Cytologia **87**(2): 163–168

Rapid Selective Proliferation of Mitochondria during Zygote Maturation in the Uniparental Inheritance of *Physarum polycephalum*

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Received December 15, 2021; accepted January 10, 2022

Summary Mitochondrial DNA (mtDNA) is uniparentally inherited in most sexual eukaryotes called maternal/uniparental inheritance. It is well known that selective elimination of paternal mitochondria and digestion of paternal mtDNA are important to this process. However, little is known about the control of maternal mitochondrial proliferation. In the isogamous *Physarum polycephalum*, haploid myxamoebae act as gametes. When two myxamoebal strains with different mating types are mated, mtDNA is inherited from only one strain, defined as the maternal strain. Here, we developed a method to distinguish uniparental mitochondria in zygotes of *P. polycephalum* by chemical staining and investigated the proliferation of maternal and paternal mitochondria during the selective digestion of mitochondrial nucleoids (mt-nucleoids) in paternal mitochondria. We stained the myxamoebae with MitoTracker Red CMXRos (MTR) or MitoTracker Green FM (MTG). MTG-stained mitochondria more specifically than MTR. When the AI35 strain was crossed with the MTG-stained DP246 strain and mtDNA was stained with *N*-aryl pyrido cyanine 3, mt-nucleoids in mitochondria derived from DP246 became shorter in the zygotes 3 h after crossing. There was no significant difference in the number of mitochondria derived from both AI35 and DP246 in early zygotes, but the number of mitochondria derived from AI35 proliferated 1.7-fold from 3 to 5 h after crossing. Similar results were obtained when MTG-stained AI35 crossed with DP246. These results indicate that the proliferation of mitochondria derived from AI35 rapidly occurred after the initiation of digestion of mt-nucleoids derived from DP246. Our results suggest the possibility of controlling the mechanism of the selective proliferation of maternal mitochondria during zygote formation for uniparental inheritance.

Keywords Mitochondrial DNA, Uniparental inheritance, *Physarum polycephalum*, *N*-aryl pyrido cyanine 3, Mitochondrial proliferation.

Mitochondria contain their DNA, which is inherited uniparentally/maternally in almost all sexual eukaryotes, including animals, plants, fungi, and protists (Birky 1995, 2001, 2008, Kuroiwa 2010). This process is called uniparental/maternal inheritance. Previous reports have indicated that both the selective digestion of paternal mtDNA and selective elimination of paternal mitochondria occur during maternal inheritance (Sasaki and Sato 2021). Conversely, maternal mtDNA and mitochondria need to proliferate to be inherited by progeny. PCR analyses show that the mtDNA copy number does not change from the oocyte to 8 cell stage in mice (Cree *et al.* 2008). Studies in *Cryptococcus neoformans*, an isogamous species, have shown that when a-cells are crossed with *α*-cells, mt-nucleoids derived from *α*-cells decrease, whereas those derived from a-cells proliferate during zygote maturation (Nishimura *et al.* 2020). However, the control of mitochondrial proliferation and the relationship between mitochondrial proliferation and mtDNA digestion remains elusive.

Since mitochondria undergo repeated fusion and fission, their shape is complex. Therefore, it is generally difficult to analyze the proliferation of maternal mitochondria after fertilization. In *Physarum polycephalum*, on the other hand, mitochondrial fusion is only observed when the mitochondria contain a mitochondrial fusion

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DOI: 10.1508/cytologia.87.163

plasmid (Kawano *et al.* 1993). Moreover, since the mitochondria contain one long rod-shaped mt-nucleoid, digestion of mtDNA is easily observed as the length of the mt-nucleoids changes. Therefore, *P. polycephalum* is suitable for analyzing the regulation of mitochondrial proliferation during uniparental inheritance. Haploid myxamoebae of *P. polycephalum* act as isogametes, containing approximately 15 simple oval-shaped mitochondria (Moriyama and Kawano 2003). Two myxamoebal strains with different mating types can be mated for zygote formation. At least 720 mating types exist, and mtDNA donors in crosses are defined as maternal parents (Moriyama and Kawano 2003). When the AI35 strain was crossed with the TU41 strain, mtDNA derived from TU41 could not be detected by PCR analysis in zygotes 3 h after mating (Moriyama and Kawano 2003). These zygotes subsequently underwent repeated mitotic cycles without cell division, to form a multinuclear plasmodium. In the plasmodium 36 h after mating, mitochondria derived from TU41 began to be digested (Moriyama and Kawano 2003).

