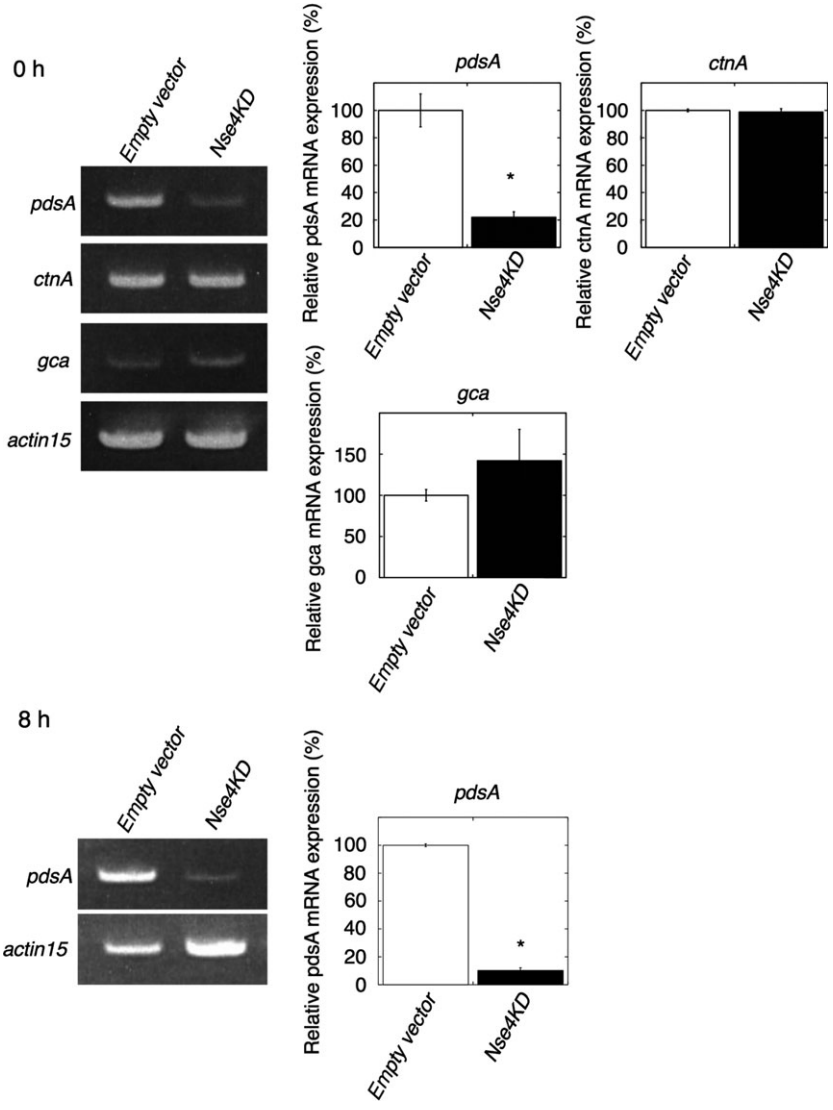
