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Research Paper

Knockdown of the coenzyme Q synthesis gene *Smed-dlp1* affects planarian regeneration and tissue homeostasis

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ABSTRACT

The freshwater planarian is a model organism used to study tissue regeneration that occupies an important position among multicellular organisms. Planarian genomic databases have led to the identification of genes that are required for regeneration, with implications for their roles in its underlying mechanism. Coenzyme Q (CoQ) is a fundamental lipophilic molecule that is synthesized and expressed in every cell of every organism. Furthermore, CoQ levels affect development, life span, disease and aging in nematodes and mice. Because CoQ can be ingested in food, it has been used in preventive nutrition. In this study, we investigated the role of CoQ in planarian regeneration. Planarians synthesize both CoQ9 and rhodoquinone 9 (RQ9). Knockdown of *Smed-dlp1,* a trans-prenyltransferase gene that encodes an enzyme that synthesizes the CoQ side chain, led to a decrease in CoQ9 and RQ9 levels. However, ATP levels did not consistently decrease in these animals. Knockdown animals exhibited tissue regression and curling. The number of mitotic cells decreased in *Smed-dlp1 (RNAi)* animals. These results suggested a failure in physiological cell turnover and stem cell function. Accordingly, regenerating planarians died from lysis or exhibited delayed regeneration. Interestingly, the observed phenotypes were partially rescued by ingesting food supplemented with α-tocopherol. Taken together, our results suggest that oxidative stress induced by reduced CoQ9 levels affects planarian regeneration and tissue homeostasis.

1. Introduction

Regeneration involves several common mechanisms that are shared among different organisms. The freshwater planarian can regenerate lost tissues from a small body part after bisection via the protection of injured epithelium, blastema formation and subsequent morphogenesis. Regeneration-with the exception of a few tissues-largely relies on neoblasts, adult somatic stem cells that are distributed throughout the body and are the only mitotic cells found in mature planarians [1]. Recent progress in planarian research has led to the identification of genes required for neoblast maintenance, and chromatin organizers are required for neoblast differentiation and for remodeling existing tissues along the anteroposterior axis [2]. The introduction of RNA interference (RNAi) has permitted investigation of the molecular mechanisms that drive planarian regeneration [3].

A large-scale RNAi screen conducted in planarians led to the identification of neoblast-specific genes required for regeneration [4]. This study also described a role for basal cell machinery proteins in regeneration. Knockdown of several basal cell machinery genes phenocopied the knockdown of neoblast-specific genes. For example, knockdown of the protein L3, an essential ribosomal peptidyltransferase, led to planarian lysis without blastema formation. This suggested that neoblast-specific genes only function in healthy animals.

CoQ is a lipophilic molecule required for mitochondrial respiration in aerobic organisms. The redox activity of CoQ also serves as an antioxidant in the membrane [5]. Therefore, it is likely that each cell synthesizes enough CoQ to maintain basic cellular activity. Interestingly, several studies have reported that defects in intrinsic CoQ biosynthesis affect an organism's development and life span [6–12]. It has also been reported that CoQ levels are associated with disease conditions [13–15] and that CoQ levels change during aging [16,17]. These changes likely involve the production of reactive oxygen species (ROS) and their scavenging by CoQ [18,19]. Therefore, CoQ plays an important role in homeostasis and disease states.

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In contrast to other basal molecules, CoQ can be obtained through the intake of dietary foods, making it an important nutrient. However, it is unclear whether CoQ plays a role in regeneration.

CoQ biosynthesis begins with the formation of a hydroxybenzoic acid head group and a lipophilic isoprenoid side chain [20,21]. The CoQ isoprenoid side chain varies in length among species and is synthesized by trans-prenyltransferase with farnesyl pyrophosphate (FPP) and several isopentenyl pyrophosphates [22]. Nematodes, rodents and most cereal crops produce CoQ9, which contains nine isoprene units, while humans, bovines and soybeans produce CoQ10. CoQ is a heterotetramer composed of two proteins, decaprenyl (DSP1) or solanesyl polyprenyl diphosphate synthase 1 (SPS1) and D-less polyprenyl diphosphate synthase (DLP1)/decaprenyl diphosphate synthase subunit 2 (PDSS2), in humans, rats, *Drosophila* and *Xenopus* [23]. Through the formation of intermediate compounds, a polyprenyl-hydroxybenzoate is enzymatically altered to produce CoQ. This pathway is also utilized to produce RQ, an aminoquinone required for anaerobic respiration in species capable of fumarate reduction [24], which is found in euglena, nematode, parasitic worms and *Rhodospirillum rubrum*. The planarian CoQ biosynthesis pathway remains to be elucidated. However, studying how CoQ levels affect planarian regeneration could inform our understanding of human biology.

Here, we address the role of CoQ in planarian tissue maintenance and regeneration. We identified the planarian trans-prenyltransferase gene and showed that its knockdown led to defects in regeneration and tissue homeostasis. We also demonstrated that the antioxidant vitamin E partially rescued the phenotypes observed in knockdown animals.

2. Materials and methods

2.1. Planarians

An asexual strain of *Schmidtea mediterranea* was used and maintained at 18 °C in 1 × Montjuïch salts (1.6 mmol/l NaCl, 1.0 mmol/l $CaCl_2$, 1.0 mmol/l $MgSO_4$, 0.1 mmol/l $MgCl_2$, 0.1 mmol/l KCl and 1.2 mmol/l $NaHCO_3$ prepared in Milli-DI water) [25]. Planarians that were 4–6 mm in length were starved for at least 1 week before experiments were performed.

