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參展科別　微生物學

作品名稱　**The state of parental mitochondria influences the replicative life span of zygotes of *Saccharomyces cerevisiae***

得獎獎項　大會獎：一等獎

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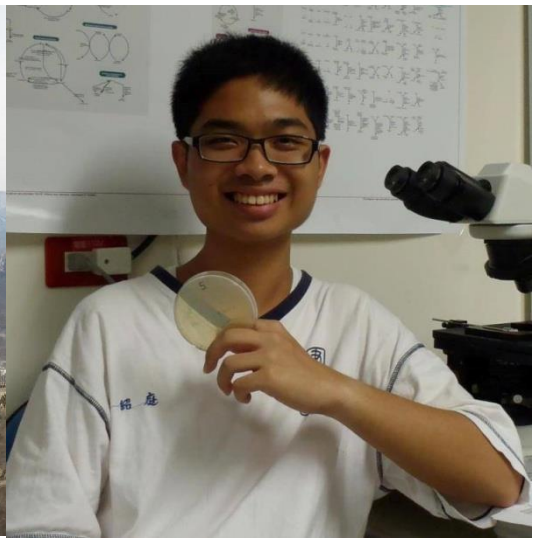
關鍵字　**Replicative Lifespan、mitochondria、sexual reproduction**

作者簡介



陳培鳴

科學不是紙上談兵，唯有自己親自經歷過探索的歷程後，才能真正了解書本上的知識是前人用鍥而不捨的努力以及嚴謹的推理而得來的。從小就對自然科學感興趣的我，在科學知識的學習上下了許多功夫，對於許多著名的理論有概略的了解。然而以往吸收的種種學術資訊對我的影響，遠不及這半年來，為了驗證自己小小的理論而付出的心血來的大。科學，終究是要從做中學。

邱紹庭

實驗是一條艱辛且少有收穫的苦路，一路上沒有太多驚喜，但每一次都令人難以忘懷。國中時，參加學校的數理彈性課程，培養了我對於實驗的興趣，我底心小小的科學夢從此發芽。高一時，因為一次意外的對話，靈光乍現，便開啟了研究的大門。由於實驗不易完成，每一次的失敗都讓我體會到接近真理的難度，但我不會停止追求，直到所有的謎團解開。

摘要

粒線體缺陷與許多人類疾病有關，尤其是關於神經退化或老化的疾病。在這項研究中，我們使用了酵母菌作為模式生物來研究粒線體對於生物老化的影響。有別於一般的基因刪除和改變基因表現量的方法，我們採用一種新的方法來解決這個問題：老x年輕合子的方法。在該方法中，我們將一個老的野生型細胞和年輕的突變細胞進行交配，並形成一個老x年輕合子。透過比較突變型合子和野生型合子的壽命，我們能夠確定粒線體中那些基因在自然老化時（即其DNA序列老化過程中發生突變）對合子的壽命具有破壞性的影響。利用這種方法，我們發現如果合子中的*COX1*，*COX2*或*COX3*基因只有老的複本，其壽命會顯著下降。另一方面，我們也發現缺乏新的*COB*基因複本對壽命的影響不大。該結果代表老細胞可藉由注射所有必需年輕基因的複本來延長壽命。

ABSTRACT

Mitochondrial defects have been associated with many human diseases, especially in the diseases related to neuron degeneration or aging. In this study, we used baker's yeast as a model to study the influence of mitochondria, especially mitochondrial DNA (mtDNA), on the aging of organisms. Different from the common gene deletion and overexpression method, we utilized a novel approach to address the question: the *old* × *young zygote* method. In this method, an replicatively old wild-type cell is mated with a young mutant cell deprived of mtDNA, respiratory function, or certain genes encoded in mtDNA, and forms an old × young zygote. By comparing the lifespan of mutant zygotes and wild-type zygotes, we are able to identify specifically which genes, when aged naturally (i.e. whose DNA sequence are mutated during aging), have destructive impact on the lifespan of zygotes. With this method, we found that if there are only old copies of *COX1*, *COX2*, or *COX3* in the zygotes, their lifespans will decreased significantly. On the other hand, we also found the absence of young copies of the gene *COB* has little effect on the lifespan. The results imply that rejuvenation of old cells might be possible by injection of young copies of all the essential genes.

INTRODUCTION

Mitochondrial defects have been associated with many human diseases, especially in the diseases related to neuron degeneration or aging. However, it is difficult to use human cells to study this kind of diseases directly since most human cells with defects in mitochondria cannot do respiration, and are unviable.

The model organism *Saccharomyces cerevisiae*, also known as baker's yeast, is well suitable for the study of mitochondrion-related diseases, because it has viable mitochondria deficient mutants. Here we used baker's yeast as a model to study the influence of mitochondria on the aging of organisms.

The lifespan of yeasts is termed RLS (replicative lifespan), defined as the number of daughter cells produced by a mother cell before it stops dividing. The sexual reproduction of *S. cerevisiae* requires two yeast cells with different mating types, a and α, to meet, detect each other, and fuse (Fig. 1). The resulting cell, called *zygote*, has the shape of a dumbbell (Fig. 2). We used zygote as a tool to investigate the impact of the *natural* aging of mitochondria on the aging of eukaryote cells.

MATERIALS AND METHODS

Plasmids and Strains

(1) *Saccharomyces cerevisiae* W303 (MATa ura3-1 ade2-1 his3-11,15 Leu2-3,112 trp1-1 can1-100 ho::HPH (hygromycin B)) and its isogenic mate type α is used in the study.

