Molecular anatomy of tunicate senescence: reversible function of mitochondrial and nuclear genes associated with budding cycles

Kaz Kawamura1,*, Seigo Kitamura1, Satoko Sekida2, Masayuki Tsuda3 and Takeshi Sunanaga1

SUMMARY
Zooids of the asexual strain of Polyandrocarpa misakiensis have a lifespan of 4-5 months; before dying, they produce many buds, enabling continuation of the strain. This study was designed to investigate the nature of gene inactivation and reactivation during this continuous process of senescence and budding. During senescence, the zooidal epidermis showed acid β-galactosidase activity, lost proliferating cell nuclear antigen immunoreactivity and became ultrastructurally worn, indicating that the epidermis is a major tissue affected by the ageing process. Semi-quantitative PCR analysis showed that the genes encoding mitochondrial respiratory chains (MRCs) engaged in decreased transcriptional activity in senescent adults compared with younger adults. The results of in situ hybridization showed that the epidermis dramatically attenuates MRC expression during ageing but restores gene activity when budding commences. During budding and ageing, the nuclear gene Eed (a polycomb group component) was activated and inactivated in a pattern similar to that observed in MRCs. In buds, RNA interference (RNAi) of Eed attenuated Eed transcripts but did not affect the gene expression of pre-activated MRCs. A tunicate humoral factor, TC14-3, could induce inactivation of Eed in adult zooids. When RNAi of Eed and Eed induction were performed simultaneously, zooidal cells and tissues failed to engage in MRC reactivation, indicating the involvement of Eed in MRC activation. Results of this study provide evidence that the mitochondrial gene activities of Polyandrocarpa can be reversed during senescence and budding, suggesting that they are regulated by nuclear polycomb group genes.

KEY WORDS: Budding, Eed, Mitochondria, Polycomb group, Senescence, Tunicate, Polyandrocarpa, P. misakiensis

INTRODUCTION
All eukaryotes inevitably undergo senescence, which is defined as the time-related deterioration of physiological functions necessary for survival and fertility (Gilbert, 2000; Kirkwood, 2005). Functional deterioration related to senescence can occur at various biological levels. At the tissue level, senescence results in deterioration of homeostatic tissue maintenance (Beerman et al., 2010) and regeneration (Timchenko, 2009). At the cellular level, senescence results in decreased cell proliferation in response to mitogen exposure, injury and other stresses (Iakova et al., 2003). In mammals, senescence-associated β-galactosidase (SA-Gal) is a reliable marker of ageing-related cell growth arrest (Ilihana et al., 2007). At the organelle level, senescence accompanies the decrease in mitochondrial respiratory chain (MRC) activity (Byrne et al., 1991). It has been confirmed that one of the main causes of such functional deterioration in mitochondria is the time-related accumulation of DNA damage (Hebert et al., 2010).

Polycomb group (PcG) proteins are multimeric and multifunctional proteins involved in histone modification, a process that leads to genome-wide gene repression (Kuzmichev et al., 2005) and plays a central role in the maintenance of embryonic stem cells (Rajasekhar and Begemann, 2007) and hematopoietic stem cells (Konuma et al., 2010). The primary PcG targets have been considered to be Hox genes; this has now extended to homeodomain-containing and other types of transcriptional regulators (Boyer et al., 2006). In the epidermis (keratinocytes), PcG proteins regulate cell growth, differentiation and senescence (Eckert et al., 2011). In a recent study, we found that TC14-3, a tunicate cytosolic factor, exerts cell growth-inhibitory activity via the gene embryonic ectoderm development (Eed), a PcG component (Kawamura et al., 2012).

Polyandrocarpa misakiensis belongs to the family Styelidae (Ascidiae, Pleurogona). However, unlike Styela and its other solitary relatives, P. misakiensis can propagate by budding (Fig. 1A, 1) (Kawamura et al., 2008a). Buds arise as a double-walled vesicle that protrudes from the parental epidermis and atrial epithelium (Fig. 1A, 2). Differentiated and undifferentiated coelomic mesenchymal cells are located between these outer and inner epithelia (Kawamura and Sunanaga, 2010). Morphogenesis occurs mainly in the inner multipotent epithelium after the buds are detached from the parent (Fig. 1A, 3) (Kawamura and Nakauchi, 1986; Kawamura and Fujinara, 1994). In ∼1 week, these buds become juvenile zooids (Fig. 1A, 4). Approximately 1 month later, the zooids begin to undergo their own budding (Fig. 1A, 1), producing a maximum of 30-40 buds (Oda and Watanabe, 1986) 4-5 months before dying by autolysis (Fig. 1B). The newly formed zooids produced by this process live for an additional 4-5 months before dying themselves. By ensuring the continuation of this process, we have been able to maintain asexual Polyandrocarpa strains in Japanese marine stations since 1970 (Fig. 1B) (Kawamura, 1984), when the animals were first collected along the coast of Sagami Bay (Watanabe and Tokioka, 1972). It is interesting to ask how zooidal senescence proceeds over the 4- to 5-month lifespan and how and to what extent senescence events can be reset at each budding event.