To distinguish between maternal and paternal mitochondria in zygotes, uniparental mitochondria should be labeled. In this study, we developed a method to visualize the uniparental mitochondria in the zygotes of *P. polycephalum* using Mito Tracker Green FM (MTG) and monitored the change in the number of maternal and paternal mitochondria during zygote maturation.

Materials and methods

Cell strains and culture

AI35 and DP246 were used as model *P. polycephalum* strains. We cultured these myxamoebae on PGY rich plates (32.3 mM KH_2PO_4, 11.5 mM K_2HPO_4, 4 mM $MgSO_4$, 0.75% glucose, 1% Bacto peptone, 0.1% Bacto yeast extract, 1.5% agarose) at 24°C with live bacteria (*Aerobacter aerogenes*) as a food source.

Zygote formation

AI35 and DP246 grown on plates were collected in distilled water (DW) and centrifuged at 1,600×*g* for 5 s. Cells were washed twice with DW and suspended in DW. To make zygotes, the same number of AI35 and DP246 were mixed, and then cells were cultured on SM30 mating plates (10.5 mM citric acid monohydrate, 19.5 mM trisodium citrate dihydrate, 10.0 mM $MgSO_4$, 2.5% agarose) at 24°C.

Vital staining of mitochondria in myxamoeba

Myxamoebae were collected and cell suspensions prepared as described above. Subsequently, 2 mM MitoTracker Red CMXRos (MTR; Thermo Fisher Scientific, Waltham, MA, USA) or MitoTracker Green FM (MTG; Thermo Fisher Scientific, Waltham, MA, USA) in dimethyl sulfoxide (DMSO) was added to the final concentration of 1 μM in the cell suspension and the cells were incubated at 20°C for 30 min. The cells were then re-plated on SM30 plates and cultured at 24°C for 4 h.

Vital staining of mtDNA in zygotes

Zygotes were collected on plates in DW. 1 mM *N*-aryl pyrido cyanine 3 (PC3) in DMSO was added to the final concentration of 1 μM in the cell suspension.

Microscopic observation

We used a fluorescence/phase-contrast microscope (BX51N-34FL; Olympus, Tokyo, Japan) connected to a 6 mega pixel color camera (Axiocam 506 color; Zeiss, Oberkochen, Germany). Excitation filters (WIB and WIG) were used for fluorescence observation.

Image analyses

Images were analyzed using the ImageJ software (National Institutes of Health, http://rsb.info.nih.gov/ij/). The length of the mt-nucleoid stained with PC3 was subsequently measured using ImageJ software.

Results and discussion

In this study, we used the AI35 and DP246 strains, neither of which contained a mitochondrial fusion plasmid. Previous PCR analysis results have shown that mtDNA derived from AI35 are selectively inherited by plasmodium when AI35 and DP246 are crossed (Moriyama and Kawano 2003). AI35 and DP246 had 12.3±3.8 and 12.3±3.3 mitochondria per cell (mean± s.d.; n_{cells}=20), respectively. For vital staining of the mitochondria in myxamoebae, we used MTR and MTG. When myxamoebae were observed on agar after staining with 1 μM MTR, the mitochondria were not visible in phase contrast and fluorescence microscopy images (Fig. 1a–c). In addition, fluorescence signals from cell debris attached to the agarose were observed extracellularly and on the cell. On the other hand, observation of myxamoebae in DW improved both images, although the mitochondria became round due to osmotic shock (Fig. 1d–i). The mitochondria were observed as black round organelles by phase-contrast microscopy. The mitochondria were visualized with MTR and MTG, but those dyes also stained other small, round objects (Fig. 1d–f). The MTR-stained cell contained many more such objects than did the MTG-stained cell. Therefore, 1 μM MTG was used for staining in subsequent analyses.

Next, we examined the effect of MTG staining on mating. When unstained AI35 and DP246 were crossed, they synchronously fused to form zygotes, and the ratio of fused cells in myxamoebae was 68.3±1.0% (Fig. 2a). When AI35 or DP246 was stained with MTG and then crossed, 53.2±1.0% and 55.0±2.7% of the ratio of fused cells in the myxamoebae were maintained, respectively.