(2) Δ*cox1*, Δ*cox2*, Δ*cox3* and Δ*cob* strains are of D273-10B strain background. They are generous gifts from Dr. Thomas D. Fox.

Mediums & Buffers

(1) Yeast Extract Peptone Dextrose Medium (YPD)

Yeast Extract 10g, Bacto-peptone 20g, Dextrose 20g (per liter)

(Solid medium contain 20g agarose)

(2) Yeast Extract Peptone Dextrose Medium (YPE)

Yeast Extract 10g, Bacto-peptone 20g (per liter)

(Solid medium contain 20g agarose)

(3) Complete Supplement Mixture (CSM)

Yeast Nitrogen Base without amino acid 7g, Dextrose 20g, Bacto-agar 20g, CSM mix 1g (per liter). We have used CSM-Ura, CSM-Arg, and CSM-Ura-Arg in this project.

(4) Phosphate buffered saline pH7.0 (PBS pH7.0)

PBS pH7.0 stock solution dilute 10X.

(5) Li/TE buffer

100mM LiOAc, 10 mM Tris-Cl (pH8.0), 1 mM EDTA (pH 8.0)

Chemicals

Sulfo-NHS-LC-Biotin, Streptavidin magnetic beads, DMSO (Dimethyl sulfoxide**)**, PEG **(**Polyethylene glycol), Ethanol.

Equipment

micropipette, bacterial-free plastic culture plate, test tube, flask, bacterial-free wood sticks, filter, strong magnet, glass slides, glass beads, parafilm, PCR machine

Statistics

Mann–Whitney U test was used to determine the significant difference. $p < 0.05$ is considered statistically significant.

Measuring RLS (Replicative Lifespan)

Spread the refreshed yeasts on Yeast Extract Peptone Dextrose Medium agar plate (YPD), and then select virgin cells by knocking off the daughter cells for the budding yeasts every 2 hours, recording times of division at the same time.

Making zygotes

Spread the refreshed a cells and α cells on the plate, and then take two of each to arrange into the shape of a clover. Wait for 2 hours. Select the cells which look like dumbbells.

Magnetic Sorting

Suspend and incubate yeasts overnight. Centrifuge and wash yeast suspension with 1mL PBS pH8.0. Add Sulfo-NHS-LC-Biotin (2mg/mL in 1X PBS pH8.0). Incubate at 4℃ for 1 hour. Wash with 4℃ 100 mM Glycine 1X PBS pH8.0 and incubate at 28℃ for 48 hours. Wash with PBS pH8.0 and sonicate for 30 seconds (low energy).

Add 5 μL Streptavidin magnetic beads and incubate at 4℃ for 2 hours. Use magnet to attract the magnetic-beads-labeled old cells and remove the rest. Then, Wash with PBS pH8.0 (Sinclair, Mills, & Guatente, 1998).

Making Petite Yeasts

Culture cells in YPD containing 10 μL/mg of Ethidium Bromide for 24 hours. Then, spread the liquid culture on YPD agar plate, culture for 2 days. Transfer to Yeast Extract Peptone glycerol agar plate (YPG), culture for 3 days. Then, choose colony which can grow on YPD but not on YPG agar plate. Stain with DAPI, making sure it does not have mitochondrial DNA.

Yeast Transformation

First, wash fresh cells with 500 μL Li/TE buffer. Then, centrifuge and remove supernatant. Add 50μL Li/TE buffer, 5μL ssDNA (salmon sperm DNA), 1 μL DNA fragment to be transformed, 350μL Polyethylene glycol (PEG). Then, incubate at 28°C for 30 minutes. Add 40μL of Dimethyl sulfoxide (DMSO), and then heat shock at 42°C for 15 minutes. Add 800μL of YPD, centrifuge, and spread the culture on selecting plate. The cells that can grow on selecting plate are successfully transformed.

RESULTS

1 Lifespan of young × young zygotes is similar to that of the two parents

We mated young a cells with young α cells, and the lifespan of the resulting young × young zygotes fell between the two parents, having no significant difference with both of them (Fig. 3A).

2 Old × young zygotes possess intermediate lifespan

Then we substituted one of the young parents with old cells (obtained with magnetic sorting), and measured the lifespan of the old × young zygotes. The results show that the old × young zygotes possess intermediate lifespan, having significant difference with both of the parents (Fig. 3B). It implies that the lifespan of old cells can be *rejuvenized* by mating with young cells, so next we wanted to find out which components in the young cells are critical for the lifespan extension of old cells.

3 Lifespan of old × young-ρ0 zygotes drops significantly

Mitochondria have been linked to the aging of yeasts, especially through the ROS (reactive oxygen species) produced by the ETC (electron transport chain). Therefore it is likely that mitochondria are involved in the lifespan extension of old × young zygotes. To test this hypothesis, we substituted the young wild-type cell in old × young zygote with the young-ρ^0 cell, which is a mutant that has no mitochondria DNA (mtDNA), but still possess mitochondria.

The lifespan of old × young-ρ^0 zygotes has significant difference with the young parent but has no significant difference with the old parent (Fig. 4A). Furthermore, the lifespan of old × young-ρ^0 zygotes is also significantly shorter than

that of the old × young zygotes (Fig. 4B).

The results imply that the young cells fail to extend the lifespan of old cell if they do not provide mtDNA. This directly shows that mitochondria indeed play a role in the rejuvenation of old cells. So then we wanted to find out which parts of mitochondria are essential for the lifespan extension.