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This study aimed to fulfill four primary goals. The first goal was to determine whether the epidermis is one of the major tissues affected by the ageing process. In doing so, we examined the activity of acid β-galactosidase, referred to as SA-Gal in mammals (Ithana et al., 2007), the nature of cell proliferation and the ultrastructural cell aspects from budding to zooid senescence in *P. misakiensis*. The second goal was to elucidate the gene activity of MRCs, such as NADH dehydrogenase subunit 1 (complex I) and cytochrome c oxidase subunit 1 (complex IV), to demonstrate that the transcriptional activity of these genes in the epidermis declines conspicuously during senescence but can be restored during budding. The third goal was to identify nuclear genes that change during budding and senescence with a gene expression pattern similar to that of MRCs. The final goal was to explore whether MRCs could be regulated by upregulating or downregulating *PmEed* gene expression. Results of this study are discussed in the context of novel nuclear and mitochondrial gene interactions in tunicate senescence events.

### MATERIALS AND METHODS

#### Nomenclature

In this study, the term bud refers only to a growing 'bud' still attached to its parent (Fig. 1A, 1; Fig. 2C), a 'juvenile zooid' to a 3- to 4-week-old zooid that has not yet begun to bud (Fig. 1A, 4; Fig. 2B) and a 'senescent zooid' to a zooid aged 18 weeks or older (Fig. 2E). In some experiments, 'developing buds' were additionally used. They separate from the parent (Fig. 1A, 2) and commence morphogenesis (Fig. 1A, 3).

#### Animals

Asexual strains of *P. misakiensis* (Kawamura, 1984) that had settled in Uranouchi Inlet near the Usa Marine Biological Institute (Kochi University) were cultured on glass slides in culture boxes.

#### Acid β-galactosidase assay

A modified conventional procedure was used to perform enzyme histochemistry (Choi et al., 2000; Ithana et al., 2007). Animals were fixed in Zamboni’s fixative (Zamboni and DeMartino, 1967) for 30 minutes to 1 hour on an ice bath. After thorough washing in chilled phosphate-buffered saline (PBS, pH 7.4), specimens were pre-incubated twice for 10 minutes in 20 mM phosphate buffer (PB, pH 6.0) or 20 mM Na-citrate buffer (pH 6.0). Samples were incubated in a staining solution (3 mM K-ferrocyanide, 3 mM K-ferricyanide, 150 mM NaCl, 1 mM MgCl2, and 0.1% X-gal in the same buffer) overnight at 37°C.

#### Immunohistochemistry

Animals were fixed in 4% paraformaldehyde in PBS (pH 7.4) for 1 hour in an ice bath, and then serially dehydrated and infiltrated in a plastic resin Technovit 8100 (Heraeus Kulzer, Wehrheim, Germany) for more than 10 hours at 4°C. After hardening, samples were sectioned at 2 μm with glass knives. Sections were pre-treated with 0.01% trypsin in 20 mM Tris Cl (pH 7.6) containing 0.1% CaCl2 for 10 minutes at 30°C, and then with the blocking solution (0.5% skimmed milk in PBS) for 10 minutes at room temperature. Anti-proliferating cell nuclear antigen (anti-PCNA) primary antibody was raised in rabbit using recombinant PCNA from *Botryllus primigenus* as an immunogen and anti-rabbit secondary antibody labelled with horseradish peroxidase (HRP) was purchased from Vector Labs (Burlingame, CA, USA). After antibody staining, sections were washed with PBS containing 0.1% Tween 20 (PBST), and the specimens then coloured with Trueblue dye (KPL, Baltimore, MD, USA). In some sections, the nucleus was counterstained for 10 minutes with 4’,6-diamidino-2-phenylindole (DAPI, 5 μg/ml).

#### Electron microscopy

Glutaraldehyde (70%) was diluted 30-fold with 0.1 M PB (pH 7.4) containing 1.5% NaCl. The specimens were prefixed with the fixative for 2 hours on ice, and then postfixed with 1% osmium tetroxide in the same buffer for 1 hour on ice. After postfixation, the specimens were rinsed several times with PB containing 1.5% NaCl, dehydrated with a graded acetone series and embedded in Spurr’s resin. The resulting resin blocks were sectioned with a diamond knife on an Ultracut UCT ultramicrotome (Leica, Vienna, Austria). Sections were mounted on formvar-coated grids and stained with 1% aqueous uranyl acetate for 1 hour and with lead citrate for 15 minutes in sequence at room temperature. The samples were observed with a Jeol JEM-1400 transmission electron microscope (JEOL, Tokyo, Japan) and photographed using a charge coupled device (CCD) camera.