Full-length *Smed-dlp1* cDNA (1-1387) was cloned into the pPR242 vector (courtesy of Peter Reddien, Whitehead Institute/Massachusetts Institute of Technology). The pPR242 vector without an insert was used as a control. Plasmids were transformed into the HT115 (DE#3) *E. coli* strain [26]. Bacterial cultures in 2 × YT medium supplemented with 10 μg/ml kanamycin and 10 μg/ml tetracycline were induced with 100 mM isopropyl β-D-1-thiogalactopyranoside for 2 h. Gene expression silencing was performed as described previously [27]. Briefly, bacteria were mixed with homogenized liver and red food coloring. The mixed food was fed to planarians (approximately 4 mm in body length along the anteroposterior axis) every three days. Fresh bovine liver was obtained from a butcher. To supplement the liver with antioxidants, vitamin E was mixed with homogenized bovine liver and red food coloring. Animals were examined under an SZX7 stereomicroscope equipped with a DP71 CCD camera (Olympus, Tokyo, Japan).

2.2. Cloning and sequence analysis

RNA was extracted using TRIZol reagent (Invitrogen, CA, USA). Full-length 5′ and 3′ ends of cDNAs were obtained using the GeneRacer Kit (Invitrogen). *Smed-sps1* gene-specific primers (CGATGTTCCTGCCGAATTCGAAAGCCGCATCT and ACCGCTTTGCAACAATTGGCAATAAGACTTGCGGT) were used for 5′ rapid amplification of cDNA ends (5′ RACE). *Smed-sps* gene-specific primers (ATGCGGCTTTCGAATTCGGCAGGAACATCGG and GCGGAACAGTTAGGCAAGCCAAGTTGCGG) were used for 3′ RACE. A *Smed-dlp1* gene-specific primer (ATTTCAGCGACACTGCGTTGTTCA) was used for 5′ RACE, and a *Smed-dlp1* gene-specific primer (ACAAATTGTGTGCTACCCATCCTCC) was used for 3′ RACE. Multiple alignments of amino acid sequences were performed using the CLUSTAL W program. The amino acid sequences of hDPS1 (NCBI accession no. AB210838), mSPS1 (accession no. AB210841), hDLP1 (accession no. AB210839) and mDLP1 (accession no. AB210840) were obtained from GenBank.

2.3. HPLC analysis

Each animal was weighed in a microtube without solution. Then, 200 μl of 2-propanol was added and mixed with the tissue by grinding. The supernatant was injected into an HPLC-ECD system consisting of a pump (NANOSPACE SI-2, Shiseido, Japan), two analytical columns (Type Mightysil RP-18GP, 5 μm, 150 × 4.6 i.d., Kantochemical, Japan), a reduction column (Type RC-10-1, Irica, Japan), an electrochemical detector (NANOSPACE SI-2), and an integrator (Model C-R7A plus, Shimadzu, Japan) as described previously [28]. The mobile phase was 50 mM sodium perchlorate in methanol/2-propanol (700/300, v/v) with a flow rate of 1 ml/min. The CoQ homolog concentrations were determined by comparing standard chromatograms of the oxidized forms of CoQ7, CoQ9 and CoQ10 (Kaneka, Tokyo, Japan); their reduced forms were prepared in our laboratory. The concentration of rhodoquinone 9 (RQ9) was determined using a standard chromatogram for RQ9 extracted from adult *Ascaris suum*. α-tocopherol was purchased from Wako (Osaka, Japan).

2.4. ATP measurement

ATP was measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). Each planarian was dissolved in CellTiter-Glo buffer using a homogenizer pestle. After centrifugation at 18,800*g* at 4 °C for 10 min, the supernatant was diluted into 5/8 Holtfreter's solution (2.188 mg/l NaCl, 31 mg/l KCl, 63 mg/ml $CaCl_2$, 125 mg/ $NaHCO_3$). The sample was incubated with 2 × CellTiter-Glo at room temperature for 10 min and was subjected to luminescence measurements using the Ultra Evolution microplate reader (TECAN, Männedorf, Switzerland). For protein measurement, a BCA Protein Assay Kit (Pierce, IL, USA) was used according to the manufacturer's protocol.

2.5. Immunofluorescence

Planarians were fixed in Carnoy's fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid), were labeled with a rabbit anti-phosphorylated-histone H3 antibody (Santa Cruz Biotechnology, CA, USA) and were detected with Alexa Fluor 488 goat anti-rabbit IgG antibody (Molecular Probes, CA, USA) as described previously [1]. All stained planarians were examined under a fluorescence stereomicroscope SZX7 equipped with a DP71 CCD camera system (Olympus, Tokyo, Japan).

2.6. Reverse transcription PCR

Equal amounts of total RNA (typically 200 ng) from each animal were used in each set of controls and RNAi samples from experiments performed on the same day. cDNA was synthesized using Superscript III reverse transcriptase (Invitrogen) with oligo(dT) primers. PCR was performed using GoTaq Hot Start Polymerase (Promega, WI, USA) with *Smed-sps1*-specific primers (GCCGCATCTACTGCACTTTCTGAC and AACTATGCATTCCGGTTTAACACCAGT), *Smed-dlp1*-specific primers (ACAAATTGTGTGCTACCCATCCTCC and

ATTTCAGCGACACTGCGTTGTTCA), and *Smed-gapdh* primers (GTTGT CATCAATCCTTCTACAATACCG and CAGTTGACGTTGTTGCTCTAAACG). To semi-quantify mRNA, linear amplification of PCR products was confirmed using several concentrations of cDNA fragments for each gene. Amplified DNA bands that did not show fluorescence saturation were used for fluorescent ratio analysis. *Smed-gapdh* and *Smed-βactin* mRNA were used to analyze the expression of housekeeping genes that were be affected by RNAi treatment. PCR products were electrophoresed and quantified using the Typhoon 9410 imager (GE Healthcare, Buckinghamshire, UK).