There are three possible reasons why young-ρ^0 cannot extend the lifespan (Fig. 4C):

1. The young-ρ^0 cells brought **dysfunctional mitochondria** into the zygotes.
2. The zygotes have **insufficient amount of mtDNA**.
3. The zygotes have **no young mtDNA**.

So next we designed experiments to discriminate the influences of these factors.

4.1 Dysfunctional mitochondria from young-ρ0 is not the cause of the shorter lifespan of old × young-ρ0 zygotes

To see if the dysfunctional mitochondria from the young-ρ^0 caused the shorter lifespan, we substituted the young-ρ^0 with young-Δ*sco1* and young-Δ*kgd1*. *SCO1* and *KGD1* are nucleus-encoded genes that are necessary for respiration, so their deletion mutants have dysfunctional mitochondria (by *dysfunctional* we mean "unable to do respiration"), but still have intact mtDNA.

The lifespan of old × young-Δ*sco1* zygotes and old × young-Δ*kgd1* zygotes are not significantly different with that of the old × young zygotes (Fig. 5A). The results indicate that dysfunctional mitochondria have no significant effects on the lifespan

(Fig. 5B).

4.2 Decreased amount of mtDNA is not the cause of the shorter lifespan

Next we continued to test the effects of mtDNA amount on the lifespan. We compared the lifespan of young × young zygotes with that of the young × young-ρ^0 zygotes, which have less mtDNA because only one of the parents contributes mtDNA. The results show that the two have no significant difference (Fig. 6A), implying that mtDNA amount also has no significant influences on the lifespan (Fig. 6B).

Taking the results together, we have excluded two of the three possible reasons of the shorter lifespan of old × young-ρ^0 zygotes. Therefore we continued to test the influence of the third factor: young mtDNA.

4.3 Three mtDNA-encoded genes, COX1, COX2, and COX3 (subunits of complex IV) are critical for the intermediate lifespan of zygotes

To test whether the absence of young mtDNA can affect lifespan, we deleted genes encoded in mtDNA from young cells and compared the lifespan of mutant zygotes with wild-type zygotes. The mitochondria of yeasts consist of about 1,000 proteins, but only eight of which are encoded in the mtDNA. We obtained the deletion mutants of four of them, *COX1*, *COX2*, *COX3*, and *COB* from Dr. Thomas D. Fox. Cox1, Cox2, and Cox3 are protein subunits of complex IV (cytochrome c oxidase), and Cob is cytochrome b in complex III. These strains are of D273-10B background, different from the W303 background that we used in the previous experiments. So first we measured the lifespan of old × young zygotes and old × young-ρ^0 zygotes to confirm that the shorter lifespan phenomenon still exists in D273-10B background before proceeding.

Then we measured the lifespans of old × young-Δ*cox1* zygotes, old × young-Δ*cox2* zygotes, old × young-Δ*cox3* zygotes, and old × young-Δ*cob* zygotes and compared them with that of the old × young zygotes and old × young-ρ^0 zygotes (Fig. 7).

The results showed that old × young-Δ*cox1* zygotes, old × young-Δ*cox2* zygotes, and old × young-Δ*cox3* zygotes have lifespans significantly different from that of the old × young zygotes, while not significantly different from that of the old × young-ρ^0 zygotes. This shows that in the absence of young copies of *COX1*, *COX2*, and *COX3*, the lifespans of zygotes decreased significantly, while the absence of young copies of *COB* has little impact on the lifespans.

The results not only prove directly that young mtDNA is critical for the young cells to extend the lifespan of old cells, but also further identify specific genes that are essential for the lifespan extension.

DISCUSSION

In the project, we first observed that old × young zygotes possess intermediate lifespan, which suggests that the lifespan of old cells can be extended through sexual reproduction with young cells. Since there are many literatures discussing the mitochondrial involvement in aging, and several mitochondria-related aging pathways has been found out through extensive researches (Lin, Ford, Haigis, Listz, & Guarente, 2003), we thought that mitochondria may be the aging factor that affect the lifespan of old × young zygotes. Therefore first we investigated the mitochondria.

Then we showed directly that mitochondria play an important role in the lifespan extension with the old × young-ρ^0 zygotes. Old × young-ρ^0 zygotes, which are old × young zygotes deprived of mtDNA from the young parent, possess significantly shorter lifespan. While the results show the involvement of mitochondria in the lifespan of zygotes, it was still not clear which parts of mitochondria make the major contribution. There are three possible candidates: (1) the dysfunctional mitochondria young-ρ^0 brought into the zygotes (2) the less amount of mtDNA the zygotes received (3) the lack of young copies of mtDNA in the zygotes. We next designed experiments to discriminate the influences of these factors.

First we measured the lifespan of old × young-Δ*sco1* zygotes and old × young-Δ*kgd1* zygotes to investigate the influence of the function of mitochondria. We chose the two genes for three criteria: (1) they are necessary for respiration, so the deletion mutants cannot do respiration (2) they are encoded in nucleus, so the deletion mutants still have intact mtDNA (3) the deletion of these genes do not affect the maintenance of mtDNA, so the deletion mutants will not lose the mtDNA after several rounds of divisions. The information is obtained from the online database

Saccharomyces Genome Database. The lifespans of these mutant zygotes have no significant difference with the wild-type old × young zygotes, implying that the function of mitochondria from the young cells has no significant effects on lifespan.