#### Reverse-transcription PCR

Poly (A)† RNA was extracted and purified from buds, juvenile zooids and senescent adult zooids using the biotinyl magnet method according to the manufacturer’s protocol (Roche, Mannheim, Land Baden-Württemberg, Germany). Single-stranded DNA complementary to poly (A)† RNA was synthesized for 1 hour at 42°C by StrataScript reverse transcriptase (Stratagene, Santa Clara, CA, USA). Reverse-transcription PCR (RT-PCR) was performed in two steps: one cycle for sense-strand synthesis (30 seconds at 94°C, 2 minutes at 52°C and 2 minutes at 72°C) and 20-30 cycles of denaturation for 30 seconds at 94°C, annealing for 60 seconds at 52°C and extension for 90 seconds at 72°C. As an internal standard, β-actin cDNA was amplified by PCR.
In situ hybridization
In situ hybridization was performed according to a method previously described (Sunanaga et al., 2007). In brief, after fixation and proteinase K treatment, specimens were hybridized with the digoxigenin (Dig)-labelled antisense ribonucleotide probe for 12-14 hours at 58°C before blocking in 1% skimmed milk in Tris-buffered salt solution containing 0.1% Tween 20 (TBST) for 6 hours on an ice bath. The samples were then treated overnight with anti-Dig monoclonal antibody labelled with alkaline phosphatase (Roche) on ice before being stained with a colour-development solution, dehydrated and embedded in Technovit 8100 resin. Sections were then prepared as described above.

RNA interference
Three different siRNAs were designed from PmEed mRNA and purchased from Sigma-Proligo (Tokyo, Japan). Their oligonucleotide sequences were as follows: siRNA-1, 5’-AUGCAAAAACGAAUCGGUUU-3’ and 5’-AGGGAUAAGGAUUUUACCUU-3’; siRNA-2, 5’-AUAUUAGAAUUUUCUUCUU-3’ and 5’-GAUGGAAUAUUCUAAUUUGU-3’; siRNA-3, 5’-AAACCAUAAAGCAUUUCAACCUU-3’ and 5’-GGUUUGAUAUGCUAUUGGUCU-3’.

The siRNA oligonucleotides were dissolved in filtered seawater. Immediately before use, the same volume of each of the three solutions was mixed at a final concentration of 50 µM. The mixed siRNA was incubated for more than 20 minutes in seawater containing lipofectamine 2000 (10:1; Invitrogen, Carlsbad, CA, USA). Animals were injured with razor blades and treated with the liposome solution for 30 minutes in moist chambers and then returned to the culture water.

RESULTS
Acid β-galactosidase activity in Polyandrocarpa
In 1-week-old zooids, no overall acid β-galactosidase activity was detected at pH 6.0, except in the digestive tract (Fig. 2A), where stomach tissue displayed the strongest enzymatic activity (Fig. 2F). This activity in the stomach could be considered the functioning of a digestive enzyme rather than that of acid β-galactosidase. In 4-week-old juvenile zooids immediately prior to budding, the posterior half was observed to have weak enzymatic activity (Fig. 2B). In sections, the pharynx contained many small dotted signals (Fig. 2G), and the epidermis and coelomic mesenchymal cells did not possess as yet any substantial signals (Fig. 2H). In 9-week-old zooids, budding was observed in many areas (Fig. 2C), and although acid β-galactosidase activity appeared to be strong throughout the zooidal body, no staining was observed anywhere in the buds (Fig. 2C, arrowheads). Under neutral pH, β-galactosidase staining entirely disappeared from the zooids, except in the digestive tract (Fig. 2D). In 18-week-old zooids, budding activity was conspicuously reduced (Fig. 2E). The zooid itself was heavily stained, whereas one of small diverticula protruding from the body was not stained (Fig. 2E, arrowhead). In sections, the zooid epidermis had strong enzyme activity (Fig. 2I).

PCNA expression during senescence
In buds, strong PCNA signals were detected in the epidermal nuclei (Fig. 3A,B). Signals were also detected in the atrial epithelium (Fig. 3C) and coelomic cells (Fig. 3D), although they were not detected in the morula cells, a type of differentiated blood cell (Fig. 3D, arrowheads). When buds separated from the parent (Fig. 1A, 2), most signals once disappeared from bud tissues (Fig. 3E), and they reappeared at the morphogenetic area of developing buds (Fig. 3F). In juvenile zooids, the epidermal PCNA signals attenuated (Fig. 3G, arrowheads) and became almost undetectable in senescent zooids (Fig. 3I), although proliferative signals were still observable in the atrial epithelial and coelomic cells of juvenile zooids (Fig. 3H) and in a region of coelomic cells of senescent zooids (Fig. 3J). Fig. S1 in the supplementary material shows immunostained pictures merged with DAPI staining. In epidermal cells of growing bud (supplementary material Fig. S1A,B), a total of 21 nuclei out of 24 (87.5%) had strong (black arrowheads) or moderate (grey arrowheads) PCNA signals. In a juvenile zooid (supplementary material Fig. S1C,D), four epidermal nuclei out of 16 were labelled (25%), and in a senescent zooid (supplementary material Fig. S1E,F) only three nuclei out of 41 were moderately labelled (7.3%). Results are statistically summarized in Table 1. In contrast to epidermal cells, the atrial epithelium kept the high score of labelling from buds to juvenile zooids. Coelomic cells also kept relatively high score on average, but the values were variable among sections, depending on the number of co-existing morula cells.