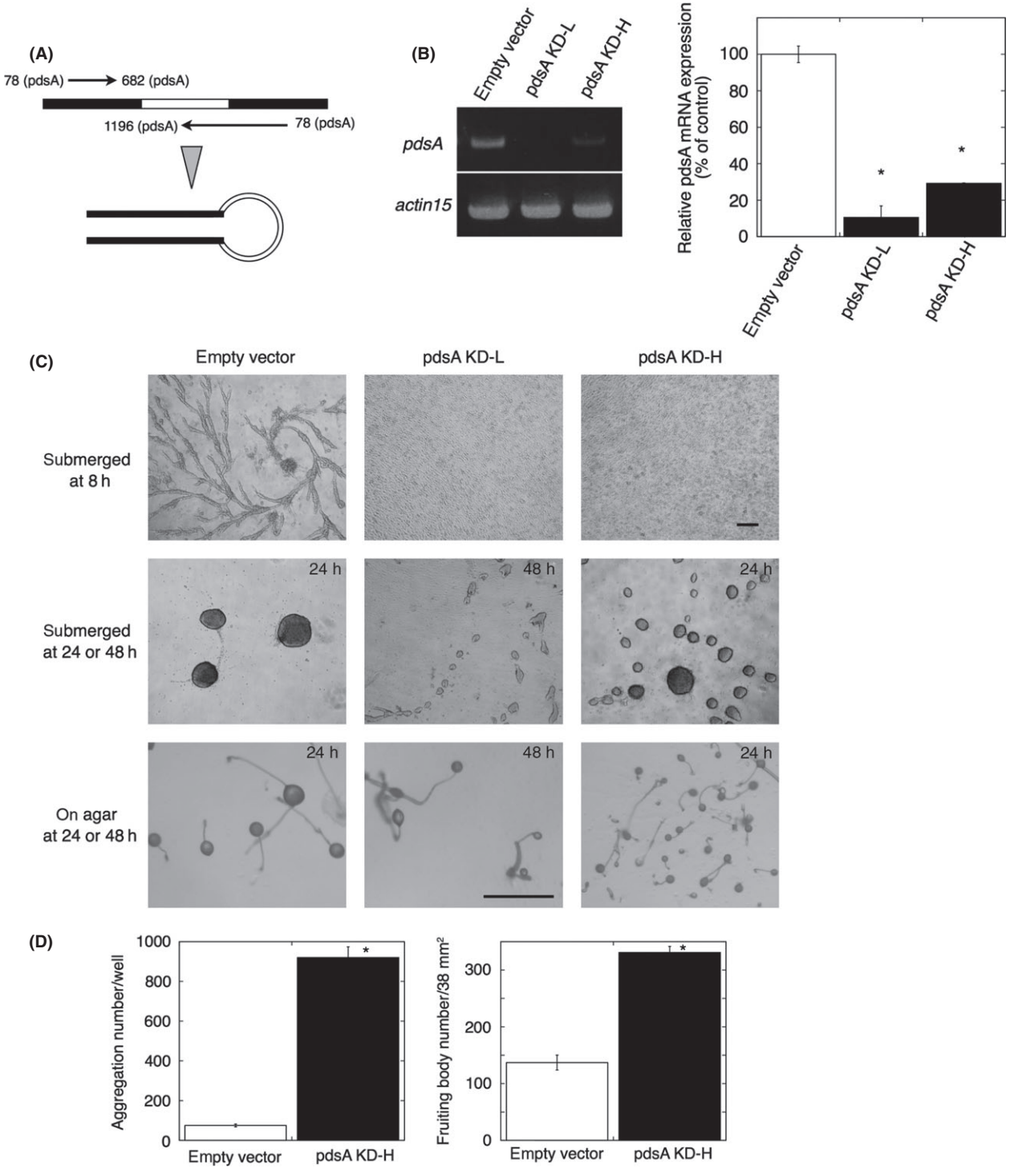


