Involvement of Elongin C in the spread of repressive histone modifications

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SUMMARY

In our previous work, we induced RNA interference (RNAi) against the spectinomycin resistance-conferring *aadA* transgene by transcribing a long inverted repeat in *Chlamydomonas reinhardtii*. However, after long-term culture, the level of transcripts of the inverted repeat was markedly decreased. In this study, we performed random insertional mutagenesis of the RNAi strain to identify the genes that contribute to the transcriptional silencing of the silencer construct. We succeeded in isolating several mutants showing derepression of transcription of the inverted repeat. One of these tag mutant strains, 148-10H, had a deletion of the Elongin C gene (*ELC*), which is a component of some E3 ubiquitin ligase complexes. In the mutant, the level of monomethyl histone H3 on lysine 9 (H3K9me1) was reduced to less than half of the parental strain, and a large portion of deacetylated H3 marks were removed from the promoter region of the silencer construct, while these repressive histone modifications and levels of methyl-CpG levels were retained in the inverted repeat region. The most probable interpretation of the above-mentioned phenomenon is that ELC is essential for stepwise extension of heterochromatin formation that is nucleated in the inverted region over the promoter region.

Keywords: methyl-CpG, histone modification, random insertional mutagenesis, RNA interference, transcriptional silencing.

INTRODUCTION

The eukaryotic genome is organized into two types of chromatin, euchromatin and heterochromatin (e.g. Farkas *et al.*, 2000). These distinct chromatin structures are characterized by DNA methylation and histone modifications which work cooperatively to regulate transcriptional activity (Chan *et al.*, 2005; Kouzarides, 2007; Vaillant and Paszkowski, 2007; Vaissière *et al.*, 2008). In land plants, cytosine methylation at the CpHpG sites (where H represents A, T, or C) is faithfully inherited by daughter cells in addition to the symmetric CpG sites (Tariq and Paszkowski, 2004; Chan *et al.*, 2005). In *Chlamydomonas reinhardtii* (Hattman *et al.*, 1978) and its closely related multicellular relative *Volvox carteri* (Babinger *et al.*, 2001), the frequency of cytosine methylation is very low (around 5%) and methylcytosine is mostly limited to CpG sites as it is in vertebrates (Hendrich and Tweedie, 2003; Feng *et al.*, 2010). Methyl-CpG-mediated transcriptional repression has been reported in *C. reinhardtii* (Babinger *et al.*, 2001, 2007; Casas-Mollano *et al.*, 2007; Yamasaki *et al.*, 2008).

Long inverted repeat DNA constructs are generally used to induce strong RNA interference (RNAi) in organisms lacking RNA-dependent RNA polymerase, which is an essential enzyme for the generation of double-stranded RNA from single-stranded RNA (for a review, see Cerutti and Casas-Mollano, 2006). *Chlamydomonas reinhardtii* and humans lack RNA-dependent RNA polymerase (Cerutti and Casas-Mollano, 2006). In those RNAi strains of *C. reinhardtii* reported so far that contain a long inverted repeat transgene (i.e. a silencer), knockdown efficiency is often attenuated after long-term culture (Rohr *et al.*, 2004; Schroda, 2006).

We recently demonstrated that a decline in efficiency of RNAi is attributable to repression of silencer transcription (Yamasaki *et al.*, 2008). Transcriptional repression of the silencer was shown to be positively related to the levels of repressive histone marks, such as H3K9me1 and deacetylation of H3, which are associated with the promoter and inverted repeat regions and also with the frequency of methyl-CpG in the inverted repeat regions (Yamasaki *et al.*,

2008). A previously generated RNAi strain, RNAi-37, contained a transcriptionally active aminoglycoside 3''-adenyltransferase transgene (*aadA*) to confer spectinomycin resistance, as well as an inverted repeat construct to induce RNAi that targets the *aadA* mRNA. This *aadA* silencer construct contains an inverted repeat composed of an N-terminal deletion of an approximately 680-bp fragment of *aadA* open reading frame (ORF). The inverted repeat is arranged such that it is driven by the *C. reinhardtii* small subunit of the ribulose-bisphosphate carboxylase gene (*RBCS2*) promoter (Yamasaki *et al.*, 2008). In RNAi-37, the level of *aadA* mRNA was downregulated to 10–60% of that of the parental strain 19-P(1030), which does not contain the *aadA* silencer construct (Yamasaki *et al.*, 2008). Therefore, the growth of RNAi-37 was clearly inhibited by spectinomycin irrespective of the active transcription of the *aadA* gene.

After long periods of successive culture and subsequent isolation of a single colony, we obtained a clone of RNAi-37 that stably showed strong transcription of the inverted repeat transgene (termed RNAi-37S, and noted as a strongly silenced RNAi-37 in our previous work; Yamasaki *et al.*, 2008), which was manifested as lowered spectinomycin resistance. Around 10% of the *aadA* mRNA remained in this strain (Yamasaki *et al.*, 2008). However, another clone of RNAi-37 showed higher spectinomycin resistance as a result of epigenetic transcriptional silencing of the silencer itself (termed RNAi-37W; previously noted as a weakly silenced RNAi-37 in Yamasaki *et al.*, 2008). Around 40% of the *aadA* mRNA remained in this strain because of the limited amount of hairpin RNA available to generate siRNA (Yamasaki *et al.*, 2008).

In this study, we obtained a tag mutant of RNAi-37W that lacks the Elongin C gene (*ELC*). The mutant showed release of transcriptional silencing of the silencer. Based on our observations, we will describe a simple model in which the *ELC* gene is essential for accomplishing the effective repression of the silencer construct by extending the repressive histone modifications that nucleated in the inverted repeat over the promoter region.

RESULTS

Isolation and characterization of a mutant that is defective in silencer silencing

In strain RNAi-37W, knockdown efficiency is significantly decreased as a result of transcriptional silencing of the silencer construct. On the other hand, in the case of another RNAi-37 derivative strain, i.e. RNAi-37S, the level of transcription of the silencer was maintained as high as that of the primary RNAi-37 transformants (Figure 1a and Table S1 in Supporting Information; Yamasaki *et al.*, 2008).