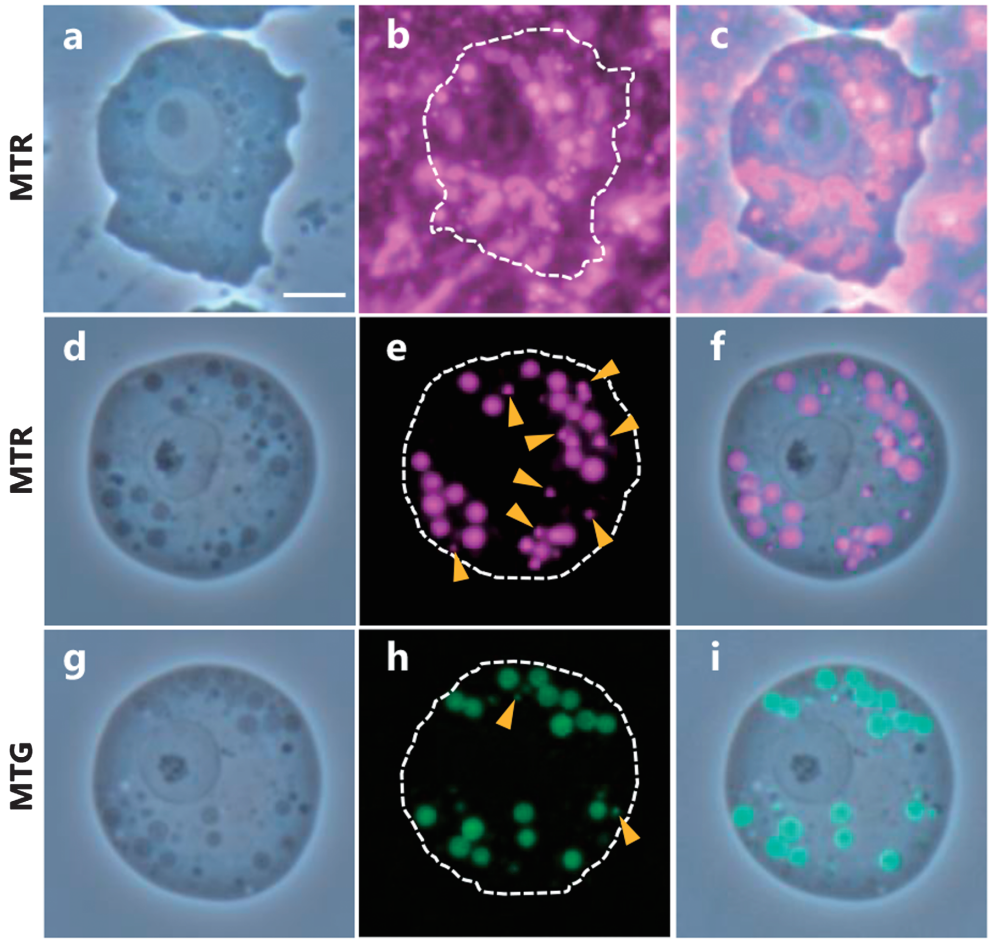

Fig. 1. Vital staining of mitochondria in myxamoebae. Myxamoebae (AI35) were stained with MTR (a–f) or MTG (g–i). AI35 on agar (a–c) and in DW (d–i) were observed with phase contrast and fluorescence microscopy. (a, d, g) Phase-contrast images. (b, e) MTR-stained images. (h) MTG-stained images. (c, f, i) Merged images. The position of the cell (white dotted line) is shown in each of the MTR- and MTG-stained images. Scare bar=5 μm.

We further observed that approximately half of the mitochondria had MTG fluorescence in zygotes 1 h after crossing, indicating that uniparental mitochondria were distinguishable in a zygote (Fig. 2b).

When AI35 and DP246 are crossed, mt-nucleoids become short or disappeared in approximately 50% of the mitochondria in each zygote 5 h after crossing and complete uniparental inheritance are occurred in zygotes 11 h after crossing (Moriyama *et al.* 2005). To confirm whether the digestion of mt-nucleoids occurred only in mitochondria derived from DP246 during zygote maturation, we further visualized mt-nucleoids using PC3, a red fluorescence DNA stain for living cells (Uno *et al.* 2021). In HeLa cells, only the nuclear DNA was stained with 10 nM PC3, whereas mtDNA was predominantly stained with 100 pM PC3. In *P. polycephalum*, mtDNA was predominantly stained with 1 μM of PC3. When AI35 was crossed with MTG-stained DP246, all mitochondria had long rod-shaped mt-nucleoids in the zygotes 1 h after crossing. (Fig. 3a–e). In contrast, in the zygote 5 h after crossing, the mt-nucleoids became shorter in the MTG-stained mitochondria, although long rod-shaped mt-nucleoids were observed in the unstained mitochondria (Fig. 3f–j). These results indicated that the digestion of mt-nucleoids was selectively undergone in the mitochondria derived from DP246 in zygotes 5 h after crossing. This is the first cytological evidence of selective digestion of mt-nucleoids in the uniparental mitochondria of *P. polycephalum*.