2.7. Statistical analysis

An unpaired Welch's *t*-test was performed to calculate *P*-values in comparison with the corresponding control.

3. Results

3.1. Planarians synthesize quinones

The CoQ biosynthesis pathway is summarized in Fig. 1a according to a previous review [29]. The length of the isoprenoid side chain depends on the trans-prenyltransferase employed. We examined the quinone compounds synthesized by planarians. Standard CoQ9, CoQ10 and RQ9 compounds showed an identical peak for each quinone (Fig. 1b, trace a and b). Planarians showed five peaks for each retention time (Fig. 1b, trace c). The two peaks representing CoQ10 disappeared in animals that were starved for 7 days (Fig. 1b, trace d). Thus, the CoQ10 peaks observed in the planarians were likely derived from food and not produced endogenously. Accordingly, bovine samples contained CoQ10 [30]. We next analyzed the electrochemical properties of the molecules represented by these peaks (data not shown). Taken together, these results clearly showed that planarians produce CoQ9 and RQ9. Free-living freshwater planarians belong to the Turbellaria subgroup of Platyhelminthes. The parasitic Cestoda subgroup of Platyhelminthes generates RQ for anaerobic respiration [31,32]. The RQ9 content of planarians could indicate a close taxonomical relationship between the Platyhelminthes subgroups, although the role of RQ9 in planarians remains unclear.

3.2. RNAi-mediated gene silencing of trans-prenyltransferase

To identify the planarian gene that encodes trans-prenyltransferase, we performed 5′ RACE and 3′ RACE RT-PCR using