We attempted to isolate the genes involved in epigenetic repression of the inverted repeat by screening tag-inserted mutants. We performed random insertional mutagenesis of
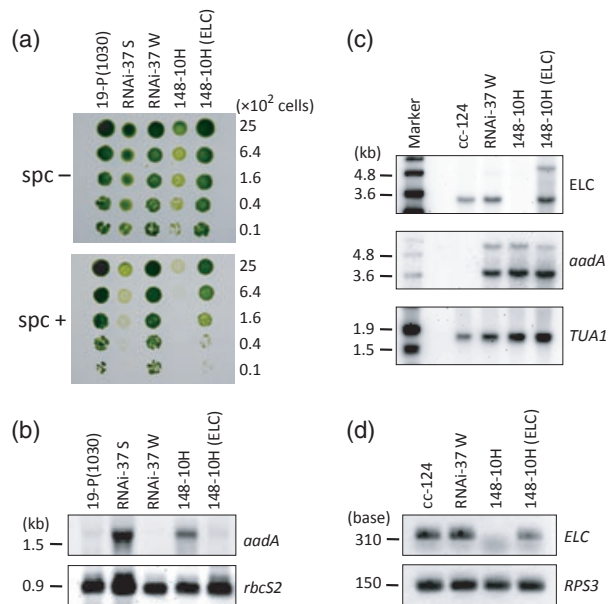

Figure 1. Characteristics of the *ELC* gene-deleted tag mutant 148-10H and the gene-complemented strain 148-10H(ELC).
(a) Spotting tests to compare relative levels of spectinomycin resistance in the indicated strains. 19-P(1030) cells are host cells of RNAi-37. RNAi-37S and RNAi-37W are subclones of the RNAi-37 strain. 148-10H is an *ELC*-deleted tag mutant derived from RNAi-37W. 148-10H(ELC) is an *ELC*-complemented strain. spc+, 2-amino-2-(hydroxymethyl)1,3-propanediol (TRIS)–acetate–phosphate (TAP) plate containing spectinomycin (100 µg ml^{-1}); spc-, TAP plate not containing spectinomycin. Numbers of spotted cells are indicated to the right of the panels.
(b) Northern hybridization of the *aadA* inverted repeat transcript. Ten micrograms of total RNAs from the indicated strains was resolved on a 1.2% agarose–formaldehyde–3-(*N*-morpholino)propanesulfonic acid (MOPS) gel and transferred to a nylon membrane. A 5′-terminal *aadA* sense RNA probe was used to detect an intact *aadA* hairpin RNA of about 1.5 kb intact. Detection of the *RbcS2* mRNA was performed using the same membrane to confirm an approximately equal loading of RNAs.
(c) Southern hybridization to confirm the deletion of the *ELC* gene in 148-10H and successful complementation of the *ELC* gene in 148-10H(ELC). To demonstrate the sensitivity of the detection system, Southern hybridizations to detect the *aadA* gene (the middle panel) and the alpha-tubulin 1 gene (*TUA1*) (the lowest panel) were also carried out. The upper faint band in the middle panel shows the *aadA* gene, while the lower dense band is the signal for the *aadA* inverted repeat construct. The same membrane was reused for these detections. cc-124 is the wild-type strain of *Chlamydomonas reinhardtii*.
(d) A RT-PCR analysis of Elongin C mRNA. DNaseI-treated total RNAs were used for reverse transcription. PCR products were resolved on a 1.5% agarose–TBE gel and visualized by SYBR Green I staining. The ribosomal protein S3 (*RPS3*) gene was amplified as a positive control for RT-PCR.

RNAi-37W using a linearized plasmid *aphVIII*-pKF18-2 as a tag, which contains an aminoglycoside 3′-phosphotransferase gene VIII (*aphVIII*) expression cassette to confer paromomycin resistance (Sizova *et al.*, 2001). To obtain prospective tag mutants in which transcriptional repression of the inverted repeat transgene was released we subsequently screened paromomycin-resistant transformants, i.e. tag plasmid-integrated cells, for a change in spectinomycin resistance. One hundred and twenty-six of 31 000 paromomycin-resistant transformants were found to display

significantly increased sensitivity to spectinomycin (data not shown), which indicates the efficiency of recovered RNAi. Simple Southern hybridization revealed that 50 of the 126 strains were single-tag-inserted transformants, while the others contained two or more tags (data not shown). Among these 50 single-tag transformants, we focused on 148-10H, which showed markedly weaker spectinomycin resistance than the parental RNAi-37W (Figure 1a). That is the expected phenotype of a strain in which silencer repression is released.

To identify the genomic locus where the tag-plasmid was integrated, we amplified the tag-flanking chromosomal DNA fragments by the restriction enzyme site-directed amplification (RESDA)-PCR method (González-Ballester *et al.*, 2005). Sequence information for the tag-flanking sites showed that a chromosomal DNA region about 11 kb long was deleted in 148-10H (Figure 2) along with the tag insertion. The deleted region corresponded to the Scaffold_23:154845-165931 (JGI *Chlamydomonas* genome browser v4.0; http://genome. jgi-psf.org/Chlre4/Chlre4.home.html). To verify whether this deletion is responsible for the phenotype of 148-10H, we performed complementation analysis using a bacterial artificial chromosome (BAC) clone, 31C19, that contains the deleted chromosomal region and its flanking regions (Figure 2). We co-transformed 148-10H with the 31C19 BAC clone along with the pHyg3 plasmid, which carries a hygromycin resistance gene (Berthold *et al.*, 2002) as a transformation marker (i.e. these two DNA molecules were not ligated). We isolated 2880 hygromycin-resistant transformants. Among them, 42 successful co-transformants reduced spectinomycin resistance compared with 148-10H, which is the anticipated phenotype of the successfully

complemented 148-10H tag mutant. On the other hand, no such transformant was obtained when only the pHyg3 co-transformation marker plasmid was used (Table S2). These results indicate that the gene(s) responsible for the 148-10H phenotype resides in the 31C19 BAC clone.

The *Chlamydomonas* genome browser (http://genome. jgi-psf.org/Chlre4/Chlre4.home.html) indicated the presence of four genes within the deleted genome region. To identify the gene responsible for the observed phenotype, we performed further complementation analyses using three partially overlapping subclones derived from this BAC (Figure 2). Among these three subclones, only one plasmid, 31C19-EX, which was predicted to contain only one intact gene that encodes the ELC protein homolog, was able to rescue the phenotype (Figure 2, Table S2). The *Chlamydomonas ELC* gene model showed a high level of amino acid sequence conservation with ELC proteins identified from a wide range of organisms (Figure S1). This protein has been shown to be a component of the E3 ubiquitin ligase complex (Pickart, 2001; Glickman and Ciechanover, 2002; Bosu and Kipreos, 2008) and it is also a component of the RNA polymerase II elongation activator, Elongin (SIII).

We selected an *ELC*-complemented clone, named 148-10H(ELC), which had a level of spectinomycin resistance as high as that in the low-efficiency RNAi strain, RNAi-37W (Figure 1a). In this transformant, the silencer transcript, 1.6-kb-long *aadA* hairpin RNA in 148-10H(ELC), was markedly reduced to the level of that in RNAi-37W (Figure 1b).