Then we continued to investigate the impact of mtDNA amount. We compared the lifespan of young × young zygotes with that of the young × young-ρ^0 zygotes, which have only mtDNA from one of the parents. The results show that the two lifespans have no significant difference. This suggests that mtDNA amount have no significant impact on the lifespan of zygotes.

From the two experiments above, we have ruled out two of the three possible reasons for the shorter lifespan of old × young-ρ^0 zygotes. However, it does not prove *directly* that the lack of young mtDNA is the cause, since there may be some other factors that are not so obvious. So we must prove it in a more direct way.

In the next experiment, we removed specific genes encoded in the mtDNA of *young* cells from old × young zygotes. We checked the influences of young mtNDA by measuring the lifespans of old × young-Δ*cox1* zygotes, old × young-Δ*cox2* zygotes, old × young-Δ*cox3* zygotes, and old × young-Δ*cob* zygotes. We found that old × young-Δ*cox1* zygotes, old × young-Δ*cox2* zygotes, and old × young-Δ*cox3* zygotes have significantly shorter lifespan than old × young zygotes while old × young-Δ*cob* zygotes have similar lifespan with old × young zygotes. The results not only provided direct evidence that the lack of young mtDNA is the cause of the shorter lifespan of old × young-ρ^0 zygotes, but also further demonstrated that there are both important and unimportant genes on mtDNA in terms of lifespan. The absence of young copies of *COX1*, *COX2*, and *COX3* has destructive effect on the lifespan of the zygotes, while the absence of young copies of *COB* seems to have little impact on the lifespan.

Since mitochondria electron transport chain (ETC) is the main source of cellular reactive oxygen species (ROS), it is very likely that ROS participates in the shorter lifespans of these mutant zygotes. It has been shown in human cells that mtDNA is more susceptible to ROS damage and has less efficient repair mechanism than nucleus DNA (nDNA) (Mandavilli, Santos, & Houten, 2002). This could help explain why the old × young-Δ*cox1* zygotes have decreased lifespan while old × young-Δ*sco1* zygotes have intermediate lifespan despite the fact that both zygotes have only old copies of the genes deleted: *COX1*, which is encoded in mtDNA, suffers more damages and has more mutations than those encoded in the nDNA during the course of aging. mtDNA mutations have been shown to cause premature aging in mutant mice through the translation of defective proteins on ETC (Edgar et al., 2009). The downstream mechanism may be related to Sir2 and ERCs (extrachromosomal rDNA circles), which have been shown to interact with ROS and affect cell aging (Bitterman, Medvedik, & Sinclair, 2003; Hwang, Jeong, & Lee, 2012).

The results also provide a means of lifespan extension of diving cells like stem cells without knowing the detailed mechanism. With this old × young zygote method, in principle we can identify essential sequences on mtDNA that when aged can decrease the replicative lifespan. In addition, genes on mtDNA of *Saccharomyces cerevisiae* have homologous genes in humans, which have essentially the same function. So in the future we may be able to assemble these important sequences into a *rejuvenation* plasmid, which can be sent into the mitochondria of aged stem cells and resume their division.

FIGURE LEGENDS & FIGURES

Fig. 1. Lifecycle of *Saccharomyces ceresiviae*

Fig. 2. Zygote and its diploid F1 offspring under optical microscope.

Fig. 3. (A) Survival curves of young × young zygotes and the parents. (B) Survival curves of old × young zygotes and the parents ($n \geqq 36$).

Fig. 4. (A) Survival curves of old × young-ρ^0 zygotes and the parents. (B) Comparison of survival curves of old × young-ρ^0 zygotes and old × young zygotes. (C) Illustration of the three possible reasons for the shorter lifespan of old × young-ρ^0 zygotes.

Fig. 5. (A) Comparison of survival curves of old × young-*Δkgd1* zygotes, old × young-*Δsco1* zygotes, and old × young zygotes. (B) Illustration of the comparison with old × young-*Δkgd1* zygotes, old × young-*Δsco1* zygotes, and old × young zygotes.

Fig. 6. (A) Comparison of survival curves of young × young-ρ^0 zygotes and young × young zygotes. (B) Illustration of the comparison with young × young-ρ^0 zygotes and young × young zygotes.

Fig. 7. Comparison of survival curves of old × young-*Δcox1* zygotes (A) old × young-*Δcox2* zygotes (B) old × young-*Δcox3* zygotes (C) old × young-*Δcob* zygotes (D) with old × young zygotes and old × young-ρ^0 zygotes.