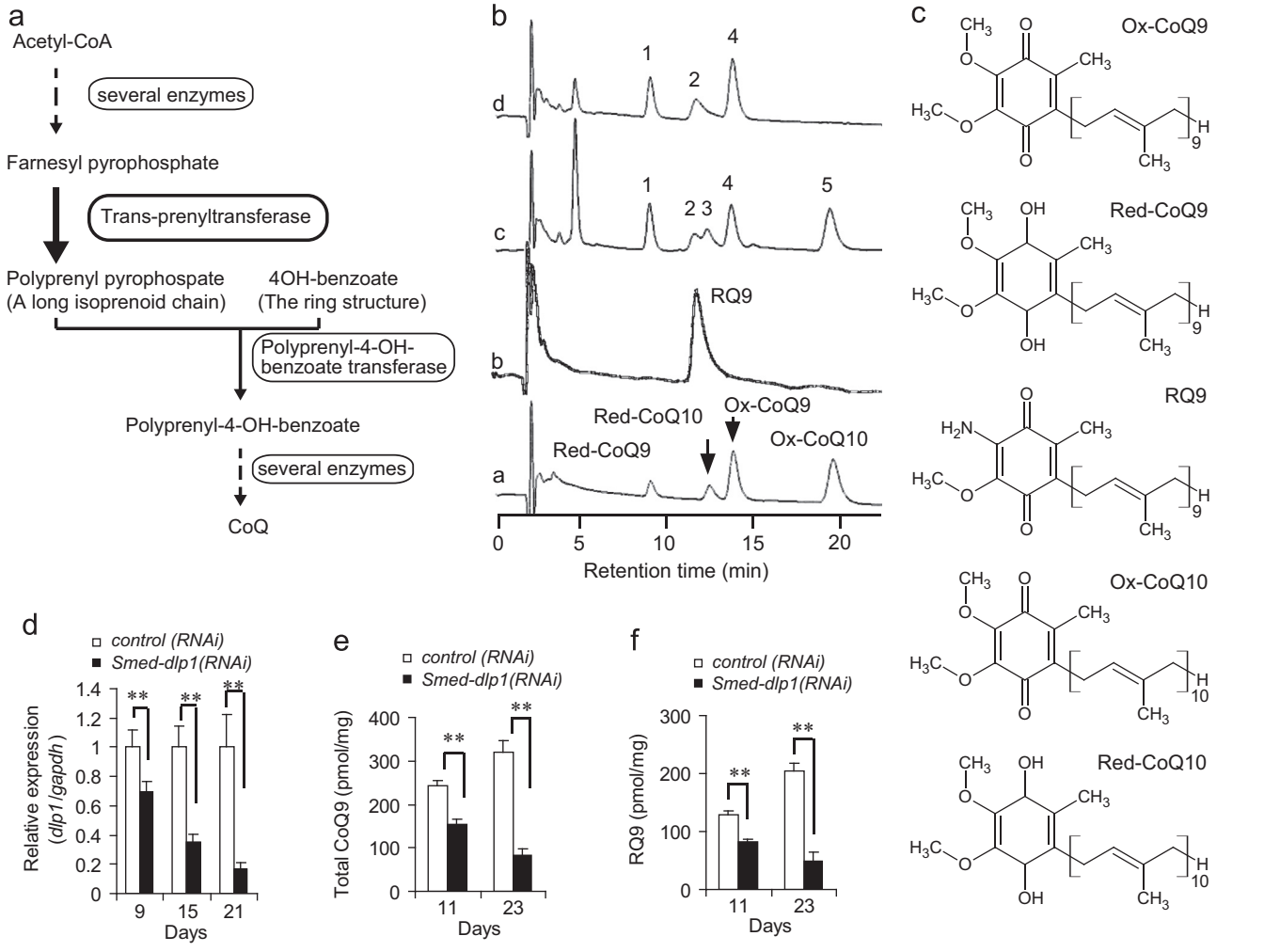

Fig. 1. *Smed-dlp1* gene expression knockdown led to CoQ deficiency. (a) Overview of the CoQ biosynthesis pathway. (b) HPLC chromatograms of quinone. Trace a: a standard mixture of oxidized CoQ9 (Ox-CoQ9), reduced CoQ9 (Red-CoQ9), oxidized CoQ10 (Ox-CoQ10) and reduced CoQ10 (Red-CoQ10). Trace b: RQ9 standard. Trace c: two days after feeding. Trace d: seven days after feeding. (c) Structures of CoQ and RQ. (d) *Smed-dlp1* gene expression knockdown. Animals were fed every three days. Relative *Smed-dlp1* mRNA levels were analyzed after three days. Mean ± s.e.m.; **: $P < 0.01$, $n = 5$. (e, f) The effect of silencing *Smed-dlp1* expression on the total CoQ9 and RQ9 levels. Animals fed every three days were starved for five days. The total CoQ9 (e) and RQ9 (f) levels were normalized by animal weight. Mean ± s.e.m.; **: $P < 0.01$, $n = 20$.

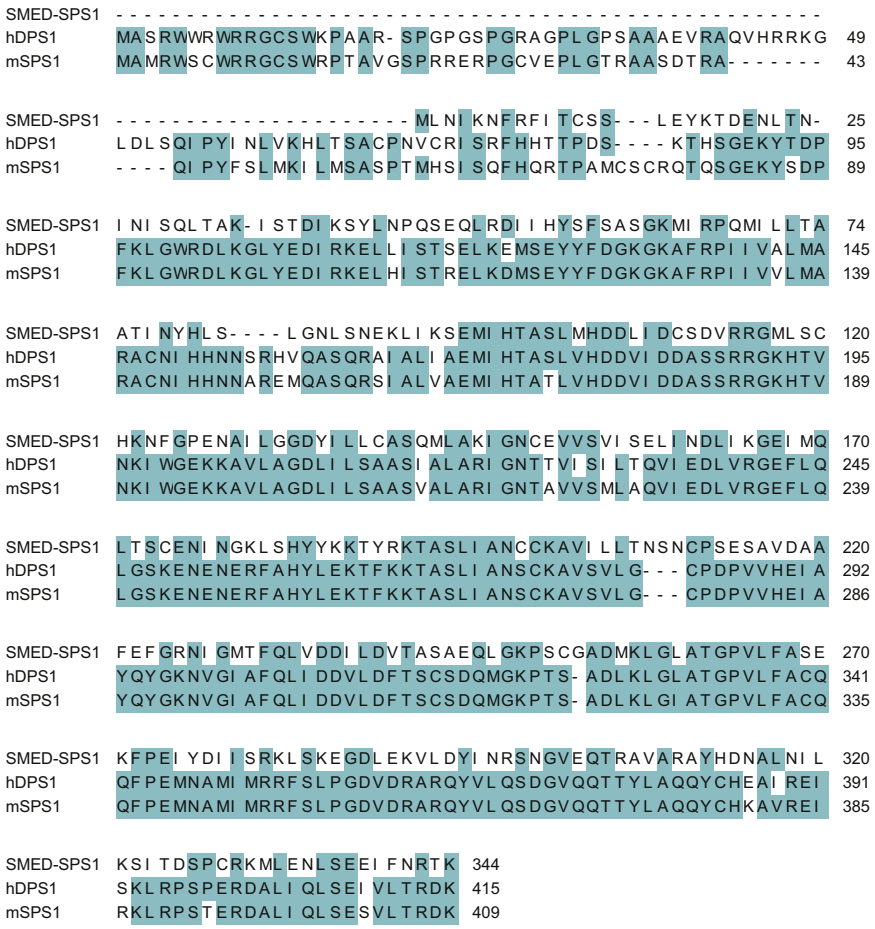

Fig. 2. Amino acid alignment of SMED-SPS1 with human and mouse sequences. Alignment of SMED-SPS1 (DDBJ accession no. AB548864) with human and mouse sequences.

PCR primers. We obtained gene sequences from the planarian genome database using the mouse trans-prenyltransferase protein sequences SPS1/DPS1 and DLP1. We denoted the planarian genes as *Smed-sps1* and *Smed-dlp1*, respectively. The planarian SMED-SPS1 amino acid sequence was approximately 43% similar to the human and mouse sequences (Fig. 2), while the planarian SMED-DLP1 amino acid sequence shared approximately 41% similarity to the human and mouse sequences (Fig. 3).

We next performed *Smed-dlp1* gene silencing by feeding animals dsRNA-containing food every three days. We did not observe a difference in the eating behavior of the *control (RNAi)* and *Smed-dlp1(RNAi)* animals. Under these conditions, we observed a decrease in the *Smed-dlp1* mRNA levels in the *Smed-dlp1(RNAi)* animals after nine days (Fig. 1c). After 21 days, the *Smed-dlp1*mRNA levels were approximately 80% decreased compared to the controls. Knockdown animals showed a decrease in the total CoQ9 content (Fig. 1d). CoQ is an intermediate in RQ biosynthesis [33]; consistently, these animals also showed a decrease in RQ9 levels (Fig. 1e). Therefore, *Smed-dlp1* gene expression directly affects quinone levels.

