THE ROLE OF GENE DUPLICATION DURING THE EVOLUTION OF AEROBIC FERMENTATION IN YEAST

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The genetic basis underlying how organisms adapt to different environments and evolve new life style is a central issue of molecular evolution. The evolution of aerobic fermentation in yeasts is one of those good examples. So far the underlying genetic basis of this phenotypic evolution remains unclear. Gene duplication, as a primary source of materials for evolutionary novelties, has long been thought to play an important role in the adaptation of organisms to their environments. It was hypothesized that whole genome duplication (WGD) led to the development of this efficient fermentative life style. However, it remains unclear how the WGD genes are regulated during the switch of energy metabolism, an issue which should be the essence of the "WGD-Fermentation hypothesis".

In this study, I first used a genome-wide expression dataset during robust metabolic oscillation in response to oxygen in budding yeast (Tu et al. 2005) to investigate the distribution of WGD genes in metabolic cycle. An enrichment of WGD genes was found underlying the physiological response of *S. cerevisiae* to oxygen change. Our results provided new evidence for the WGD-Fermentation hypothesis. In the next, I explored in more detail about what were the important WGD genes that contributed greatly to the evolution of aerobic fermentation. Two WGD genes are brought to the front to study this issue due to their importance on regulating energy metabolism.

One of the two genes is pyruvate kinase (PYK), and the other is the target of rapamycin (TOR). In both cases, I revealed positive correlations between the copy number of these genes and the strength of aerobic fermentation on a yeast phylogenetic tree, which implies that gene duplication events of PYK and TOR are possible to facilitate the evolution of aerobic fermentation. In the PYK experiment, I found that yeasts with higher capabilities on the allosteric regulation of PYK tend to have higher fermentation abilities. I then assumed that the strengthened allosteric regulation of PYK after gene duplication might be important in the development of fermentation lifestyle in yeast. Further study on T403E mutant which is defect in allosteric regulation on PYK supported this assumption, as T403E mutant showed raised oxidative phosphorylation and decreased fermentation rate compared with the control. In the TOR experiment, I mimicked the TOR gene duplication during yeast history and doubled the TOR gene in a Crabtree-negative yeast *K.lactis.* I observed that the fermentation ratio in the doubled TOR mutant was significantly increased compared with the control, which supported that TOR gene duplication contributed greatly to the evolution of fermentative life style. In the view of the similar features shared by fermenting yeast and cancer cells on aerobic fermentation, our studies on PYK and TOR may shed lights on the mechanism how the Warburg effect is regulated in tumor cells.

BIOGRAPHICAL SKETCH

Hong Chen was born and raised in Suzhou, China. She graduated from Shengze High School in 2000 and began her undergraduate studies at Nanjing Agriculture University, Nanjing, China. In college she received a Fan Qing-sheng Scholarship and was funded by SRT (Student Research Training) program to lead a project named: Developing putative rice SNPs through bioinformatics approaches.

Hong graduated with honors from Nanjing Agriculture University and then moved to Fudan University, Shanghai, in 2004, for a genetics master degree. Under the supervision of Dr. Keke Huo, she participated in a national 973 program of constructing Human Liver Proteome Project Database by a large-scale yeast two hybrid screening method.

After that she moved to Ithaca, New York, in 2007, to attend graduate school at Cornell University to pursue a Ph. D. in Nutritional Sciences. Throughout the past five years, she has worked under the mentorship of Dr. Zhenglong Gu to explore the evolution of aerobic fermentation in yeast.

To my parents,

for your support and encouragement,

friendship, and never-ending love.

And to my husband,

for pushing me further than I thought possible,

for your tremendous patience,

for making me laugh every day,

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CHAPTER ONE

Introduction for aerobic fermentation and gene duplication

1.1. Aerobic fermentation in yeast

The evolution of aerobic fermentation in yeasts is a good example of phenotypic evolution. In eukaryotes, glucose is mainly assimilated through the respiration pathway in mitochondria to produce CO_2 and H_2O for maximum energy yield in the presence of oxygen. However, some yeast such as *Saccharomyces cerevisiae* undergo aerobic fermentation in which glucose is predominantly fermented to ethanol even in the presence of oxygen. Those are called as Crabtree-positive yeasts (1). In fact, fermenting yeasts grow at higher rates compared to those that grow oxidatively (2). These yeasts are subjected to fluctuations of nutrient availability in their natural environment, thus, must be able to successfully adapt their metabolism in order to meet the energy demand and survive.