FIGURE 1

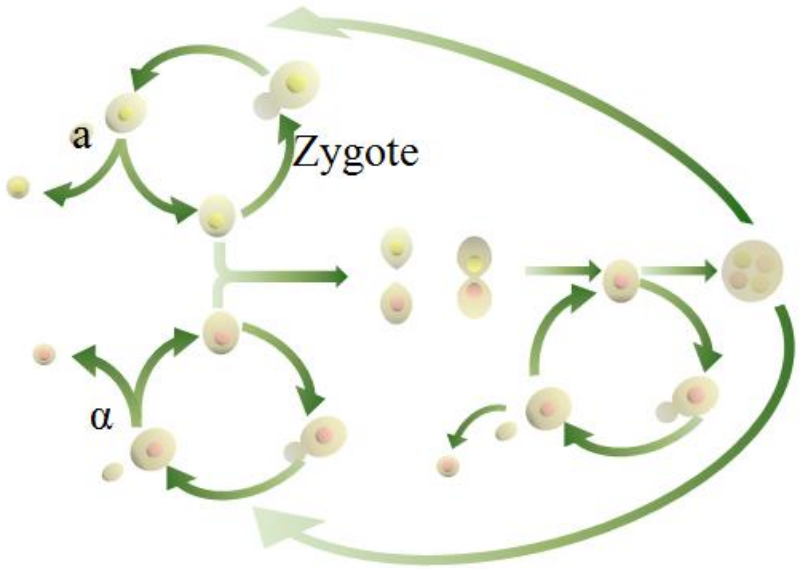


FIGURE 2

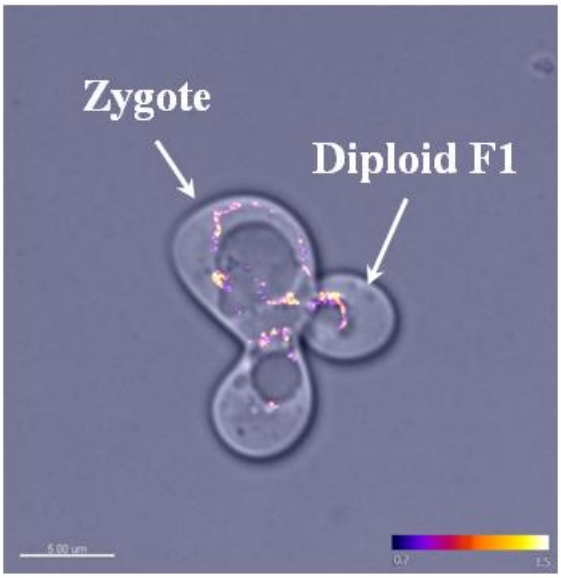
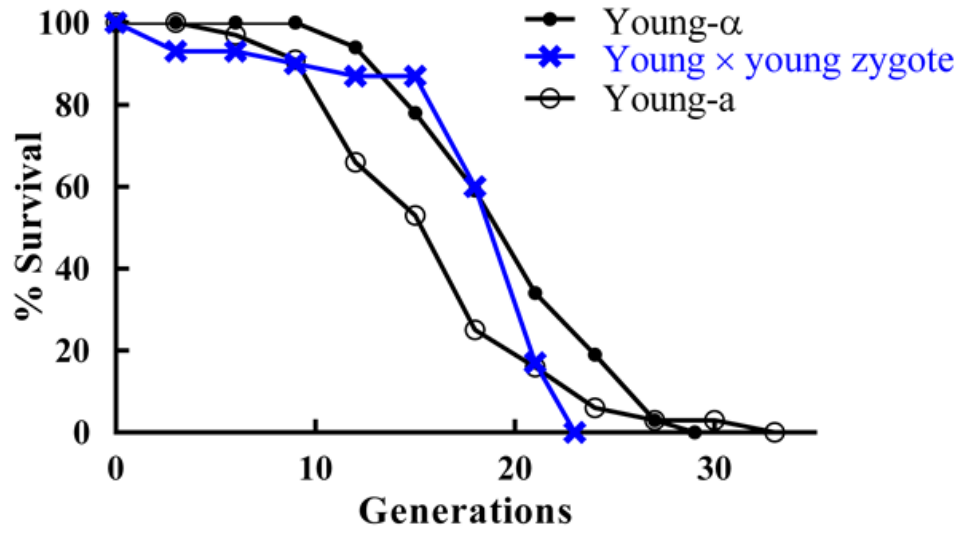


FIGURE 3

A


Young-α
Young × young zygote
Young-a
% Survival
100
80
60
40
20
0
0
10
20
30
Generations

B

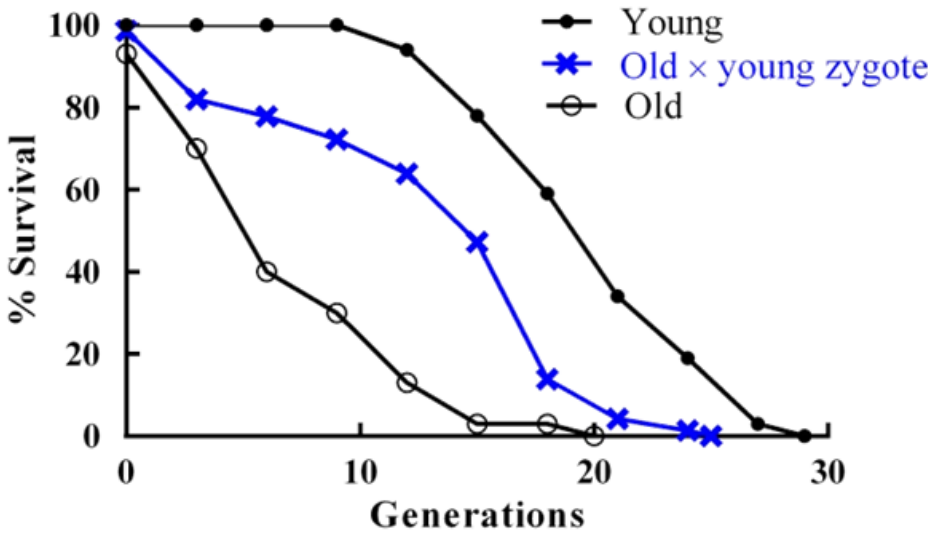
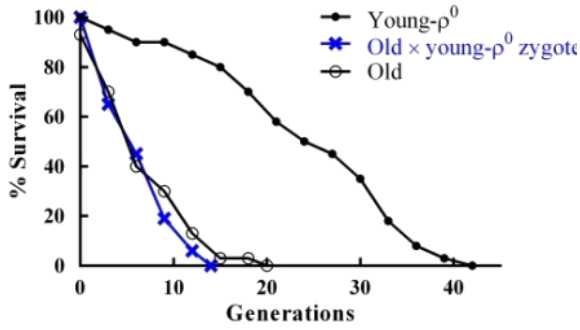