Furthermore, we investigated the timing of initiation of mt-nucleoid digestion. As the length of the mt-nucleoid is proportional to the amount of mtDNA in *P. polycephalum* (Sasaki *et al.* 1994), we measured the length of the mt-nucleoids during zygote maturation (Fig. 4a, b). At 1 h after crossing of AI35 with MTG-stained DP246, the length of the mt-nucleoids in mitochondria derived from AI35 and DP246 were 1.50± 0.50 μm and 1.56±0.47 μm, respectively (Fig. 4a). The lengths of mt-nucleoids in mitochondria derived from AI35 did not change in zygotes 3 h and 5 h after crossing. Conversely, in mitochondria derived from DP246, the length of mt-nucleoids began to shorten in the zygote 3 h after crossing, reaching 0.57±0.28 μm in the zygote 5 h after crossing. In addition, when MTG-stained AI35 was crossed with DP246, the mt-nucleoids derived from DP246 became shorter in zygotes 3 h after crossing (Fig.

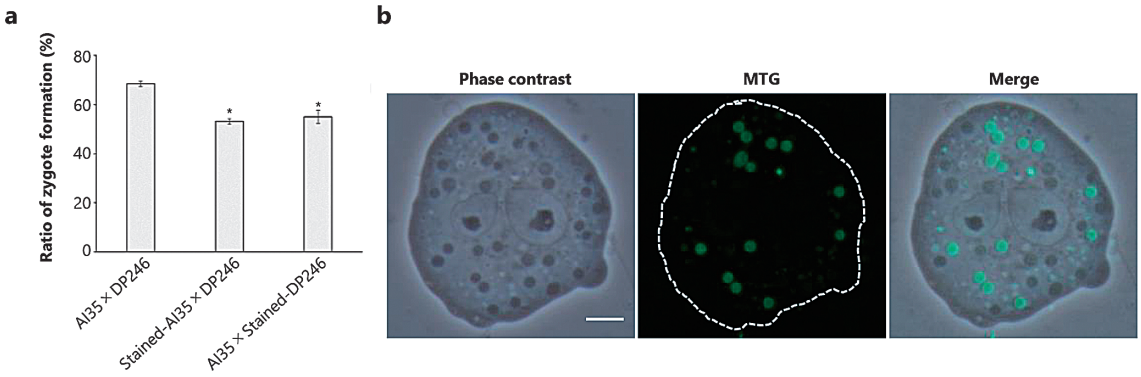

Fig. 2. Zygote formation using MTG-stained myxamoebae. (a) Effect of MTG staining on the mating of myxamoebae. Unstained AI35 and DP246 (AI35×DP246), MTG-stained AI35 and unstained DP246 (stained-AI35×DP246), and unstained AI35 and MTG-stained DP246 (AI35×MTG-stained DP246) were crossed. Cells were observed 1 h after crossing and the ratio of fused cells in myxamoebae was calculated; n_{cells}=272–561. Error bars represent mean±SEM from three independent experiments. Significance differences, defined as $*p<0.05$, were tested using the Dunnett's test. (b) Observation of MTG-stained mitochondria derived from DP246 in a zygote 1 h after crossing. AI35 was crossed with MTG-stained DP246 and zygotes were observed 1 h after crossing. (i) Phase contrast image. (ii) MTG-stained image. (iii) Merged images. Half of the mitochondria in the zygote showed MTG fluorescence. The position of the cell (white dotted line) is shown in the MTG-stained image (ii). Scale bar=5 μm.