Epidermal morphology during senescence
The bud epidermis was observed to have a cuboidal shape (Fig. 4A,B) and the cytoplasm to contain electron-lucent granules of various sizes (Fig. 4B), which are known to be thepigmented granules that give the epidermis of Polyandrocarpa its orange colour (Kawamura and Fujiwara, 1994). Golgi bodies were found to have developed on the apical side facing the tunic (Fig. 4B),
Fig. 3. PCNA expression at different ageing stages, anti-PCNA immunohistochemistry. (A-D) Growing buds. (A) Lower magnification. Scale bar: 100 μm. (B) Epidermis. Scale bar: 25 μm. (C) Atrial epithelium. Scale bar: 25 μm. (D) Coelomic cells. Arrowheads show morula cells having no PCNA signals. Scale bar: 25 μm. (E,F) Buds after separation from the parent, merged with DAPI images. (E) Epidermis and coelomic cells. A single epidermal cell (arrowhead) had PCNA signal. Scale bar: 25 μm. (F) Morphogenetic area of 2-day-old developing bud. Note that PCNA signals reappear. Scale bar: 25 μm. (G,H) Juvenile zooids. (G) Epidermis. Several nuclei (arrowheads) were PCNA positive. Nonspecific signals were detected in the cytoplasm of body wall muscle. Scale bar: 25 μm. (H) Atrial epithelium and coelomic cells. Many cells were still PCNA positive. Scale bar: 25 μm. (I,J) Senescent zooids. (I) Epidermis. No signals were observable. Scale bar: 25 μm. (J) Pharynx. Coelomic cells in cluster showed the PCNA signal. Scale bar: 50 μm. a, atrial epithelium; c, coelomic cells; e, epidermis; m, body muscle cell.

whereas most mitochondria were located on the opposing basal side (Fig. 4B). At the cell boundary, the intercellular junction was observed at the apical end (Fig. 4C, arrowhead), and the projections from adjacent cells were tightly interdigitated in the middle (Fig. 4C, star). In juvenile zooids, epidermal cells appeared slender to some extent (Fig. 4D). The cytoplasm was similar to that of the bud epidermis (Fig. 4E). Mitochondria were observed at the basal and middle areas, and the interdigitated junction became loose at the cell boundary (Fig. 4F, star). In senescent zooids, the epidermal cells were observed to have assumed a slender, conical shape (Fig. 4G,H). The adjacent cells lost the indented junction and adhered to one another only by the intercellular junctions, leading to wide spreading of the intercellular space (Fig. 4H). Lysosomes were abundant in the cytoplasm (Fig. 4H). The epidermal mitochondria appeared normal in morphology but the matrix became electron dense in the senescent epidermis, when compared with that of bud and juvenile zooids (Fig. 4I).

When the number of epidermal mitochondria was counted on micrographs of the sections (supplementary material Table S1), it was found to be 8.9±1.4 per 20 μm² in growing buds (Fig. 4B,C), 6.9±1.9 per 20 μm² in juvenile zooids (Fig. 4E,F) and 6.2±2.0 per 20 μm² in senescent adults (Fig. 4H,I).

MRC gene expression during senescence

The cDNA for cytochrome c oxidase subunit 1 (PmCOX1, AB591739) was 1526 bp long and it encoded 507 amino acid residues. The cDNA for NADH dehydrogenase subunit 1 (PmND1, AB591740) was 915 bp long, encoding 304 amino acid residues. The RT-PCR analyses showed that PmCOX1 and PmND1 transcripts were abundant in buds and juvenile zooids but remarkably weak in senescent adults (supplementary material Fig. S2).

Spatiotemporal gene expression of MRCs was examined by in situ hybridization. In growing buds, PmCOX1 was widely expressed in the epidermis (Fig. 5A,B), coelomic cells and the atrial epithelium (Fig. 5C). Juvenile zooids showed similar but somewhat lower staining of the epidermis, atrial epithelium and coelomic cells (Fig. 5D-F). In senescent zooids, the epidermal signal declined to an undetectable level (Fig. 5G). In the coelomic space, signals attenuated, but in places they remained in cell clusters (Fig. 5H). Among differentiated tissues, PmCOX1 was weakly expressed in the oesophagus, stomach and intestine (supplementary material Fig. S3A-D). In the gonad, germline precursor cells and oocytes continuously expressed PmCOX1 (supplementary material Fig. S3E,F).

To determine whether decreased PmCOX1 signals in the epidermis was due to artefactual infiltration of probes in adult tissues, the expression of Pmβ-Catenin was examined in senescent adult zooids as a positive control. The epidermis and coelomic cells emitted clear signals (Fig. 5I).