Fig. 8. mRNA expression levels of *pdsA*, *ctnA* and *gca* in Nse4 KD cells. Total RNA extracted from vegetative and 8 h-starved control (empty vector) and Nse4 KD cells under submerged culture was used for oligo(dT)-primed reverse transcription–polymerase chain reaction (RT–PCR). Expression levels of *pdsA*, *ctnA*, *gca* and *actin15* in vegetative cells are shown in the left upper panel (0 h), and the expression levels of *pdsA* and *actin15* in 8 h-starved cells are shown in the left lower panel (8 h). The quantified results were normalized against *actin15* RNA levels and expressed as percentage of the control (right panel). Each value represents the mean ± standard error (SE) ($n = 3$). The asterisk indicates a value that significantly differs ($P < 0.005$) from the value of the control (empty vector).

Fig. 9. PdsA KD cells development upon starvation. (A) Construction of a stem-loop RNA directed against pdsA. One fragment containing nucleotides +78 to +682 of the coding region of pdsA (+1 at the beginning of the ATG start codon; 605 bp) and another fragment containing nucleotides +78 to +1196 of the coding region (1169 bp) were fused in reverse orientation and cloned into the pTX expression vector under the control of the actin 15 promoter. The pdsA-RNAi vector was introduced into Ax-2 cells, and stable transformants were selected with G418. Empty vector was introduced into Ax-2 cells as a control. (B) Reverse transcription–polymerase chain reaction (RT–PCR) analysis of pdsA knockdown cells (pdsA KD-L and pdsA KD-H cells). Total RNA extracted from vegetative cells of control (empty vector), pdsA KD-L and pdsA KD-H cells was used for oligo(dT)-primed RT–PCR (left panel). *Actin15* expression in vegetative cells was used as an internal standard. The quantified results were normalized against *actin15* RNA levels and expressed as percentage of the control (right panel). Each value represents the mean ± standard errpr (SE) ($n = 3$). The asterisk indicates a value that significantly differs ($P < 0.005$) from the value of the control. (C) Development of pdsA KD cells. Control (empty vector), pdsA KD-L (pdsA KD-L) and pdsA KD-H (pdsA KD-H) cells were developed under submerged culture and were imaged at 8 h, and 24 or 48 h after the onset of starvation as indicated. Control (empty vector), pdsA KD-L (pdsA KD-L) cells and pdsA KD-H (pdsA KD-H) cells were also developed on non-nutrient agar plates and were imaged at 24 or 48 h after the onset of starvation as indicated. Scale bars: 200 μm (submerged) and 500 μm (on agar). (D) Quantification of aggregation or fruiting body number. The number of aggregates that formed under submerged culture/well (1.9 cm^2/well) and the number of fruiting bodies that formed on non-nutrient agar plates in a 38 mm^2 field derived from control cells (empty vector) and pdsA KD-H cells were counted. Each value represents the mean ± standard error (SE) ($n = 3$). The asterisk indicates a value that significantly differs ($P < 0.005$) from the value of the control (empty vector).



Nse1, Nse3 and Nse4 proteins are expressed predominantly in nuclei in yeast (McDonald *et al.* 2003; Pebernard *et al.* 2004; Hu *et al.* 2005). Nse1 may play a role in the cytoplasm in *Dictyostelium* cells. Nse1, Nse3 and Nse4 are the non-Smc elements in the Smc5–Smc6 complex, and form a subcomplex in yeast and mammals (Hudson *et al.* 2011). Although only the interaction between Nse3 and Nse1 was detected among the combinations using yeast two-hybrid assays, all of the interactions among the three proteins were recognized by co-immunoprecipitation assay, suggesting that Nse1, Nse3 and Nse4 also

form a complex in *Dictyostelium* cells. GFP-tagged Nse1 was enriched in nuclei when co-expressed with Nse3 or Nse4, suggesting that the Nse1/Nse3/Nse4 complex could play a role in nuclei. However, we could not detect such an altered distribution of GFP-Nse1 in the cells during development or upon UV irradiation (data not shown). Because all the components of the Smc5–Smc6 complex are essential in yeast, we chose an RNAi-mediated knockdown approach for investigating the functional role of Nse proteins. The Smc5–Smc6 complex together with Nse1, Nse3 and Nse4, has been shown to mitigate the effects of various forms of DNA damage in yeast (Harvey *et al.* 2004; Pebernard *et al.* 2004; Hu *et al.* 2005). Consistently, we found that Nse4 and Nse1 KD cells were more sensitive to UV irradiation than were control cells. These results suggest that these Nse proteins derived from *D. discoideum* have characteristics similar to those that had been reported in yeast.