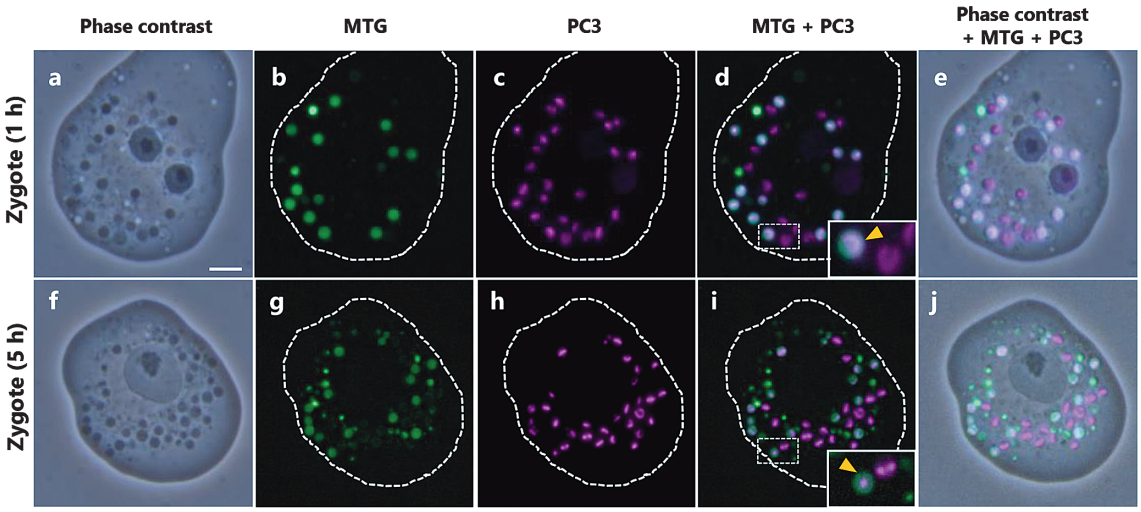

Fig. 3. Selective digestion of mt-nucleoids derived from DP246 in zygotes. AI35 was crossed with MTG-stained DP246 and the zygotes 1 h (a–e) and 5 h (f–j) after crossing were stained with PC3. (a, f) Phase-contrast images. (b, g) MTG-stained images. (c, h) PC3-stained images. (d, i) Merged images of MTG-stained images and PC3-stained images. (e, j) Merged images of phase-contrast images, MTG-stained images, and PC3-stained images. Insets in panels "d and i" are enlarged images from the dashed rectangle areas, and the arrowheads mark MTG-stained mitochondria derived from DP246. The position of the cell (white dotted line) is shown in each of the MTG- and PC3-stained images. Scare bar=5 μm.

4b), suggesting that MTG staining did not affect the digestion of mt-nucleoids. These results indicate that the selective digestion of mt-nucleoids derived from DP246 began in the zygote 3 h after crossing.

To clarify the timing of mitochondrial proliferation, we counted the number of mitochondria derived from AI35 and DP246 in zygotes 1 h, 3 h, and 5 h after crossing. When AI35 was crossed with MTG-stained DP246, the number of mitochondria derived from AI35 and DP246 were 12.6±1.2 and 12.5±3.0 per cell in the zygote 1 h after crossing, respectively (Fig. 4c). As expected, these numbers were similar to those observed in the amoeba described above. There was no significant difference in the number of mitochondria derived from AI35 and DP246 within 3 h after mating. However, in the zygote 5 h after crossing, the mitochondria derived only from AI35 proliferated, reaching 20.8±1.6 mitochondria per cell (Fig. 4c). When MTG-stained AI35 was crossed with DP246, the similar proliferation of mitochondria derived from AI35 was observed (Fig. 4d), suggesting that MTG staining did not affect the proliferation of mitochondria. These results suggest that the control of mitochondrial proliferation during zygote formation differs between AI35 and DP246.

Although mitochondria derived from DP246 did not proliferate during zygote formation, the number of mitochondria derived from AI35 proliferated 1.7- (Fig. 4c) and 1.8-fold (Fig. 4d) from 3 to 5 h after crossing. This