Fig. S4 in supplementary material shows PmND1 expression. In buds, the epidermis was stained most strongly and extensively (supplementary material Fig. S4A,B). Coelomic cells and the atrial epithelium were also stained (supplementary material Fig. S4C). In juvenile zooids, epithelial tissues and coelomic cells were stained to an extent similar to that of buds (supplementary material Fig. S4D-F). In senescent adults, signals became very weak in the epidermis, atrial epithelium, coelomic cells, pharynx and endostyle (supplementary material Fig. S4G-K). In the gonad, punctuated strong signals emitted from the oocyte cytoplasm (supplementary material Fig. S4L).

MRC reactivation during budding

Fig. 6A shows whole-mount staining of PmCOX1 in an adult zoid piece that has two protruding buds. In the bud primordium (Fig. 6A, right open box), the epidermis already has a strong signal, as demarcated by arrowheads from the parent epidermis (Fig. 6B,C). Signals were also evident in coelomic cells (Fig. 6D). In growing buds, the epidermal slope (Fig. 6A, left open box) showed a gradual increase in signal from the adult zoid (with no signal) to the bud (with a strong signal) (Fig. 6E). The bud primordium could also
cells were heavily stained (Fig. 7J). The germinal vesicle of young oocyte, follicle cells and small coelomic other tissues, except the gonad (Fig. 7H-J). In the gonad, the adults, however, the signal disappeared from the epidermis and most

7F, arrowhead) and pharyngeal epithelium (Fig. 7G). In senescent undifferentiated cells (Fig. 7E, broken circle), gonad rudiment (Fig. 7F), arrowhead) but not from morula cells (Fig. 7C). The expression was expressed in juvenile zooids (Fig. 7D-G), especially in the coelomic cytoplasm (Fig. 7B, arrowheads) and from granular cells (Fig. 7B,C, arrows) but not from morula cells (Fig. 7C). The expression was maintained in juvenile zooids (Fig. 7D-G), especially in the coelomic undifferentiated cells (Fig. 7E, broken circle), gonad rudiment (Fig. 7F, arrowhead) and pharyngeal epithelium (Fig. 7G). In senescent adults, however, the signal disappeared from the epidermis and most other tissues, except the gonad (Fig. 7H-J). In the gonad, the germinal vesicle of young oocyte, follicle cells and small coelomic cells were heavily stained (Fig. 7J).

**PmEed expression during senescence and budding**

A cDNA for Eed (PmEed, AB617630), a Polyandrocarpa polycomb group component, was 920 bp long and its deduced amino acid sequence was most similar to that of Ciona Eed on the molecular phylogenetic tree (supplementary material Fig. S5). The RT-PCR analyses showed that in senescent adults, the PmEed expression was substantially less than that of buds and juvenile zooids (supplementary material Fig. S2). By in situ hybridization of buds, the epidermis, atrial epithelium and coelomic cells extensively expressed PmEed (Fig. 7A-C). Signals in the coelomic space mainly emitted from undifferentiated cells with a large nucleus and narrow cytoplasm (Fig. 7B, arrowheads) and from granular cells (Fig. 7B,C, arrows) but not from morula cells (Fig. 7C). This expression was maintained in juvenile zooids (Fig. 7D-G), especially in the coelomic undifferentiated cells (Fig. 7E, broken circle), gonad rudiment (Fig. 7F, arrowhead) and pharyngeal epithelium (Fig. 7G). In senescent adults, however, the signal disappeared from the epidermis and most other tissues, except the gonad (Fig. 7H-J). In the gonad, the germinal vesicle of young oocyte, follicle cells and small coelomic cells were heavily stained (Fig. 7J).

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**Table 1. The number of PCNA-expressing nuclei in the epidermis, atrial epithelium and coelomic cells during budding and senescence of *P. misakiensis***

<table>
<thead>
<tr>
<th>Tissues</th>
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<th>Epidermis</th>
<th>Atrial epithelium</th>
<th>Coelomic cells</th>
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<tbody>
<tr>
<td>Buds</td>
<td>125*</td>
<td>84.8±3.25</td>
<td>74.5±4.4</td>
<td>45.2±15.5</td>
</tr>
<tr>
<td>Juvenile zooids</td>
<td>100</td>
<td>28.1±3.5</td>
<td>75.5±8.2</td>
<td>40.6±15.8</td>
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<tr>
<td>Senescent zooids</td>
<td>100</td>
<td>7.5±0.5</td>
<td>10.1±5.6</td>
<td>25.6±16.5</td>
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*Number of sections examined.

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**Effect of PmEed on mitochondrial gene expression**

When buds were treated with siRNA(PmEed), PmEed signals conspicuously diminished from the buds (Fig. 8A-C) and, inversely, PCNA signals were extended ectopically from morphogenetic, proximal area of bud (31 nuclei out of 38; Fig. 8D,E,F') toward the non-morphogenetic distal area (12 nuclei out of 26; Fig. 8D,F,F') through the lateral body wall (Table 2). The results indicated that the RNAi of PmEed efficiently acted on buds. By contrast, the siRNA(PmEed) treatment was found not to attenuate the gene expression of PmCOX1 at the proximal and distal areas of bud (Fig. 8G-I), indicating that RNAi has no effect on mitochondrial gene activity that has already been enhanced during budding.