3.3. Tissue homeostasis is altered in Smed-dlp1(RNAi) animals

Cells in planarian tissues are constantly replaced by newly differentiated cells derived from neoblasts. When this mechanism is damaged, planarians exhibit morphological abnormalities [4]. We did not observe any morphological defects in the *Smed-dlp1 (RNAi)* animals cultivated for 13 days when the animals exhibited decreased CoQ9 levels (Fig. 4a). Interestingly, animals cultivated over 19 days exhibited tissue regression around the head. Most of the animals curled and showed locomotion defects after 25 days (Fig. 4b), which are phenotypes that are similar to those of irradiated planarians [34]. We next analyzed the mitotic cell number via phospho-Histone H3 immunofluorescence. We did not observe any difference in the phospho-Histone H3 distribution between *control(RNAi)* and *Smed-dlp1(RNAi)* animals cultivated for seven days (Fig. 4c). Mitotic cells were distributed throughout the mesenchyme, with the exception of the pharynx and the region in front of the photoreceptors of control animals. However, the mitotic cell population decreased significantly in *Smed-dlp1(RNAi)* animals cultivated for 19 days (Fig. 4d). Interestingly, animals exhibiting an onset of impaired tissue homeostasis showed a decrease in mitotic cell number. Additionally, ATP levels were increased in *Smed-dlp1(RNAi)* animals cultivated for 10 days (Fig. 4e). Conversely, animals cultivated for 22 days exhibited decreased ATP levels compared to controls. Thus, ATP levels were not correlated with CoQ levels.

3.4. Smed-dlp1 RNAi affects planarian regeneration

To address whether *Smed-dlp1(RNAi)* animals can regenerate, we examined head regeneration after amputation. *Control(RNAi)* animals cultivated for seven days formed unpigmented blastemas and regenerated eyes in the new tissue (Fig. 5a). In contrast, blastemas remained small in *Smed-dlp1(RNAi)* animals (Fig. 5a) and did not develop pigmented eyes in the new tissue. Interestingly, half of the

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SMED-DLP1 -------------------------------------MWKTP-------W   6
hDLP1     MNFRQLLLHLPRYLGASGSPRRLWWSP-SLDTISSVGSWRGRSSKSPAHW  49
mDLP1     MSLRQLLLRLSGYLGASGPPSRHWWYFRSLDSISSAGSWRGRSSRSPAHW  50

SMED-DLP1 QKIIKRSEQIVCYPSSLVNIRYLISDEITSVASQVRKLVGTHHPLLQTAK  56
hDLP1     NQVVSEAEKIVGYPTSFMSLRCLLSDELSNIAMQVRKLVGTQHPLLTTAR  99
mDLP1     NQVVSEAEKIVGYPASFMSLRCLLSDELSNIAMQVRKLVGTGHPLLTTAR 100

SMED-DLP1 TLISDTQGVTELRGLIVLLISKTFTRPPFAINAE-----------QRSVA  95
hDLP1     GLVHDSWNSLQLRGLVVLLISKAAGPSSVNTSCQNYDMVSGIYSCQRSLA 149
mDLP1     ALVHDSRHNLQLRGLVVLLISKAAGPSTRNASCQNYDMVSGVYSCQRSLA 150

SMED-DLP1 EIVETAHTAICIHKDLVNIEKSLQNKPTNWYYDMQFGNKMATLCGDYLLA 145
hDLP1     EITELIHIALLVHRGIVNLNELQSSDGP--LKDMQFGNKIAILSGDFLLA 197
mDLP1     EITELIHTALLVHRGIVNLSELQSSDGP--LKDMQFGNKIAILSGDFLLA 198

SMED-DLP1 SVSKALAEFQNTNIVNTVSKGIGDTIESLFCKVNVDNEENYR-------- 187
hDLP1     NACNGLALLQNTKVVELLASALMDLVQGVYHENSTS-KESYITDDIGIST 246
mDLP1     NACNGLALLQNTKVVELLSSALMDLVHGVYQENSASTKENSIPDDIGIST 248

SMED-DLP1 --RYVYLSSGSLLSKCCKISVSLRTNVNDFVDLTKSD-EDLAEKWSIHWC 234
hDLP1     WKEQTFLSHGALLAKSCQAAMELAKHDAEVQNMAFQYGKHMAMSHKINSD 296
mDLP1     WKEQTFLSHCALLAKSCQAAMELAKHDAAVQDMAFQYGKHMAMSHKINAD 298

SMED-DLP1 ELYNICEHIN---ALVIQLSNEKITSKIFPISKKLLQSDNLSDDLQFLHE 281
hDLP1     VQPFIKEKTSDSMTFNLNSAPVVLHQEFLGRDLWIKQIGEAQEKGRLDYA 346
mDLP1     LQPFIKDKASDSKTFNLNSAPVVLHQEFLGRDLWIKQIGEAQEKGSLNYS 348

SMED-DLP1 YLCKTKILVMNSHKSLPEYDLQSKSILAEMVELLYQDAIHSLNLCRMLP- 330
hDLP1     KLRERIKAGKGVTSAIDLCRYHGNKALEALESFPPSEARSALENIVFAVT 396
mDLP1     KLRETIKAGKGVTSAIDLCRYHGNKALEALESFPPSEARSALENIVFAVT 398

SMED-DLP1 ---  330
hDLP1     RFS  399
mDLP1     RFS  401
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Fig. 3. Amino acid alignment of SMED-DLP1 with human and mouse sequences. Alignment of SMED-DLP1 (DDBJ accession no. AB548865) with human and mouse sequences.

Smed-dlp1(RNAi) animals died ($n=15/30$) due to lysis between 4 and 21 days post-amputation (Fig. 5b). These animals curled and subsequently died, indicating the importance of *Smed-dlp1* function during regeneration. Accordingly, the surviving *Smed-dlp1(RNAi)* animals presented slow blastema growth (Fig. 5c) and delayed eye pigmentation ($n=15/30$) compared to control animals (Fig. 5d).