Careful Southern hybridization analysis indicated the presence of an intact (3.6-kb band in Figure 1c) and a truncated copy of the *ELC* (4.8-kb band in Figure 1c) in 148-10H(ELC). Furthermore, using the RT-PCR method, a
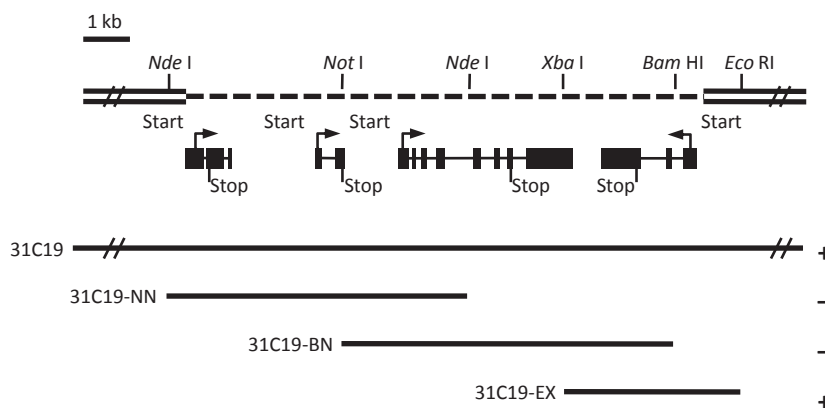

Figure 2. Schematic diagram of the deleted genomic region in strain 148-10H and the DNA fragments used for the complementation test.
The deleted genomic region is indicated by a broken line. Positions of several restriction enzyme sites used for subcloning of the 31C19 bacterial artificial chromosome (BAC) clone are also shown. Predicted gene structures in the deleted region are shown together with the translational start and stop codon sites. Solid boxes indicate predicted exons, and solid lines denote predicted introns. The BAC clone 31C19 carries the DNA fragment that covers the deleted region in 148-10H. The three subclones of BAC 31C19 are shown by solid lines. Plasmid 31C19-NN contains a 6.6-kb *Nde*I-digested fragment. Plasmid 31C19-BN has a 6.2-kb *Bam*HI–*Not*I fragment of the BAC 31C19. Plasmid 31C19-EX includes a 3.8-kb *Eco*RI–*Xba*I fragment that contains the *ELC* gene. '+' indicates the ability of a subclone to rescue the mutant phenotype; '–' indicates that the subclone was unable to rescue the mutant phenotype.

portion of the transcript corresponding to *ELC* mRNA (310-bp band) was detected in 148-10H(ELC) as well as in the wild type CC-124 and RNAi-37W. However, it was not detected in the *ELC* gene-deleted 148-10H tag mutant (Figure 1d). We further performed Northern hybridization using a digoxigenin (DIG)-labeled *ELC* RNA probe in 148-10H(ELC) and detected a signal corresponding to the full-length *ELC* mRNA at a level comparable to that in the wild type CC-124 and RNAi-37W. However, no truncated *ELC* transcripts were detected in 148-10H(ELC) (Figure S2a). These results show that only the intact *ELC* derived from the BAC subclone, 31C19-EX, is actively transcribed in 148-10(ELC). Taken together, the above-mentioned results indicate that *ELC* is responsible for the repression of the epigenetic silencing on the silencer observed in the 148-10H tag mutant. Irrespective of the complete deletion of the original *ELC* gene, a faint hybridization signal was observed in the 148-10H tag mutant by northern blot analysis using an antisense *ELC* RNA probe (Figure S2a), whereas *ELC* mRNA was not detected by the RT-PCR analysis in 148-10H (Figure 1d) as described above. This is reasonably explained by the presence of an *ELC* homologous S-phase kinase-associated protein 1 gene (*SKP1*) that also encodes an adaptor protein for the cullin 1 type ubiquitin ligase (Figure S2b). Considering that *ELC* was completely deleted from the 148-10H genome (Figure 1c), the observed signal is probably the result of cross-hybridization of the *ELC* probe to *SKP1* mRNA.

Analyses of the level of methyl-CpG in the inverted repeat transgene

We previously demonstrated that in RNAi-37W the frequency of methyl-CpG was markedly high in the inverted repeat region of the silencer, and no cytosine methylation at the CpHpG and CpHpH sites was detected in the analyzed region by the bisulfite genomic PCR method (Yamasaki *et al.*, 2008). The level of methyl-CpG in the promoter region was much lower than that in the inverted repeat. This was confirmed in the present study (Figure 3). In this strain, the repressive histone marks H3K9me1 and deacetylated H3 were present at a high level in the inverted repeat and extremely abundant in the promoter region. Therefore, repressive histone marks were present at a high level in the promoter region, irrespective of the low level of methyl-CpG (Figures 3 and 4; Yamasaki *et al.*, 2008).

We thus investigated the levels of methyl-CpG in 148-10H and 148-10H(ELC), using the methylcytosine-sensitive isoschizomeric restriction enzymes *Msp*I and *Hpa*II. Both enzymes recognize the same target sequence CCGG but differ in their sensitivity to methylcytosine (*C). *Hpa*II does not cleave *CCGG and C*CGG, whereas *Msp*I is able to cleave C*CGG but not *CCGG. We detected only a band of 482 bp in both strains, irrespective of the restriction enzymes used to digest the genomic DNA (Figure 3). Moreover, careful analyses showed that the bands detected in the

*Msp*I samples were significantly stronger than those in the *Hpa*II samples for both strains. This implies that methyl-CpG exists in a limited quantity in the promoter region in these strains as observed in RNAi-37S and RNAi-37W (Figure 3). Using the *aadA* probe, we detected a prominent 553 bp long band in addition to a 520 bp long band in the *Hpa*II-digested samples of 148-10H, 148-10H(ELC), and RNAi-37W, but not in the RNAi-37S sample (Figure 3). In the *Msp*I-treated samples, the 553 bp bands were not detected, irrespective of the type of strain. Moreover, the intensity of the 553 bp bands was nearly the same in 148-10H and 148-10H(ELC), and the levels of signals in these strains were lower than those in RNAi-37W (Figure 3). Thus methyl-CpGs may be present in substantial amounts at the 5′-CCGG-3′ sites in the inverted repeat region of 148-10H and 148-10H(ELC), and at a much higher frequency in RNAi-37W (Figure 3). In the inverted repeat, the levels of methyl-CpG were almost the same in 148-10H and 148-10H(ELC), in spite of the significant difference in the transcript level (Figure 1b). Thus, *ELC* deletion may have caused marked release of the transcriptional silencing of the silencer but no dramatic decrease in methyl-CpGs in the inverted repeat in 148-10H.