A recent study has suggested that TC14-3, a tunicate cytostatic factor, is able to induce gene expression of both PmEed and PmCOX1 in adult zooids (Kawamura et al., 2012). Similar results were obtained in the present study (supplementary material Fig. S6; Fig. 9A). Further experiments were designed to disclose whether or not siRNA(PmEed) is able to cancel TC14-3-induced enhancement of PmCOX1 gene expression in Polyandrocarpa adult zooids. In the negative control, in which zooids were only cut into pieces, both PmEed and PmCOX1 showed faint signals by RT-PCR (Fig. 9A, lane 1). By in situ hybridization, zooid pieces did not exhibit PmCOX1 signal (Fig. 9B,C). In the positive control,
zooid pieces were treated with TC14-3. They expressed both genes of PmEed and PmCOX1 (Fig. 9A, lane 2). The pharynx and coelomic cells strongly expressed PmCOX1 (Fig. 9D-F), whereas epidermal cells showed moderate signals in some places and no signals in other places on the same section (Fig. 9G,H), which would be because of the low infiltration of TC14-3 within zooid tissues. However, when the TC14-3-treated zoooid pieces were further treated with siRNA(PmEed), the induction of PmCOX1 was suppressed to a large extent (Fig. 9A, lane 3). The epidermis and pharynx did not show PmCOX1 signals (Fig. 9I), and coelomic cells showed very weak signals (Fig. 9J), suggesting that PmEed is necessary for TC14-3-mediated mitochondrial gene activation.

DISCUSSION
Impact of ageing on epidermal tissue in P. misakiensis

In mammals, acid β-galactosidase, which works efficiently in the lysosome at pH 6.0 (Lee et al., 2006), is a reliable marker of senescent cells (Itahana et al., 2007). In P. misakiensis, acid β-galactosidase activity became histochemically stronger with ageing, although, under neutral pH, it disappeared from all tissues with the exception of the digestive tract. Growing buds did not have the enzyme activity, even though the parent zooid exhibited strong signals. It should also be noted that several bud primordia from senescent adult zooids still have the enzyme activity, suggesting that the disappearance of acid β-galactosidase is not a necessary pre-requisite for bud formation.

In juvenile zooids, the pharynx had many small dotted signals of probably lysosomal origin. The epidermis of senescent adult zooids emitted the strongest signals from the whole cytoplasm that was ultrastructurally abundant in lysosome, indicating enhanced lysosomal activities in aged epidermis. These results suggest that acid β-galactosidase activity in Polyandrocarpa is homologous to that of SA-Gal in mammals.

In many organisms, senescence accompanies cell-growth arrest, which results in defects in tissue repair and regeneration (Iakova et al., 2003; Yang and Fogo, 2010). Previous autoradiographic studies of P. misakiensis using [3H]thymidine showed that in growing buds, the labelling index of epidermal cells was 3.7±0.4% (Kawamura et al., 1988), a value considerably higher than that measured in adult zooids. Labelled cells remarkably decreased in number temporarily after bud isolation from the parent. In this study, most cells of the bud epidermis were found to emit PCNA signals. They lost the signals temporarily at the transition period between the bud growth phase and the developmental phase, and they resumed the signals at the morphogenetic area of developing buds, consistent with the results of [3H]thymidine incorporation. By contrast, the epidermis of senescent adults has stopped engaging in extensive PCNA signals, consistent with the previous autoradiographic studies.

In P. misakiensis, the epidermis has severely undergone cell morphological changes during senescence. Specifically, adult epidermal cells become lean; the intercellular space has widened by the loss of interdigitated junction; many neighbouring cells remain connected only by the adherens intercellular junction; epidermal cells contain a larger number of lysosomes in the cytoplasm; the number of mitochondria decreases to some extent; and the mitochondrial matrix becomes darker than the translucent cytoplasm; the number temporarily after bud isolation from the parent. In this study, most cells of the bud epidermis were found to emit PCNA signals. They lost the signals temporarily at the transition period between the bud growth phase and the developmental phase, and they resumed the signals at the morphogenetic area of developing buds, consistent with the results of [3H]thymidine incorporation. By contrast, the epidermis of senescent adults has stopped engaging in extensive PCNA signals, consistent with the previous autoradiographic studies.

In P. misakiensis, the epidermis has severely undergone cell morphological changes during senescence. Specifically, adult epidermal cells become lean; the intercellular space has widened by the loss of interdigitated junction; many neighbouring cells remain connected only by the adherens intercellular junction; epidermal cells contain a larger number of lysosomes in the cytoplasm; the number of mitochondria decreases to some extent; and the mitochondrial matrix becomes darker than the translucent matrices in the bud epidermis, suggesting the accumulation of electron-absorbing wastes in the mitochondrial matrix in the senescent epidermis. Most interestingly, the epidermis has attenuated the transcriptional activity of several mitochondrial and nuclear genes during senescence.