3.5. Effect of α-tocopherol on tissue homeostasis and regeneration in Smed-dlp1(RNAi) animals

We next examined the effect of α-tocopherol on tissue maintenance and regeneration by feeding animals food supplemented with α-tocopherol. These animals showed an increase in α-tocopherol levels (Fig. 6a). The food did not affect the animals' eating behavior. *Smed-dlp1(RNAi)* animals supplemented with α-tocopherol exhibited improved viability and improved tissue regression (Table 1). No *Smed-dlp1(RNAi)* animals died during regeneration when fed α-tocopherol (Fig. 6b). Accordingly, the blastema grew similarly to those of the *control(RNAi)* animals after amputation (Fig. 6c). These results suggest that α-tocopherol supplementation effectively rescues the phenotypes observed in *Smed-dlp1(RNAi)* animals.

4. Discussion

Our results show that decreased CoQ levels do not immediately lead to morphological abnormalities in intact planarians. Instead, prolonged CoQ deficiency leads to a decrease in the number of neoblasts. Neoblasts are the only mitotic cells in planarians, where all differentiated cells are the progeny of neoblasts [1]. We found that CoQ-deficient planarians rarely survived. However, α-tocopherol supplementation partially prevented tissue regression and death. In planarians regenerating after bisection, CoQ deficiency leads to a defect in blastema formation. Blastema formation is dependent on the presence of an appropriate number of mitotic cells. In wounded tissue, many basal genes are responsible for blastema formation [4]. Remarkably, α-tocopherol pre-supplementation in surviving animals partially prevented death and promoted blastema formation. These results indicate that CoQ antioxidant activity is important for regeneration and the maintenance of tissue homeostasis in planarians.

The beneficial effects of vitamin E and α-tocopherol on CoQ deficiency caused by the *dlp1*/*Pdss2* mutation have been reported in mice and fission yeast [35,36]. *Pdss2* missense mutant mice developed kidney disease; however, the damage was ameliorated by the ingestion of vitamin E, CoQ or the antioxidant probucol [35]. *dlp1* deletion mutant yeast did not grow in minimal medium without α-tocopherol, antioxidant glutathione or cysteine [36]. It has been suggested that the antioxidant activity of CoQ plays a role in basic cellular activity. A sufficient supply of CoQ, an intrinsic membrane antioxidant, protects the cell membrane from oxidation. It has been suggested that proper ROS levels are required to maintain cellular functions. Our observation that α-tocopherol has a protective effect supports this hypothesis. Further study using α-tocoquinone, the oxidized form of α-tocopherol, would be required to confirm this hypothesis. It was interesting to determine whether CoQ ingestion rescued the CoQ-deficient planarians.