Analysis of histone modifications associated with the promoter region

We investigated the histone modifications associated with the promoter region of the silencer by chromatin immunoprecipitation (ChIP) assays. In the promoter region, the level of H3Ac was dramatically increased in 148-10H compared with that in the parental RNAi-37W, whereas in the *ELC*-complemented strain the level was similar to that in RNAi-37W (Figure 4). While the repressive histone marker H3K9me1 was prominently decreased in 148-10H, and in the *ELC* complement strain 148-10H(ELC), it was increased to the comparable level that in RNAi-37W (Figure 4). Thus, histone modifications in the promoter of 148-10H were changed to the active type by deletion of the *ELC* gene. On the other hand, the levels of H3Ac and H3K9me1 in the promoter region of 148-10H(ELC) were restored to levels similar to those in RNAi-37W by complementation of the *ELC* gene. Therefore, transcriptional derepression and repression of the silencer were easily explained by changes in histone modification in the promoter region where no dramatic change in the level of methyl-CpG occurred (see above).

Analysis of histone modifications associated with the inverted repeat region

In contrast to the above results, the levels of H3Ac and H3K9me1 in the inverted repeat region were not markedly changed among the tag mutant 148-10H, the *ELC*-complemented strain 148-10H(ELC), and the parental strain RNAi-37W (Figure 4). Therefore, the presence of *ELC* had no marked effects on histone modifications in the inverted repeat region.
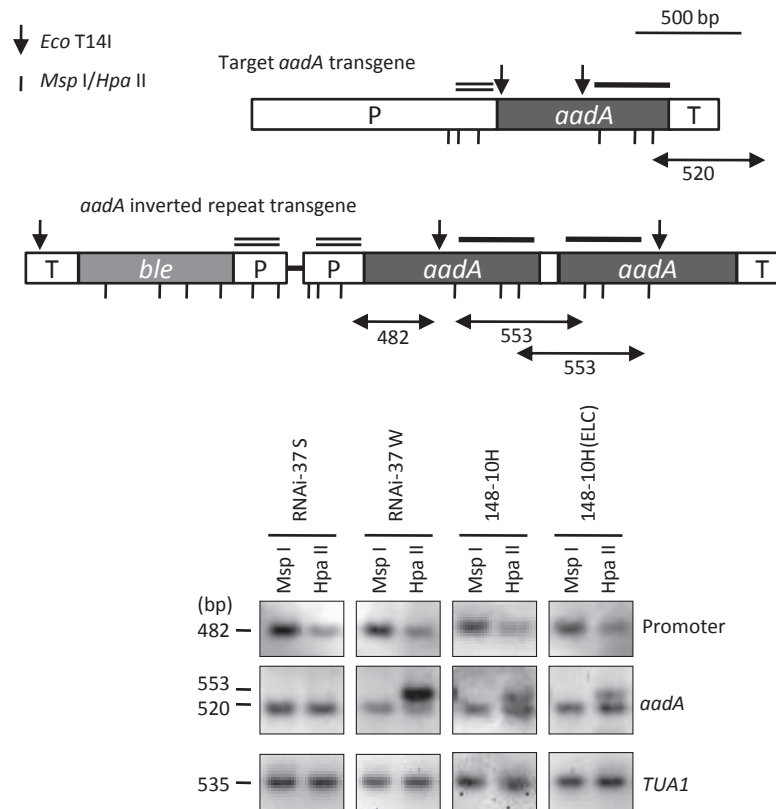
Figure 3. Analyses of the levels of methyl-CpG using methylation-sensitive restriction enzymes.
Southern hybridization analyses were performed to estimate the frequency of methyl-CpG in the inverted repeat transgene. Genomic DNA of the indicated strains was digested with the methyl-CpG-insensitive restriction enzyme *Eco*T14I, and subsequently treated with a 5-methylcytosine-sensitive restriction enzyme (*Msp*I or *Hpa*II). Schematic diagrams of the target *aadA* transgene and the inverted repeat transgene are shown at the top. 'P' indicates the *RbcS2* promoter, 'T' indicates the *RbcS2* terminator. '*ble*' is a transformation marker gene that confers bleomycin resistance. Arrows indicate *Eco*T14I sites. Short vertical bars indicate *Msp*I/*Hpa*II sites. The promoter probe, indicated by a double line, was used to evaluate the frequency of methyl-CpG in the *RbcS2* promoter region of the inverted repeat. The probe hybridizes to digested fragments of the promoter of the *aadA* transgene, the inverted repeat transgene, and the endogenous *RbcS2* gene. A 482-bp fragment detected by this probe corresponds to the completely digested fragment of the *rbcS2* promoter–*aadA* region in the inverted repeat transgene (Yamasaki *et al.*, 2008). The decreased levels of 482-bp signal in *Hpa*II-digested samples show the presence of methyl-CpGs in the *rbcS2* promoter region of the inverted repeat transgene. The *aadA*-C probe, indicated by a single black line, revealed 520- and 553-bp fragments. The 553-bp signal in *Hpa*II-digested samples shows the presence of methylated CpG in the *aadA* IR region. As an internal control, complete digestion of the alpha-tubulin 1 gene (*TUA1*) was confirmed by detection of the 535-bp fragment.

Analysis of changes in histone modifications in endogenous genes

We also investigated the changes in histone modification in endogenous genes in *Chlamydomonas*. The *TOC1* retrotransposon and DNA-transposon *Gulliver* were selected as representatives of stably repressed genes, while the ribosomal protein gene *RPS3* was chosen as a representative of actively transcribed genes. In contrast to the tremendous changes in histone modification seen in the silencer promoter region, H3K9me1 and H3Ac levels associated with the promoters of *TOC1* and *Gulliver* as well as that of the *RPS3* were not found to be different, irrespective of the absence of the *ELC*. We constantly observed marked accumulation of the silent marker H3K9me1 and a limited level of the active marker H3Ac in the *TOC1* long terminal repeat (LTR) region, even in the *ELC*-deleted strain 148-10H (Figure 4 for *TOC1*, and data not shown for *Gulliver*).

Moreover, when changes in these modifications were analyzed using whole-cell histones, no significant difference was observed among the four analyzed strains: RNAi-37S, RNAi-37W, 120-9H, and 148-10H (Figure S3). These results suggest that the ELC-mediated effect is very likely to be restricted to some specific promoters, such as those that are located in front of the inverted repeats or repetitive genes.