We also found that Nse4 and Nse1 KD cells showed developmental alterations, displaying large increases in the number of smaller fruiting bodies that formed on agar plates or aggregates that formed under submerged culture upon starvation. Developing *Dictyostelium* cells form aggregation streams that break into groups of $\sim 2 \times 10^4$ cells. Fruiting bodies have an optimal number of stalk and spore cells. We observed that Nse4 and Nse1 KD cell streams had delayed formation and aberrant fragmentation, suggesting that this impairment in cell streaming is a primary defect in Nse4 and Nse1 KD cells during development under starvation conditions. We found a reduction in the mRNA level of *pdsA* in Nse4 KD cells under vegetative and 8 h-starved conditions. Aggregate formation is mediated by the cyclic nucleotide cAMP, which serves as a chemoattractant. Starving cells also secrete a phosphodiesterase (*pdsA*), which degrades the extracellular cAMP signal to bring its concentration back to a basal level in the interval between pulses. The generated cAMP waves help the cells to form aggregation streams (Jang & Gomer 2008). Null mutants of the pdsA gene have streaming defects, and do not aggregate or enter multicellular development (Sucgang *et al.* 1997; Garcia *et al.* 2009). Therefore, we examined the developmental alterations of pdsA KD cells to determine whether they show a defect similar to Nse4 KD cells. PdsA KD-L cells (low expression clone) formed no cell streaming after 24 h and developed a few small aberrant fruiting bodies on agar plates after 48 h upon starvation conditions. This finding is consistent with the predicted phenotype based on the pdsA mutants (Sucgang *et al.* 1997). PdsA KD-H cells (high expression clone) also exhibited impaired cell streaming but a less severe phenotype. These cells displayed a large increase in the number of smaller fruiting bodies that formed on agar plates or aggregates that formed under submerged culture at 24 h after starvation. This finding suggests that the reduction of pdsA expression is a primary cause of the developmental alteration of Nse4 KD cells. High levels of phosphodiesterase have been shown to decrease cAMP pulse size, resulting in cells forming small aggregates (Faure *et al.* 1988). The amplitude of the cAMP wave generated by pdsA may be important for aggregation size. Neither the overexpression of Nse1, Nse3 or Nse4 nor triple overexpression of the three proteins caused any developmental alterations upon starvation (data not shown), suggesting that the upregulation of the pdsA expression above endogenous levels by Nse protein overexpression had no effect. Nse3 is related to the MAGE family of proteins (De Piccoli *et al.* 2009; Hudson *et al.* 2011), and Nse4 is related to the EID family of transcriptional repressors (Palecek *et al.* 2006; Hudson *et al.* 2011). Although the EID and the MAGE family proteins have been reported to interact with transcription factors and to be involved in mammalian cell differentiation (MacLellan *et al.* 2000; Kuwajima *et al.* 2006), this report is the first to demonstrate that Nse proteins, components of the Smc5–Smc6 complex, regulate gene expression. MAGE- and EID-like function in Nse proteins may have been preserved during evolution. *pdsA* gene expression is controlled by promoters that are specific to vegetative growth, aggregation or late development (Faure *et al.* 1990; Hall *et al.* 1993). Our results suggest that the Nse4 expression level affects the activity of both of vegetative growth- and aggregation-specific promoters. According to one report, the growth-specific promoter activity is highest in vegetative cells and rapidly disappears in starving cells. In contrast, the aggregation-specific promoter activity increases during aggregation (Faure *et al.* 1990). Because the aggregation-specific promoter is inducible by cAMP, its promoter activity is affected by other cAMP signaling molecules. We cannot exclude the possibility that the Nse4 protein also contributes to the regulation of other cAMP-related genes. The Nse1/Nse3/Nse4 subcomplex or the Smc5–Smc6 complex in *Dictyostelium* cells may regulate the gene expression through interactions with promoter-specific transcriptional factors and/or transcription co-activators. Smc5 or Smc6 knockdown experiments will help to determine whether Nse proteins play a role in the development similar to that of the Smc5–Smc6 complex. We also prepared Nse3 knockdown mutants (Nse3 KD cells); however, because developmental alteration upon starvation of Nse3 KD cells was not stable, we could not include these results in this manuscript. A short period after selecting the colonies,

Nse3 KD cells showed the same phenotype as Nse4 KD cells; however, soon after passages began, this phenotype disappeared. Further studies are necessary to understand how Nse proteins regulate gene expression and integrate between DNA repair and cell development functions.

References

Brock, D. & Gomer, R. H. 1999. A cell-counting factor regulating structure size in *Dictyostelium*. *Genes Dev.* **13**, 1960–1969.

De Piccoli, G., Torres-Rosell, J. & Aragon, L. 2009. The unnamed complex: what do we know about Smc5-Smc6? *Chromosome Res.* **17**, 251–263.

Faure, M., Podgorki, G. J., Franke, J. & Kessin, R. H. 1988. Disruption of *Dictyostelium discoideum* morphogenesis by overproduction of cAMP phosphodiesterase. *Prod. Natl. Acad. Sci. USA* **85**, 8076–8080.

Faure, M., Franke, J., Hall, A. L., Podgorski, G. J. & Kessin, R. H. 1990. The cyclic nucleotide phosphodiesterase gene of *Dictyostelium discoideum* contains three promoters specific for growth, aggregation, and late development. *Mol. Cell. Biol.* **10**, 1921–1930.