Young
Old × young zygote
Old
% Survival
100
80
60
40
20
0
0
10
20
30
Generations

FIGURE 4

A



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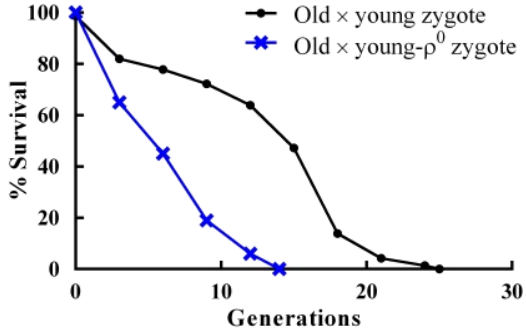
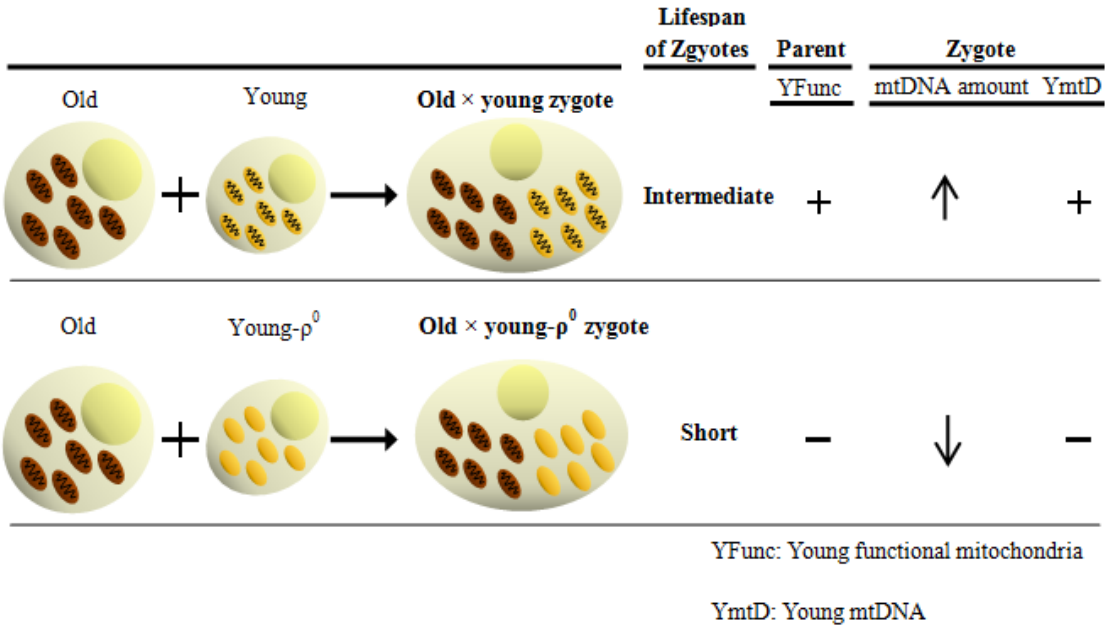