Analysis of the link between the level of methyl-CpG and histone modifications in the inverted repeat in the *ELC*-deficient condition

In RNAi-37S, showing good matching with the active transcription of the silencer, the promoter region was poor in methyl-CpGs and repressive histone marks. The abovementioned characteristics are also observed in the inverted repeat region (Figures 3 and 4; Yamasaki *et al.*, 2008). In the parental strain of 148-10H, i.e. RNAi-37W, the inverted repeat region was rich in methyl-CpGs and repressive histone
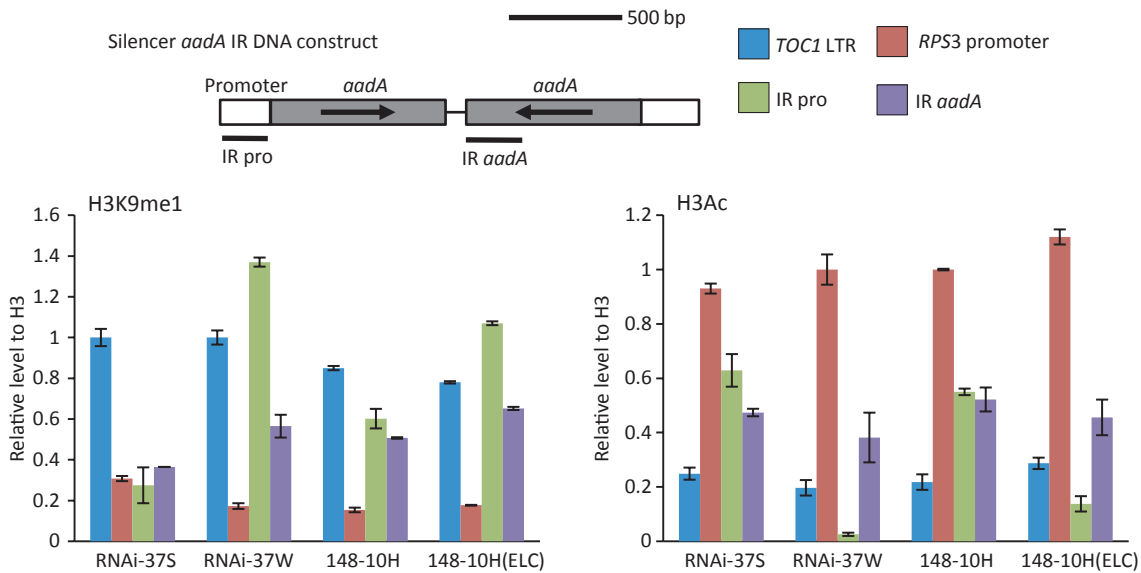
Figure 4. Changes in repressive histone modifications in the *ELC*-deficient strain 148-10H and the *ELC*-complemented strain 148-10H(ELC).
A schematic diagram of the inverted repeat transgene is shown at the top. The PCR-amplified regions are indicated by horizontal bars under the diagram. Levels of H3K9me1 and H3Ac were evaluated for those associated with the promoter region of the inverted repeat transgene (IR pro), the middle region of the inverted repeat transgene (IR *aadA*), the long terminal repeat (LTR) region of the *TOC1* retrotransposon (*TOC1* LTR), and the promoter region of the active *RPS3* gene (*RPS3* promoter). To compare histone modification states, antibodies against H3, H3K9me1, and H3Ac were used for the chromatin immunoprecipitation (ChIP) assay. Immunoprecipitated DNA was examined by a real-time PCR method. For illustration purposes, relative frequency was calculated based on anti-H3 immunoprecipitated DNA. Levels of H3K9me1 were normalized to the level of the *TOC1* LTR of RNAi-37W (left panel), whereas levels of H3Ac were normalized to the level of the *RPS3* promoter of RNAi-37W (right panel). Analyzed strains are indicated below the graph. No precipitated DNA was detected using normal mouse IgG. Results are shown as mean ± SD from three independent experiments.

markers, while its promoter region was poor in methyl-CpGs and rich in repressive histone markers (Figures 3 and 4; Yamasaki *et al.*, 2008). In 148-10H, the frequencies of repressive histone modifications H3K9me1 and deacetylated H3Ac in the promoter were changed to active types, despite the fact that such repressive histone modifications were retained along with the significant level of methyl-CpG in the inverted repeat region (Figures 3 and 4).

To closely analyze the linkage between the histone modifications and levels of methyl-CpG in the inverted repeat region under the *ELC*-deficient condition, we newly isolated a 148-10H derivative clone in which the level of methyl-CpG in the inverted repeat was shifted low enough to prevent detection of the 553 bp-long DNA fragment by Southern hybridization, leaving the level of methyl-CpG in the promoter unchanged (Figure 5c) (hereafter, 148-10H(Low-*CpG) strain). Profiles of this strain were analyzed in comparison with those of 148-10H in which the inverted repeat has a significant level of methyl-CpG.

Ignoring the different levels of methyl-CpG in the inverted repeat region (Figure 5c), these two strains showed almost the same level of hairpin transcript (Figure 5b). In accordance with the levels of transcript, histone modifications in the promoter region were retained as active types and there was no significant difference between 148-10H and 148-10H(Low-*CpG) (Figure S4). The levels of H3Ac were much

higher than those in the parental RNAi-37W, and the levels of H3K9me1 were about half of those in RNAi-37W, irrespective of the different levels of the methyl-CpG in the inverted repeat region (Figure 5c).

Unlike the levels of modified histones in the promoter region, the level of H3Ac in the inverted repeat region showed a positive correlation with the level of methyl-CpG. The H3Ac level was significantly higher in 148-10 H(Low-*CpG) than in 148-10H in that region. However, the levels of H3K9me1 in that region were almost the same in the two strains (Figure S4). In the inverted repeat region, the above-mentioned results suggest that the positive relationship between the frequency of methyl-CpG and the level of H3Ac was retained even in the *ELC*-deficient condition.