Garcia, G. L., Rericha, E. C., Heger, C. D., Goldsmith, P. K. & Parent, C. A. 2009. The group migration of *Dictyostelium* cells is regulated by extracellular chemoattarctant degradation. *Mol. Biol. Cell* **20**, 3295–3304.

Hall, A. L., Franke, J., Faure, M. & Kessin, R. H. 1993. The role of the cyclic nucleotide phosphodiesterase of *Dictyostelium discoideum* during growth, aggregation and localization studies with the separate promoters of the *pde*. *Dev. Biol.* **157**, 73–84.

Harvey, S. H., Sheedy, D. M., Cuddihy, A. R. & O'Connell, M. 2004. Coodination of DNA damage responses via the Smc5/Smc6 complex. *Mol. Cell. Biol.* **24**, 662–674.

Hu, B., Liao, C., Millson, S. H., Mollapour, M., Prodromou, C., Pearl, L. H., Piper, P. W. & Panaretou, B. 2005. Qri2/Nse4, a component of the essential Smc5/6 DNA repair complex. *Mol. Microbiol.* **55**, 1735–1750.

Hudson, J. J. R., Bednarova, K., Kozakova, L., Liao, C., Guerineau, M., Colnaghi, R., Vidot, S., Marek, J., Bathula, S. R., Lehmann, A. R. & Palecek, J. 2011. Interactions between the Nse3 and Nse4 components of the SMC5-6 complex identify evolutionaly conserved interactions between MAGE and EID families. *PLoS ONE* **6**, e17270.

Jang, W. & Gomer, R. H. 2008. Combining experiments and modelling to understand size regulation in *Dictyostelium discoideum*. *J. R. Soc. Interface* **5**, S49–S58.

Kuwajima, T., Nishimura, I. & Yoshikawa, K. 2006. Necdin promotes GABAergic neuron differentiation in cooperation with Dlx homeodomain proteins. *J. Neurosci.* **26**, 5383–5392.

Levi, S., Polyakov, M. & Egelhoff, T. T. 2000. Green fluorescent protein and epitope tag fusion vectors for *Dictyostelium discoideum*. *Plasmid* **44**, 231–238.

MacLellan, W. R., Xiao, G., Abdellarif, M. & Schreider, M. D. 2000. A novel Rb- and p300-binding protein inhibits transactivation by MyoD. *Mol. Cell. Biol.* **20**, 8903–8915.

Martens, H., Novotny, J., Oberstrass, J., Steck, T. L., Postlethwait, P. & Nellen, W. 2002. RNAi in Dictyoatelium: the role of RNA-directed RNA polymerase and double-stranded RNase. *Mol. Biol. Cell* **13**, 445–453.

McDonald, W. H., Pavlova, Y., Yates, J. R. III & Boddy, M. N. 2003. Novel essential DNA repair proteins Nse1 and Nse2 are subunits of the fission yeast Smc5–Smc6 complex. *J. Biol. Chem.* **278**, 45460–45467.

Palecek, J., Vidot, S., Feng, M., Doherty, A. J. & Lehmann, A. R. 2006. The Smc5-Smc6 DNA repair complex bridging of the Smc5-Smc6 heads by the Kleisin, Nse4 and non-Kleisin subunits. *J. Biol. Chem.* **281**, 36952–36959.

Pebernard, S., McDonald, W. H., Pavlova, Y., Yates, J. R. III & Boddy, M. N. 2004. Nse1, Nse2, and a novel subunit of the Smc5-Smc6 complex, Nse3, play a crucial role in meiosis. *Mol. Biol. Cell*, **15**, 4866–4876.

Sucgang, R., Weijer, C. J., Siegert, F., Franke, J. & Kessin, R. H. 1997. Null mutations of the *Dictyostelium* cyclic nucleotide phosphodiesterase gene block chemotactic cell movement in developing aggregates. *Dev. Biol.* **192**, 181–192.

Taniura, H., Sanada, N., Kuramoto, N. & Yoneda, Y. 2006. A metabotropic glutamate receptor family gene in *Dictyostelium discoideum*. *J. Biol. Chem.* **281**, 12336–12343.

Veltman, D. M. & van Haastert, P. J. M. 2008. The role of cGMP and the rear of the cell in *Dictyostelium* chemotaxis and cell streaming. *J. Cell Sci.* **121**, 120–127.