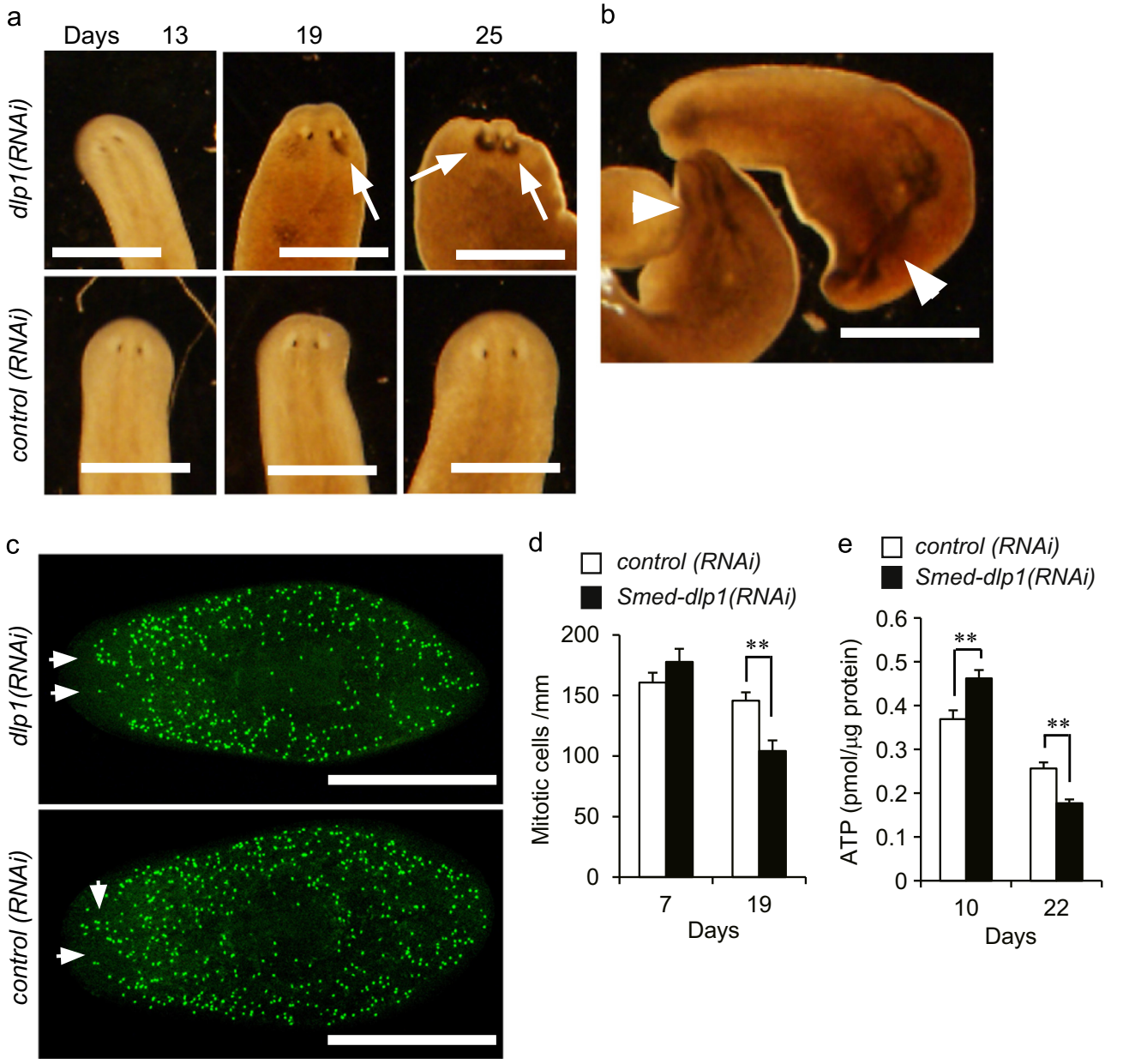

Fig. 4. Effect of *Smed-dlp1* gene silencing on tissue homeostasis. (a, b) Representative morphology of *Smed-dlp1(RNAi)* planarians fed every three days. (a) Arrows show the dorsal view of head regression. (b) Arrowheads show the ventral view of curling after 25 days. Bars: 1 mm. (c) Immunofluorescence of phospho-histone H3. Representative distribution of mitotic cells after seven days. Arrowheads denote eyes. Bars: 1 mm. (d) A population of mitotic cells. The mitotic cell number in whole animals was normalized to body length along the anteroposterior axis. Mean ± s.e.m.; **: $P<0.01$, $n=10$. (e) Cellular ATP level. Mean ± s.e.m.; **: $P<0.01$, $n=15$.

Because the food used to cultivate planarians consisted of bovine liver and food coloring, the animals incorporated a similar amount of CoQ10 from food as the amount of CoQ9 synthesized intrinsically. However, although the *Smed-dlp1* knockdown animals ingested this food, the animals still developed a phenotype. It is known that the antioxidant efficiency of CoQ does not depend on the isoprenoid chain length [37]. Therefore, the amount of CoQ10 supplied in food might not be sufficient to rescue the phenotypes of *Smed-dlp1* knockdown animals.

Impaired tissue maintenance in planarians is consistent with mouse CoQ deficiency models. *Pdss2* missense mutant mice and *Pdss2* missense mice targeting the kidney developed kidney disease [38–40]. In addition, selective degradation of the substantia nigra occurred in mice with a conditional *Pdss2* missense mutation targeted to dopaminergic neurons [41]. Therefore, the CoQ levels must be maintained within an appropriate range to allow cells carry out basal cellular activity. The CoQ content in the *Pdss2* missense mutant mouse was approximately 10–20% that of the control mouse [38–40], while the CoQ content in the *Smed-dlp1* knockdown planarian was approximately 20% of the control. These CoQ levels were lethal. It would be interesting to determine what level of CoQ is sufficient for basal cellular activity. Aged mouse and human heart showed decreased CoQ content by approximately 70% and 43% of the levels observed in the corresponding young organisms, respectively [16,17]. Although further studies are required, a wide range of CoQ levels could allow cells to perform their basal cellular functions.

5. Conclusions

Planarians produce CoQ9 and RQ9. *Smed-dlp1* gene knockdown in adult planarians leads to CoQ deficiency, which causes impaired