C

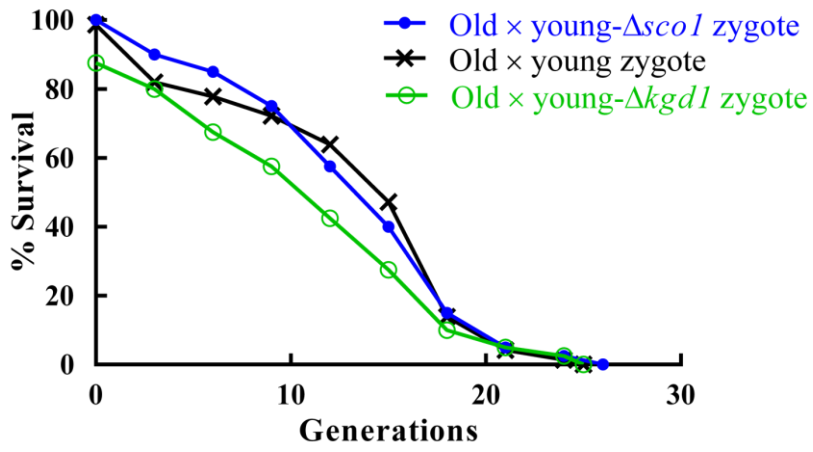


YFunc: Young functional mitochondria

YmtD: Young mtDNA

FIGURE 5

A



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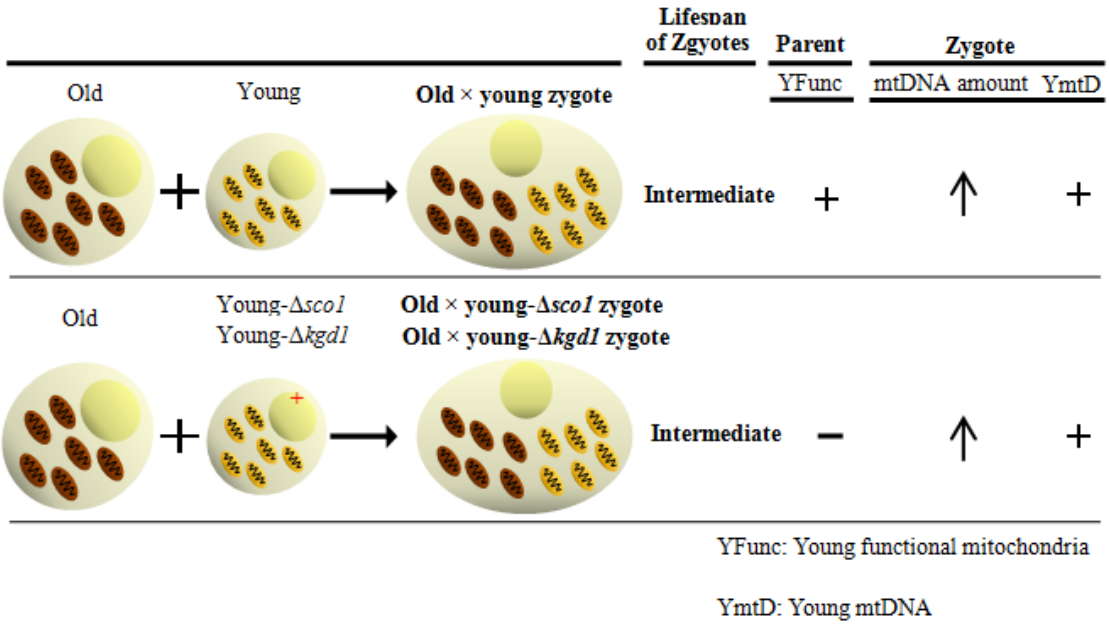
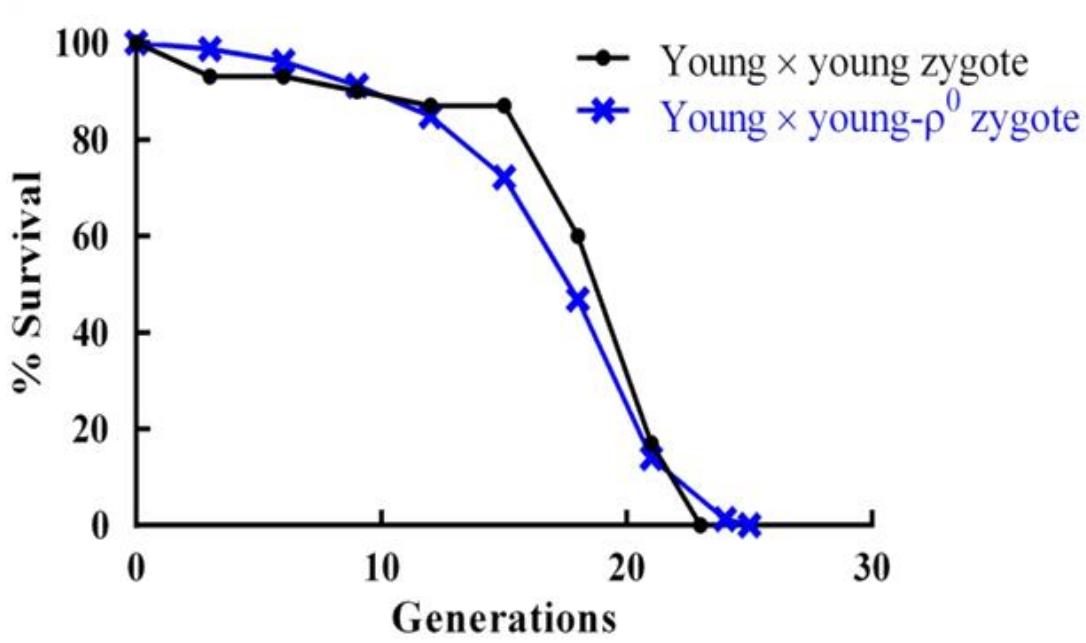


YFunc: Young functional mitochondria

YmtD: Young mtDNA

FIGURE 6

A


100
80
60
40
20
0
% Survival
0
10
20
30
Generations
Young × young zygote
Young × young-ρ0 zygote