These findings indicate that in P. misakiensis, the epidermis is one of the major tissues affected by the ageing process. This indication is further highlighted by comparison with two types of Polyandrocarpa multipotent cells: hemoblasts, which are undifferentiated cells in the hemocoel (Kawamura and Sunanaga, 2010); and cells of the atrial epithelium, which conducts cell proliferation and transdifferentiation during budding and regeneration (Kaneko et al., 2010; Kawamura and Fujiwara, 1994; Kawamura and Nakauchi, 1986; Kawamura et al., 2008a). These two types of cells do not express SA-Gal, even when the epidermis shows strong enzyme activity. PCNA signals were still observable in the atrial epithelium of the juvenile zooids, and even in a small number of the undifferentiated coelomic cells of senescent adult
zooids. Nodules of undifferentiated coelomic cells continued to express mitochondrial genes in the hemocoel of senescent adult zooids.

**Reversible function of mitochondrial genes during budding**

In senescent adult zooids of *P. misakiensis*, the epidermis was found to have almost entirely ceased the gene expression of both *PmCOX1* and *PmND1*. As previously described, the number of epidermal mitochondria has decreased to some extent during senescence, but it is unlikely that this decrease is the main cause of the conspicuous decrease in MRC gene activity in *Polyandrocarpa*. In this connection, we observed that during senescence of *P. misakiensis*, Cu/Zn-superoxide dismutase (*SOD1*) but not *SOD2* decreased gene activity specifically in the epidermis (K.K., unpublished). To mimic the decrease in *SOD1* gene activity during senescence, we investigated the expression of *PmEed* at different ageing stages, in situ hybridization.
senescence, *Polyandrocarpa* juvenile zooids were treated with siRNA(*PmSOD1*) and the resultant zooids were observed to prematurely attenuate MRC activity (K.K., unpublished). It is, therefore, possible to assume that during normal senescence of *P. misakiensis*, the decrease in *PmSOD1* gene expression should be one of main causes of the downregulation of MRC gene activities.

In human skeletal muscle, it is well known that MRC functions deteriorate with ageing (Boffoli et al., 1994; Byrne et al., 1991; Cooper et al., 1992). This decrease in MRC functions may be caused by mutations in mitochondrial genes (Byrne et al., 1991; Hebert et al., 2010). Indeed, cytochrome oxidase has been observed to alter polypeptide patterns during ageing (Boffoli et al., 1994). However, it is unlikely that during senescence of *P. misakiensis*, irreversible deterioration, such as a genetic mutation, has occurred in the epidermal mitochondrial genome, because epidermal MRCs can be activated at every budding. We found that this reactivation has already begun in the bud primordium. The repeatable mitochondrial gene function related to budding (asexual reproduction) is quite a novel finding.

**Reversible function of nuclear gene during budding**

Recently, we suggested that budding in *P. misakiensis* reinforced the gene activity of *Eed* (Kawamura et al., 2012). In this paper, we present further evidence that *PmEed* is activated in most bud tissues and attenuated in most senescent zooid tissues with an exception of gonads. The tunicate cytostatic factor, TC14-3, is a

![Fig. 8. Effects of siRNA(PmEed) on 2-day-old developing buds of *P. misakiensis*.](image-url)

Table 2. Effect of siRNA(PmEed) on PCNA expression in 2-day developing buds of *P. misakiensis*

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Proximal</th>
<th>Lateral</th>
<th>Distal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, siLacZ-treated</td>
<td>100 (4)</td>
<td>80.2±5.9</td>
<td>12.8±2.8</td>
<td>4.1±1.8</td>
</tr>
<tr>
<td>Experiment, siPmEed-treated</td>
<td>100 (4)</td>
<td>78.9±4.0</td>
<td>58.8±3.8</td>
<td>55.6±5.1</td>
</tr>
</tbody>
</table>

*Number of sections examined.

†Number of animals sectioned.

§Averages±d.

¶Nuclei expressing PCNA strongly or moderately were counted per 100 cells.
budding-specific, humoral polypeptide (Matsumoto et al., 2001). It can induce PmEed in vivo and in vitro (Kawamura et al., 2012), which would explain how PmEed is reactivated during budding.

In mammals, Eed is expressed in undifferentiated embryonic cells and is undetectable in differentiated cells (Kuzmichev et al., 2005). Eed constitutes polycomb repressive complex 2 (PRC2) and stimulates histone methyltransferase activity of PRC2 (Konuma et al., 2010). Likewise, in P. misakiensis, the induction of PmEed stimulated trimethylation of histone H3 at Lys27 (Kawamura et al., 2012) and, as shown in this paper, RNAi of PmEed can induce senescence in budding tunicates (Kawamura et al., 2012). Senescence in budding tunicates is reactivated during budding. PmEed is expressed in undifferentiated embryonic cells in P. misakiensis, a Polyandrocarpa-like stem cell. RNAi of PmEed has been found to cause the in vivo ectopic expression of PCNA in developing buds, findings that are consistent with the well-known cell growth-inhibitory effect of Pgc.