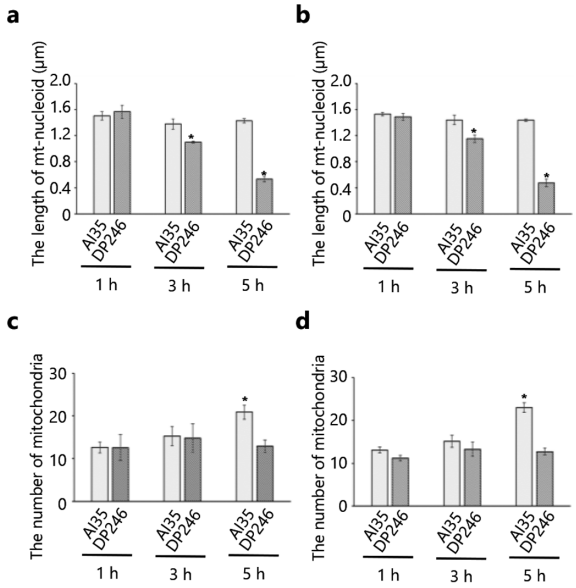

Fig. 4. Timing of the digestion of mt-nucleoids and mitochondrial proliferation during zygote maturation. (a, b) Timing of the digestion of mt-nucleoids during zygote maturation. Zygotes were formed by crossing AI35 with MTG-stained DP246 (a) or MTG-stained AI35 with DP246 (b) and stained with PC3. Subsequently, the length of mt-nucleoids derived from AI35 and DP246 were measured in zygotes 1 h, 3 h, and 5 h after crossing. n_{cells}=8. Error bars represent mean±SEM from three independent experiments. Significance differences, defined as $*p<0.05$, were tested using the Dunnett's test. (c, d) Timing of mitochondrial proliferation during zygote maturation. The number of mitochondria derived from AI35 and DP246 was counted in the zygotes, formed by unstained AI35 crossed with MTG-stained DP246 (c) or MTG-stained AI35 crossed with unstained DP246 (d), at 1 h, 3 h, and 5 h after crossing. n_{cells}=7. Error bars represent mean±SEM from three independent experiments. Significance differences, defined as $*p<0.05$, were tested using the Dunnett's test.

rate of mitochondrial proliferation is faster than that in the plasmodium of *P. polycephalum*, in which the number of mitochondria doubles within 14 h (Kuroiwa *et al.* 1978). The proliferation of mitochondria derived from AI35 occurred after the initiation of selective digestion of mt-nucleoids in mitochondria derived from DP246, which began to be observed in the zygote 3 h after crossing (Fig. 4). The digestion of mt-nucleoids in half of the mitochondria might induce a severe decrease in energy production as the mtDNA of *P. polycephalum* encodes essential subunits of the respiratory complex (Takano *et al.* 2001). Therefore, the rapid proliferation of mitochondria derived from AI35 may be important in compensating for energy production in zygotes.

Each mitochondrion derived from AI35 contained mt-nucleoids, indicating that the number of mt-nucleoids also rapidly proliferated from 3 to 5 h after crossing (Fig. 4c, d). Such proliferation of the remaining mt-nucleoids has also been observed in isogamous *C. neoformans* during zygote maturation (Nishimura *et al.* 2020). In addition, we showed that the length of mt-nucleoids derived from AI35 in zygotes 5 h after the crossing was similar to that in zygotes 3 h after crossing (Fig. 4a, b). This suggests that mtDNA replication might also occur rapidly from 3 to 5 h after crossing. In contrast, in oogamous species, the number of mitochondria and mtDNA proliferate during oogenesis, and mature oocytes have 100-fold higher mtDNA copy numbers (El Meziane *et al.* 1989, Jansen and De Boer 1998, Cree *et al.* 2008). In the early cleavage stages of embryogenesis, mtDNA replication does not occur, suggesting that mitochondrial proliferation might not occur. The mechanism of the elimination of paternal mtDNA and mitochondria varies among species. Therefore, there may be diverse mechanisms that control the proliferation of maternal mitochondria during maternal/uniparental inheritance.

Our results suggest that the proliferation of maternal mitochondria is controlled during zygote formation for uniparental inheritance in *P. polycephalum*. The mitochondrial division must occur to allow the proliferation of maternal mitochondria. It has been reported that FtsZ is necessary for mitochondrial and chloroplast division in some organisms (Strepp *et al.* 1998, Beech *et al.* 2000, Gilson *et al.* 2003). A gene similar to ftsZ exists in the transcriptome data of *P. polycephalum*. The observation of FtsZ in maternal and paternal mitochondria during zygote maturation might be useful in understanding the control mechanism of maternal mitochondrial proliferation for uniparental inheritance.

Acknowledgements

We thank Dr. Taeko Sasaki for her helpful comments and discussion. We are also grateful to Ms. Yoriko Tanaka for her technical assistance. We would like to thank Editage (www.editage.com) for the English language editing. This work was supported by a Grant-in-Aid for Scientific Research (2518K06313 to N.S.) and Grants-in-Aid for Scientific Research on Innovative Areas (Nos. JP16H06465 and JP16H06464 to T.H.). The authors declare no competing financial interests.

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