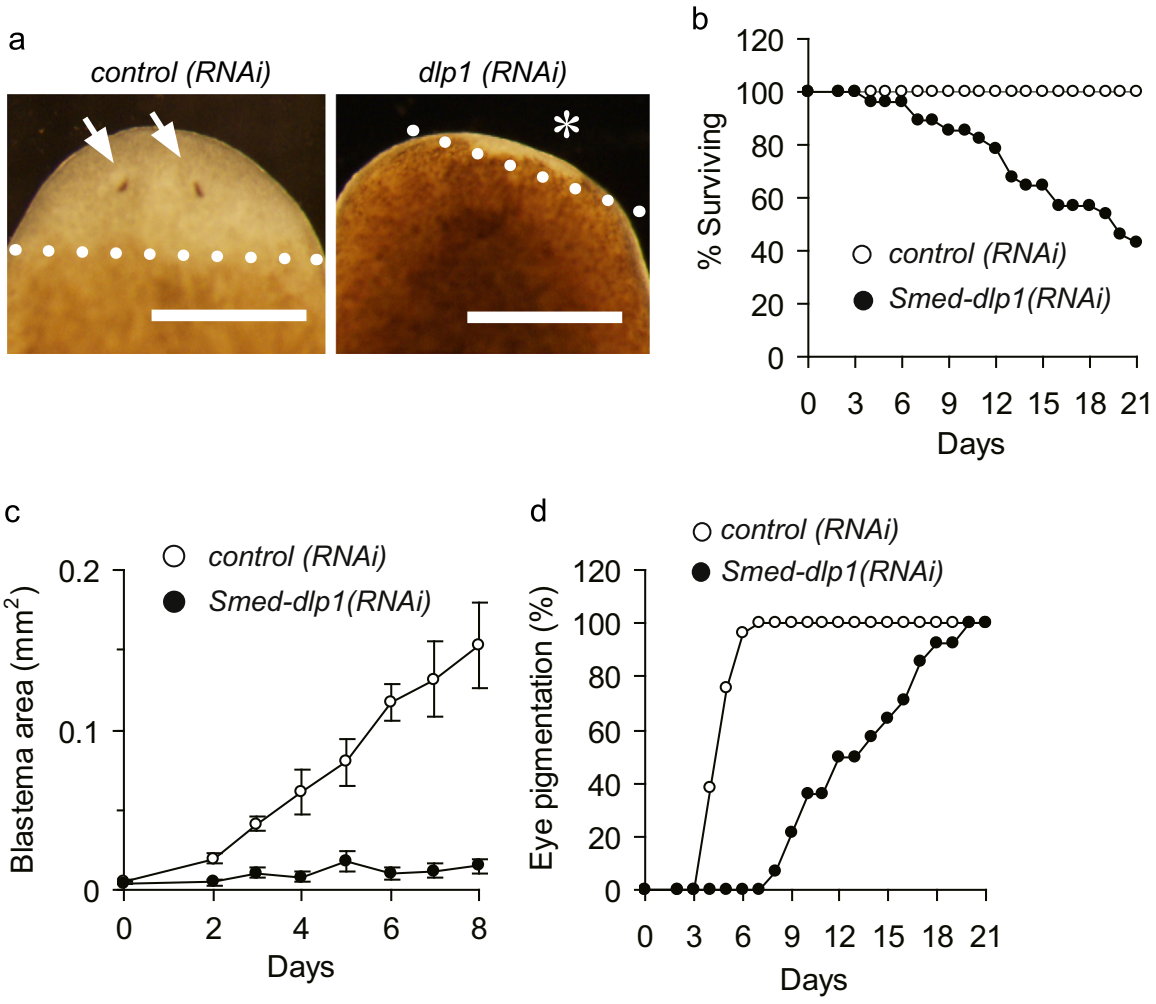

Fig. 5. Effect of *Smed-dlp1* gene silencing on regeneration. Animals were fed every three days and amputated after 21 days. (a) Representative morphology of regenerating RNAi-treated planarians seven days after amputation. Arrows denote eyes with pigmentation. The dotted line denotes an unpigmented blastema boundary. The asterisk indicates a small blastema. Bars: 0.5 mm. (b) Viability of amputated animals. (c) Regeneration of lost tissues after amputation. A blastema grew after amputation. Mean ± s.e.m.; $n=5$. (d) Eye pigmentation in the blastema. Eye pigmentation was delayed in *Smed-dlp1(RNAi)* animals. $n=30$.

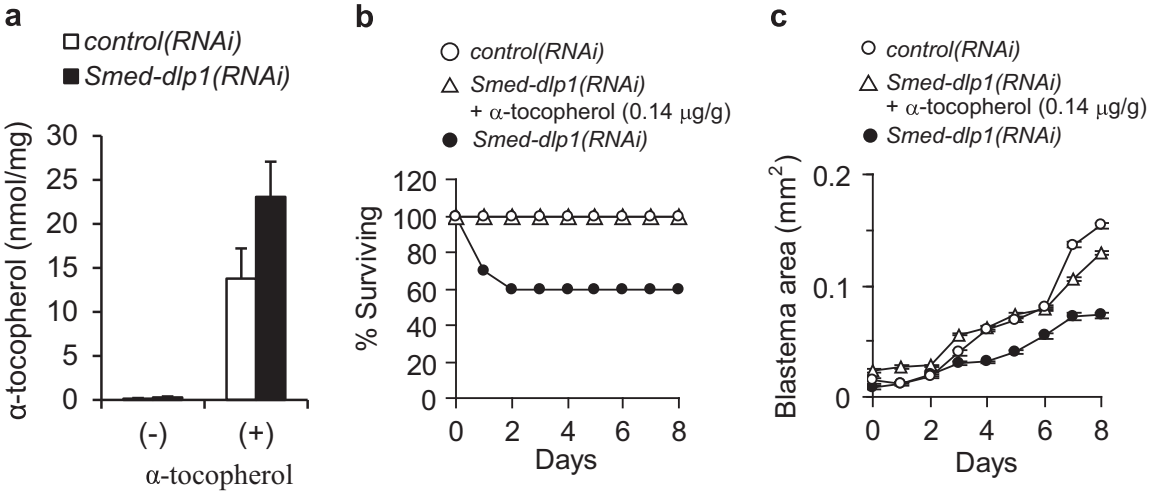

Fig. 6. Effect of α-tocopherol on regenerating *Smed-dlp1(RNAi)* animals. (a) Ingestion of α-tocopherol by *Smed-dlp1(RNAi)* animals. Animals were fed food supplemented with α-tocopherol every three days. Mean ± s.e.m.; $n=8$. (b, c) Regeneration of lost tissue after amputation. Animals were fed every three days and amputated at day 19. Viability (b) and eye pigmentation (c) were examined. $n=10$.

Table 1
Effect of α-tocopherol on *Smed-dlp1(RNAi) animals.*

	15 days		21 days	
α-tocopherol (mg/g)	Survival (%)	Head regression (%)	Survival (%)	Head regression (%)
None	70 (n=14/20)	29 (n=4/14)	0 (n=0/20)	0 (n=0/0)
3.3×10^{-5}	90 (n=18/20)	11 (n=2/18)	60 (n=12/20)	67 (n=8/12)
9.3×10^{-5}	100 (n=20/20)	0 (n=0/20)	100 (n=20/20)	35 (n=7/20)
1.4×10^{-4}	100 (n=20/20)	0 (n=0/20)	100 (n=20/20)	20 (n=4/20)

Smed-dlp1(RNAi) planarians were fed bovine liver supplemented with α-tocopherol every three days.

regeneration after bisection. Amelioration of these phenotypes using α-tocopherol suggests the importance of oxidative stress for the phenotypes.

Conflicts of interest

The authors declare that there are no conflicts of interest and that no ethical approval was required for this work.

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