**Involvement of nuclear genes in reversible mitochondrial gene activities**

Pgc proteins regulate not only cell growth and differentiation but also senescence (Eckert et al., 2011). As already described, tunicate senescence is deeply associated with MRC gene activity. As first suggested by Kawamura et al. (Kawamura et al., 2012) and confirmed in the present study, TC14-3 enhances the gene expression of PmCOX1 as well as PmEed. To determine whether PmEed was directly involved in MRC gene regulation, growing buds were treated with siRNA(PmEed) and strong PmCOX1 expression was observed to remain in the epidermis, atrial epithelium, and coelomic cells, indicating that RNAi of PmEed does not affect MRCs that are pre-activated during budding. By contrast, when zoid pieces were treated with both siRNA(PmEed) and TC14-3, MRC activation did not occur although it was observed in zoid pieces treated solely with TC14-3. This finding provides the first piece of evidence that Eed, a component of Pgc, is involved in the rejuvenation of mitochondria. An intriguing issue is whether TC14-3 can contribute to zoidal longevity in P. misakiensis. As most of senescent zoid pieces become dormant instead of completing regeneration in the presence or absence of TC14-3, it is uncertain at present whether TC14-3 can influence the zoidal lifespan.

It is unlikely that Eed and/or Pgc directly act on the mitochondrial genome, as the mitochondrial genome does not have histones. In humans, transcription levels of mitochondrial genes are stably maintained by the nuclear transcriptional regulatory factor Tbam (Kelly and Scarpulla, 2004; Scarpulla, 2008). In P. misakiensis, a Ptm-like gene belonging to the high mobility group genes was highly expressed during budding and this transcription level decreased conspicuously during senescence (K.K., unpublished). This finding suggests that the PtmTbam-like gene may be related to MRC gene regulation through TC14-3-induced Pgc and/or histone methylation. It is probable, therefore, that in P. misakiensis, several nuclear genes appear to be involved in reversible MRC functions in association with budding cycles.

**Other features of tunicate senescence**

As already mentioned, PmSOD1 is a senescence-dependent nuclear gene. SOD protects cells and tissues from oxidative stress due to reactive oxygen species (ROS), and human SOD1 can extend the normal lifespan of Drosophila by up to 40% (Parkes et al., 1998). In P. misakiensis, PmSOD1 expression has been observed to cease in the epidermis, while continuing weakly in other tissues, including the pharynx, endostyle and stomach and, exceptionally, continuing strongly in germ cells (K.K., unpublished). Such strong expression helps protect germ cells from oxidative stress, genetic mutation and cell death due to ROS production (Liu et al., 2000).
Botryllus schlosseri is a colonial tunicate related to *P. misakiensis*. In a colony, adult zooids are taken over by the younger generation every 5-7 days. At this takeover stage, tissues and organs of the adult zooids disintegrate and fragment into apoptotic bodies, followed by the ingestion by coelomic macrophages or intraepithelial phagocytes (Burghiel and Schiavinato, 1984; Lauzon et al., 1993; Tiozzo et al., 2006). In *B. primigenius*, most cells and tissues of adult zooids become mitotically quiescent before apoptosis (Kawamura et al., 2008b). In a previous investigation of *B. primigenius*, we detected SA-Gal activity in the apoptotic cell mass of degenerating zooids but not in the epidermis and other tissues of functional adult zooids or younger generations (K.K., unpublished).

In *Botryllus*, aged colonies accumulate pigment cells in the hemocoel until they can grow no further, leading to colonial death in a phenomenon referred to as nonrandom colonial senescence (Lauzon et al., 2000). When we examined SA-Gal activity during non-random colonial senescence in *B. primigenius*, the enzyme activity was observed to increase remarkably in the coelomic space but, unlike *P. misakiensis*, no signals were detected in the epidermis (K.K., unpublished). Taken together, the findings regarding zooidal takeover and colonial senescence in *Botryllus* appear to take cellular features different from zoodial senescence in *Polyandrocarpa*.

Conclusions

Senescence inevitably deteriorates physiological functions necessary for survival and fertility. It accompanies the mitochondrial dysfunction caused by the time-related accumulation of DNA damage. Only germ cells and stem cells are considered as exceptional cells that can escape from such dysfunction. In *P. misakiensis*, senescence events accompany mitochondrial dysfunction similar to other biological systems, but senescence in this species is unique because all ageing events examined are reversible and repeatable during budding, even in epidermal non-stem cells. Several nuclear genes are involved in such reversible senescence events. A candidate nuclear gene is *Eed*, which is a component of PcG. *Eed* can indeed mediate mitochondrial reactivation in senescent adult zooids. In conclusion, our results afford new insights into the function of PcG concerning the resetting or reprogramming of cellular senescence and mitochondrial rejuvenation.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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