DISCUSSION

In this study, we have shown that deficiency of the Elongin C in *Chlamydomonas* induces transcriptional derepression of the long inverted repeat transgene. This protein was originally identified in a heterotrimer complex named Elongin (SIII), which is one of the five elongation activators for RNA polymerase II (Aso *et al.*, 1995). Elongin (SIII) strongly stimulates the rate of elongation by suppressing transient pausing of RNA polymerase II along the DNA molecule (Aso *et al.*, 1995). Elongin (SIII) is composed of Elongin A, B, and C subunits. Elongin A has been shown to function as the main
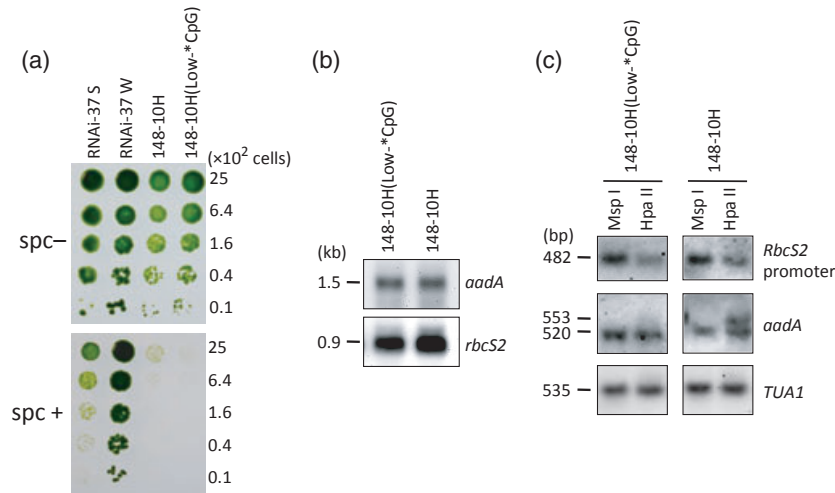
Figure 5. Analysis of accumulated silencer transcript in *ELC*-deleted148-10H and *ELC*-complemented 148-10H(ELC).
(a) Spotting test to compare relative levels of spectinomycin resistance. 148-10H(Low-*CpG) is a derivative strain of 148-10H having a low level of methyl-CpG in the inverted transgene. (See the legend of Figure 1 for details.)
(b) Northern hybridization of the *aadA* hairpin RNA transcript. The upper panel shows the hairpin RNA levels. After detection of the hairpin RNA, the 5′-terminal *aadA* sense RNA probe was stripped, and a second hybridization was performed with an antisense *RbcS2* RNA probe as a control (lower panel) to confirm the approximately equal loading of RNAs.
(c) Southern hybridization analyses to detect methyl-CpGs in the inverted repeat transgene. Frequency of methyl-CpGs in the inverted repeat region was evaluated as described in the legend of Figure 3. A *TUA1* probe was used (bottom panel) to confirm complete digestion.

transcription-activating protein, while Elongin B and C have roles as regulatory subunits (Takagi *et al.*, 1996; Conaway *et al.*, 2002). Furthermore, Elongin B and C were recently shown to act as adaptor proteins of some cullin-type E3 ubiquitin ligases (Ribar *et al.*, 2006; Yasukawa *et al.*, 2008).

Ubiquitin is conjugated to substrate proteins in a series of reactions mediated by three enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-protein isopeptide ligase (E3). In the final step, ubiquitin is transferred from the ubiquitin-conjugating enzyme (E2) to the target substrate by the E3 ligase complex (for a review see Deshaies and Joazeiro, 2009). Some multisubunit E3 ubiquitin ligases, i.e. cullin 2 and cullin 5-based complexes, are known to contain Elongin B and C as their adaptor proteins (for a review, see Hotton and Callis, 2008).

Considering the accumulating evidence on transcriptional regulation mediated by ubiquitination, we described three possible scenarios to explain the derepression of the inverted repeat mediated by Elongin C deficiency.

We previously showed that various lengths of incomplete transcripts of the silencer construct accumulated abundantly in RNAi-37 strains (Yamasaki *et al.*, 2008). This implies that RNA polymerase II often stalls at various points during transcription of the inverted repeat. Moreover, polyubiquitination of RNA polymerase II has been proved to act efficiently to remove the arrested RNA polymerase II from the template DNA. In the mouse, cullin 5/*Rbx2* is recruited to Elongin (SIII) (Elongin A/B/C complex) located on the arrested RNA polymerase II (Yasukawa *et al.*, 2008). Then active E3 ubiquitin ligase is assembled on the Elongin B/C

complex to exert polyubiquitination for proteolytic removal of the arrested RNA polymerase II (O'Connell and Harper, 2007), thereby enabling the next RNA polymerase II to transcribe the gene.

The above-mentioned reports suggest that in an Elongin C-deficient strain, arrested RNA polymerase II may not be promptly removed from the inverted repeat. Therefore, decrease in silencer transcripts and the reduced levels of RNAi are typical characteristics that should be observed in 148-10H. However, our observation was that transcripts of the inverted repeat were markedly increased, enhancing the RNAi effect in the *ELC*-deficient tag mutant 148-10H. Therefore, Elongin C must have another role that is related to strong transcriptional repression of genes besides polyubiquitination of arrested RNA polymerases.

The second scenario is as follows. It has been reported that ubiquitination of H2A and H2B has a marked effect on the regulation of gene transcription and remodeling of chromatin structure (Hicke, 2001; Osley, 2006; Latham and Dent, 2007; Sridhar *et al.*, 2007). Ubiquitinated H2B (Ub-H2B) was found to be present in *C. reinhardtii* (Shimogawara and Muto, 1992), but Ub-H2A was not detected in the same investigation in Arabidopsis and *Saccharomyces cerevisiae* (Zhou *et al.*, 2008). We will therefore restrict our discussion to a mechanism that may be related to the Ub-H2B-mediated transcriptional regulation in which Elongin C may be implicated.

In *S. cerevisiae*, the *Rad6* (E2)/*Bre1* (E3) complex is responsible for Ub-H2B formation; this ubiquitination induces the methylation of histones H3K4 and K79, both of

which are representative activating marks for chromatin remodeling and transcription (Robzyk *et al.*, 2000, Sun and Allis, 2002; Kim and Roeder, 2009). Therefore, even on the assumption that the Elongin B/C complex in *Chlamydomonas* is the adaptor for H2B ubiquitin ligase, deficiency of H2B ubiquitin-ligase activity is the least likely to lead to the release of silencer transcription, as observed in 148-10H.

The third scenario is as follows. Our data show that a deficiency of Elongin C positively changed the histone modifications in the promoter to active types, and the deficiency exerted no significant effect on histone modifications in the inverted repeat region. The level of H3K9me1 in the inverted repeat was as high as it was in its parental strain RNAi-37W in 148-10H, irrespective of the enhanced transcription. This led us to speculate that repressive histone modifications, especially H3K9me1, which nucleated in the inverted repeat, were unable to extend over the promoter region in the Elongin C-deficient cell.

Thus, stepwise self-propagating heterochromatin formation can be summarized briefly as follows. Deacetylations of H3K9, K14, and K16 induce methylation of H3K9, and methylated H3K9 then increases its affinity to *Swi6/Chp2* and localizes them to the nucleosome. Subsequently, *Swi6/Chp2* recruits histone deacetylases to the adjacent nucleosomes to establish a positive feedback loop for heterochromatin formation (Nakayama *et al.*, 2001).