B

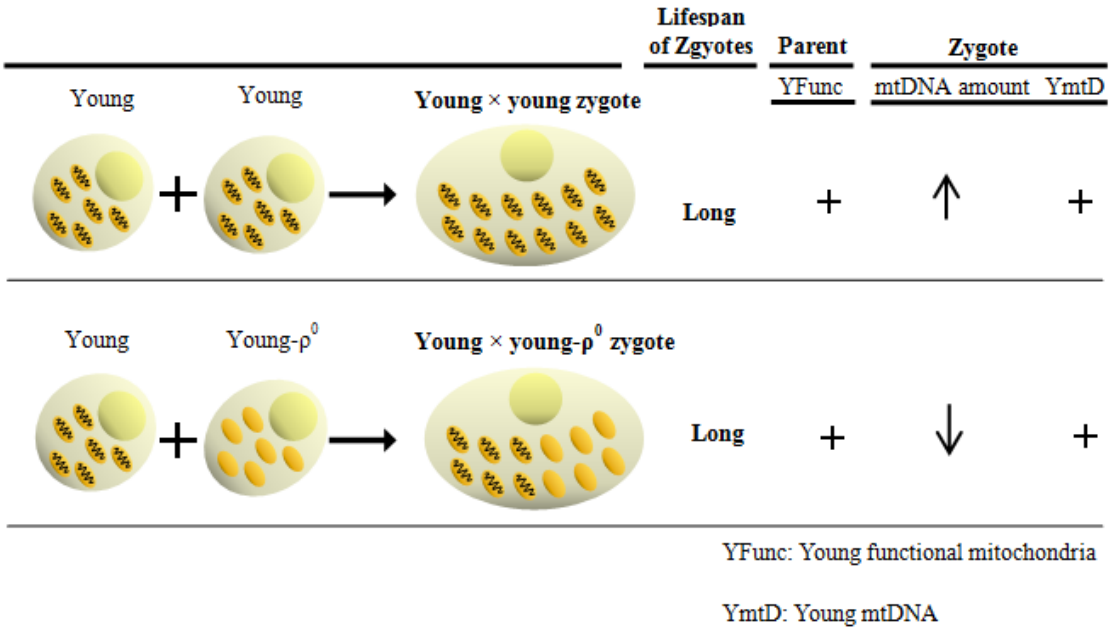
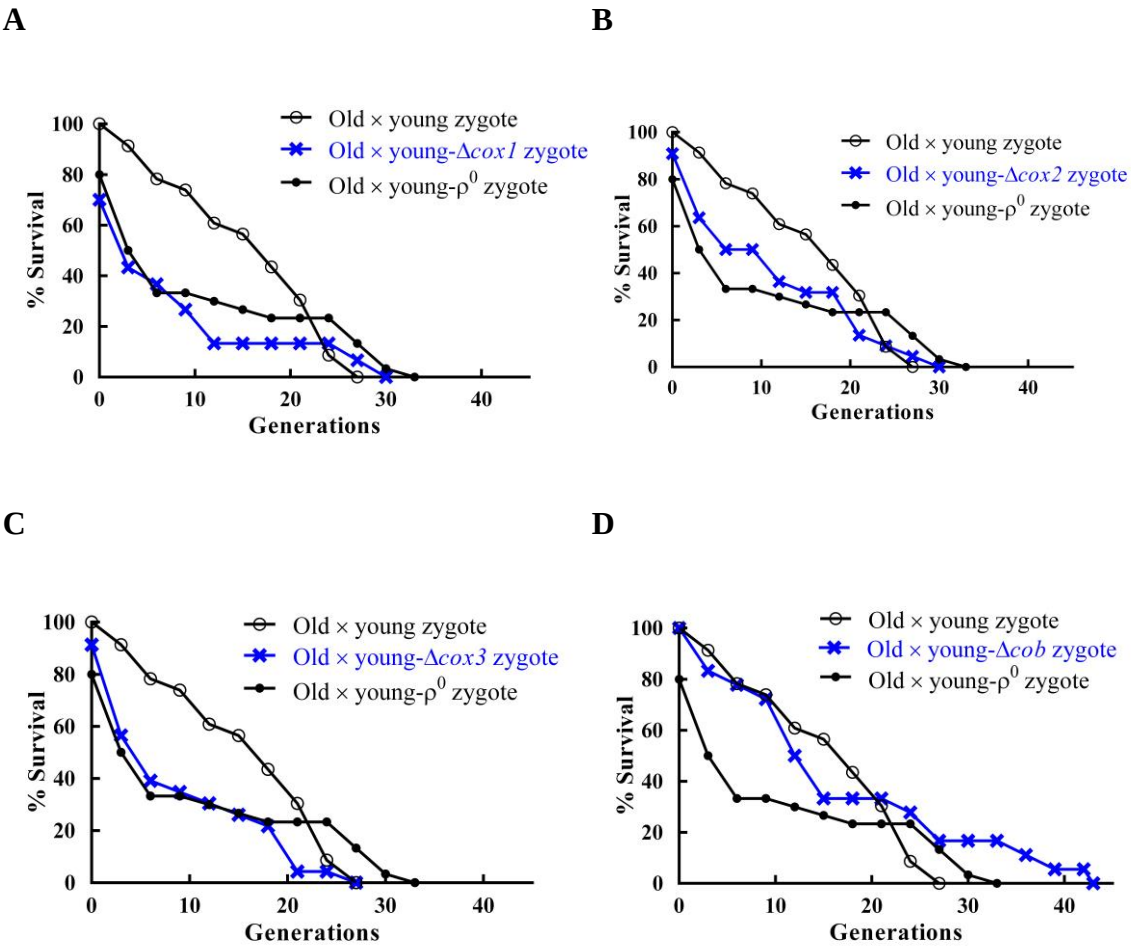

Lifespan of Zgyotes
Parent
Zygote
YFunc
mtDNA amount
YmtD
Young
Young
Young × young zygote
Long
+
+
Young
Young-ρ0
Young × young-ρ0 zygote
Long
+
+
YFunc: Young functional mitochondria
YmtD: Young mtDNA

FIGURE 7


A
Old × young zygote
Old × young-Δcox1 zygote
Old × young-ρ0 zygote
% Survival
Generations
B
Old × young zygote
Old × young-Δcox2 zygote
Old × young-ρ0 zygote
% Survival
Generations
C
Old × young zygote
Old × young-Δcox3 zygote
Old × young-ρ0 zygote
% Survival
Generations
D
Old × young zygote
Old × young-Δcob zygote
Old × young-ρ0 zygote
% Survival
Generations

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評語

1. 本研究所呈現的數據能證明研究的目的，酵母菌的 life span 與粒線體抗氧化相關性，皆有合理的結果，是一個很完整的研究。

2. 如果能配合上生化數據，將會使本研究成果更完美，如 peroxidase, SOD 等酵素上的變化，有必要加以探討。