Moreover, accumulating evidence in fission yeast shows that enzymes involved in heterochromatin formation are assembled in a large complex, which contains cullin 4/*Rik1*-based E3 ubiquitin ligase. The target substrate of this ubiquitin ligase has not been identified. However, mutations in cullin 4 or *Rik1* cause a severe defect in heterochromatic silencing in *Saccharomyces pombe* (Horn *et al.*, 2005; Jia *et al.*, 2005). Therefore, normal activity of this ubiquitin ligase is essential for the formation of a heterochromatic region.

Assuming that cullin 4/*Rik1*-based ubiquitin ligase in *Chlamydomonas* is replaced by Elongin C-containing E3 ubiquitin ligase, derepression of silencer transcription may occur in the cell lacking Elongin C because of the defect in self-propagating heterochromatin formation.

In the case of wild-type *Chlamydomonas*, heterochromatin formation very likely spreads over the promoter region, irrespective of the present level of methyl-CpG in it. Moreover, in *Chlamydomonas*, a high level of methyl-CpG in the inverted repeat region may be an effective trigger for heterochromatin formation. On the other hand, small interfering RNA (siRNA) may not work as the main trigger for nucleating heterochromatin formation, because *aadA*-siRNAs were unable to exert efficient transcriptional silencing of the *aadA* gene in *trans* in this organism (Yamasaki *et al.*, 2008). Taken together, the observed characteristics of the transformants are interpreted as follows: RNAi-37W, which has a high level of methyl-CpG in the inverted repeat,

showed limited transcription because of the repressive histone markers and heterochromatin formation in the promoter. Such repressive histone modifications were most probably nucleated in the inverted repeat and extended over the promoter region. On the other hand, the methyl-CpG content in the inverted repeat region was much lower in RNAi-37S, and this probably led to the limited nucleation of heterochromatin formation in the silencer construct, so that the histone modifications in the promoter were active types. In the case of 148-10H, nucleation of heterochromatin formation might be initiated in the inverted repeat region because of the substantial level of methyl-CpG; however, this process was unable to spread over the promoter region because of a deficiency of *ELC*. The above-mentioned model is the simplest one to explain the release of transcriptional silencing of the inverted repeat in 148-10H, irrespective of the retained methyl-CpG level in the inverted repeat region.

In *Chlamydomonas*, H3 histone monomethyltransferase SET3 is known to regulate the expression of repetitive *aadA* transgenic genes arranged in inverted repeat and direct repeats (Casas-Mollano *et al.*, 2008). RNAi-mediated suppression of the gene markedly reduced the global levels of H3K9me1 and reactivated the expression of repetitive *aadA* transgenic arrays. Interestingly, this reactivation was accompanied by an increase in methyl-CpG in the reactivated repetitive transgenes. The authors interpreted this phenomenon as an opening of the chromatin structure and increased accessibility of CpG methyltransferases to the DNA, which was caused by reduction of the H3K9me1 in the *aadA* coding region. To investigate how Elongin C is implicated in the spreading of heterochromatin formation, analysis of complexes that contain this protein will be essential and informative. Elongin C and SET3 may be included in the same complex, as it is often the case that enzymes with opposite effects are included in the same complex to accomplish active tuning of gene expression.

EXPERIMENTAL PROCEDURES

Strains and culture conditions

Unless noted otherwise, all *Chlamydomonas* strains used were grown mixotrophically in liquid 2-amino-2-(hydroxymethyl)1,3-propanediol (TRIS)–acetate–phosphate (TAP) medium with vigorous shaking or on a TAP agar plate under constant white fluorescent light (20 μmol m^{-2} sec^{-1}) at 25°C.

The stable spectinomycin resistant strain, 19-P(1030), is harboring a single copy of an exogenous *aadA* gene expression cassette (Cerutti *et al.*, 1997). 19-P(1030) was used for generation of the RNAi-37 strain by further introduction of a single copy of an *aadA* inverted repeat construct (Yamasaki *et al.*, 2008). Robust knockdown was induced against *aadA* in RNAi-37 immediately after transformation; however, after long periods of successive cultivation, individual clones of RNAi-37 displayed variable knockdown efficiency that showed a correlation with the degree of transcriptional silencing of the inverted repeat construct. RNAi-37S and RNAi-37W are clones of RNAi-37, but they exhibit different levels of sensitivity to spectinomycin. RNAi-37S shows strong knockdown of

aadA, while *aadA* is only weakly suppressed in RNAi-37W (Yamasaki *et al*., 2008).

Spotting test to compare the relative spectinomycin resistance

Independents were cultured to reach stationary phase in liquid TAP medium and subsequently diluted with TAP medium. Five microliters of the diluted samples (about 25×10^2, 6.4×10^2, 1.6×10^2, 0.4×10^2, and 0.1×10^2 cells) were spotted on plates with or without 100 µg ml^{-1} of spectinomycin, then incubated for 7–10 days under constant white fluorescent light (20 µmol m^{-2} sec^{-1}) at 25°C.

Transformation and screening of transformants

The *aphVIII*-pKF18-2 plasmid used for insertional mutagenesis of the RNAi-37W was generated by subcloning the pSI103 *aphVIII* cassette (Sizova *et al*., 2001), which confers paromomycin resistance, into the pKF18-2 plasmid (Takara, http://www.takara-bio.com/). Transformants were selected on TAP plates containing paromomycin (Sigma, http://www.sigmaaldrich.com/) (10 µg ml^{-1}). Subsequently, paromomycin-resistant transformants were screened on TAP plates containing spectinomycin (Sigma) (100 µg ml^{-1}), and spectinomycin-sensitive mutants were identified. Chromosomal DNA sequences flanking the inserted *aphVIII*-pKF18-2 plasmid were determined by the RESDA-PCR method (González-Ballester *et al*., 2005).

The BAC clone 31C19, containing the region which had been deleted from the 148-10H strain, was obtained from the *Chlamydomonas* BAC library (http://www.genome.clemson.edu/). Three 31C19 subclones were generated by recloning the fragments created by the digestion of BAC clone 31C19 DNA with appropriate restriction enzymes: 6.6 kb *Nde*I, 6.2 kb *Bam*HI–*Kpn*I, and 3.8 kb *Eco*RI–*Xba*I. These individual fragments were then ligated into the pT7Blue-1 plasmid (Takara) (Figure 2). Plasmid pHyg3 (Berthold *et al*., 2002), which contains the hygromycin B-resistant marker gene *aphVII*, was used for co-transformation with BAC clone 31C19 or one of its three subclones. Transformants were selected on TAP plates containing hygromycin B (Wako, http://www.wako-chemicals. de/Wako-Gruppe/) (20 µg ml^{-1}). Complemented strains were identified on TAP plates containing spectinomycin (100 µg ml^{-1}).

Preparation of genomic DNA and total RNA

Total DNA was extracted by the cetyl trimethyl ammonium bromide (CTAB) method as described previously (Yamasaki *et al*., 2008). Total RNA was isolated using TRIzol reagent (Invitrogen, http:// www.invitrogen.com/) following the manufacturer's instructions.

Reverse transcription PCR

Total RNA was treated with DNase I (Takara) to remove contaminating genomic DNA. First-strand cDNA was extended from an oligo-dT primer using PrimeScript reverse transcriptase (Takara) following the manufacturer's protocol. The PCR products were resolved on 1.5% agarose/TBE gel and visualized by SYBR Green I staining (Takara). The primer sequences were as follows: ELC-F (5′-TACTGGCAGACCTTCTCCAGGATG-3′) and ELC-R (5′-TCTATTGG CAGGGAGAATAGGTCGG-3′) were used for Elongin C gene; RPS3-F (5′-CGTCCAGATCAGCAAGAAGCG-3′) and RPS3-R (5′-GGATGA TAATCTCAGTGCGCATGG-3′) were used for the *RPS3* gene.

Southern and Northern hybridization

Preparation of membranes and probes for Southern and Northern hybridization using the DIG system were performed as described previously (Yamasaki *et al*., 2008). To determine the copy number of genome-integrated tag-DNA, *Nhe*I-digested and *Xba*I-digested genomic DNA samples were used for Southern hybridization. To examine the methylation status of cytosines in the CCGG sequence, isoschizomeric restriction enzymes, *Hpa*II and *Msp*I, were used to digest genomic DNA. The DIG labeled DNA probes were generated by PCR using the PCR DIG probe synthesis kit (Roche, http://www.roche.com/). The Elongin C DNA probe was generated from BAC clone 31C19 DNA using the primers ELC-F and ELC-R. The DNA probe for *C. reinhardtii* alpha tubulin-1 (GenBank accession number XP001691876) was generated from a cloned alpha tubulin-1 DNA fragment using the primers atub-F (5′-CGCCTCGCTTCGCTTTG-3′) and atub-R (5′-CTGCTCGTGGTACGC-CTTCTC-3′). The DIG-labeled probes to hybridize with the *RbcS2* promoter region, *aadA*, *RbcS2* mRNA and 5′-terminal sense *aadA* RNA were generated as described before (Yamasaki *et al*., 2008). The RT-PCR product of the Elongin C gene was cloned into pT7Blue-1 vector (see section on Reverse transcription PCR), and an anti-sense Elongin C RNA probe was generated from this plasmid using T3 RNA polymerase and DIG RNA labeling mix (Roche). A luminescent image analyzer, LAS-1000 (Fujifilm, http:// www.fujifilm.com/), was used to detect hybridization signals.

Chromatin immunoprecipitaion (ChIP) assay

The ChIP assays and analyses of immunoprecipitated DNA by the real-time PCR method were performed as previously described (Yamasaki *et al*., 2008). Histone H3 antibody (ab1791; Abcam, http:// www.abcam.com/), histone H3K9me1 antibody (ab8896; Abcam), and histone H3Ac antibody (06-599; Millipore, http://www.milli-pore.com/) were used for immunoprecipitations, while normal rabbit IgG (PP64B, Millipore) was used as a negative control.

Immunoblot analysis

Approximately 5×10^8 cells were harvested and resuspended in 2.5 ml of nucleus isolation buffer [20 mM PIPES-KOH (pH 7.0), 0.25 M sucrose, 10 mM MgCl$_2$, 0.1% Triton X-100, 5 mM beta-mercaptoethanol, and 2 µl ml^{-1} of plant protease inhibitor cocktail (Sigma)]. Cells were disrupted twice using the Mini-Bom Cell (Kontes, http://www.kontes.com/) at 10.5 MPa under nitrogen (Shneyour and Avron, 1970), then centrifuged for 10 min at 30 000 *g*. The pellet was resuspended with 0.5 ml of high-salt buffer [20 mM HEPES-KOH (pH 7.5), 2 M NaCl, 1 mM EDTA (pH 8.0), 1 mM DTT, and 2 µl ml^{-1} of plant protease inhibitor cocktail (Sigma)]. The sample was centrifuged for 10 min at 10 000 *g*, and the supernatant containing soluble proteins including histones was collected. Proteins were separated on a SDS-polyacrylamide gel (15%) and electroblotted onto polyvinylidene fluoride (PVDF) membrane (Hybond-P; GE Healthcare, http://www.gehealthcare.com/). Specific modifications on the N-terminal tail residues of histone H3 protein were detected using antibodies against mono-methylated H3K9 (ab8896; Abcam) or acetylated H3 (06-599; Millipore) and the ECL plus Western blotting detection system (GE Healthcare Bioscience). Luminescence was visualized using an LAS-1000 image analyzer (Fujifilm). After stripping membranes of modified histone-specific antibodies, the amount of total histone H3 was determined using a modification-insensitive anti-H3 antibody (ab1791; Abcam).

Alignment of Elongin C

Amino acid sequences of Elongin C from various organisms were obtained from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/in-dex.html) and aligned using ClustalX software (Thompson *et al*., 1997). Their accession numbers are as follows; *S. cerevisiae* Elongin C (GenBank accession number BAA22612), *S. pombe* Elongin C (CAB52743), *Ostreococcus tauri* Elongin C (CAL57216), *Homo*

sapiens Elongin C (NP005936), *Drosophila melanogaster* Elongin C (BAA24287), *Caenorhabditis elegans* elc-1 (NP497405), *Arabidopsis thaliana* putative Elongin C (AAK67779), *C. reinhardtii* Elongin C (XP 001700238).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Alignment of Elongin C amino acid sequences from a wide range of organisms.

Figure S2. Analysis of *ELC* mRNA in the *ELC* gene-deleted strain 148-10H and the gene-complemented strain 148-10(ELC).

Figure S3. Analysis of the levels of H3K9me1 and H3Ac in 148-10H whole cell histones.

Figure S4. Analysis of H3K9me1 and H3Ac in the *ELC*-deficient 148-10H strains that have different levels of methyl-CpG.

Table S1. List of strains and their features.

Table S2. Complementation test of 148-10H using BAC 31C19 and its subclones.

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