In a glucose-rich environment, fermentation is their main metabolic pathway for carbon and energy metabolism. In contrast, when a non-fermentable carbon source such as glycerol, ethanol or lactate is present, respiration will be the main energy-yielding pathway (3). If glucose is added during yeast aerobic growth, they immediately arrest respiration and accelerate glycolysis at the same time that ethanol is produced (4). This phenomenon is called as Crabtree effect. The transition is modulated by short-term and long-term events. The latter involves the down-regulation of gluconeogenic and oxidative metabolism-related proteins and the enhanced transcription of glycolytic enzymes and glucose transporters (5, 6). Recent studies indicated that the evolution of aerobic fermentation in the *Saccharomyces cerevisiae* lineage was associated with regulatory reprogramming of genes involved in respiration and mitochondrial functions (7,

8). The loss of a specific cis-regulatory element in many genes coding for mitochondrial proteins in the *Saccharomyces cerevisiae* lineage was speculated to have contributed to the transcriptional reprogramming process (7).

1. 2. Tumor cell metabolism: The Warburg effect

One of the major differences observed between cancer cells and normal cells is in how they metabolize glucose; most cancer cells primarily metabolize glucose by glycolysis, most normal cells completely catabolize glucose by oxidative phosphorylation (9). The phenomenon in tumor cells, as described as even in the presence of a normal oxygen level, glycolysis is highly elevated and accompanied by high lactate production as well as drastically reduced mitochondrial respiration, is termed as "the Warburg effect" (10-12). As a matter of fact, aerobic glycolysis is an inefficient way to generate adenosine 5'-triphosphate (ATP); however, the advantage it confers to cancer cells has been unclear. It is probable that this phenotype endows tumor cells with some advantages in order to survive and proliferate in harsh microenvironments, such as in hypoxic conditions and within poorly vascularized tumors (13). This metabolic adaptation could also help malignant cells to overcome programmed cell death mechanisms (14). For some tumor cells a correlation between invasiveness and their glycolytic capacity was observed (15, 16). It is also reported that this shift to aerobic glycolysis with lactate production, coupled with increased glucose uptake, is likely used by proliferating cells to promote the efficient conversion of glucose into the macromolecules needed to construct a new cell (17).

The molecular mechanisms underlying the "Warburg effect" are still unknown. Nevertheless, it is possible that the enhanced expression of glycolysis enzymes and glucose transporters along with a down-regulation of mitochondrial metabolism could be at the basis of the glycolytic phenotype of tumor cells (10). In that case, the hypoxia-induced metabolic reprogramming

mediated by the HIF-1 transcription factor has been pointed out as the key regulator for the“aerobic glycolysis” in tumors, as the expression of glycolysis enzymes, glucose transporters and several other tumor-related genes are controlled by HIF-1 (18). It is also proposed that Warburg effect appears as consequence of an irreversible damage to mitochondria and to the proteins involved in oxidative metabolism (10). Some recent studies do suggest that many cancers have mitochondrial DNA mutations, which might result in dysfunctional mitochondria and promote glycolysis (19, 20). However, this hypothesis was met with significant skepticism specifically because some tumors do not have defects in respiration (21-23). In particular, Chance et al. (24, 25) found that ascites tumor cells used by Warburg had in fact, higher respiration than cardiac or yeast mitochondria. From these observations it was construed that respiratory deficiency could not be the cause of high glycolytic rates, despite the observations that many cancer cells do indeed have morphologically abnormal mitochondria. There might be other mechanisms underlying the “aerobic glycolysis” in cancer cell.

Since the “aerobic glycolysis” is considered the paradigm of tumor cell metabolism, it seems reasonable that some features of tumor aerobic glycolysis can actually be used as a marker for cancer diagnosis, and disruption of glycolysis might be a promising candidate for specific anti-cancer therapy. The fluorodeoxyglucose-based positron emission tomography (FDG-PET scan) is widely employed for these purposes. This technique is based on the increased capacity of tumor cells for glucose uptake, thus facilitating the identification of tumors and potentially malignant overgrowths (26). Some studies show that the tumor cell lines are more sensitive to

oxidative phosphorylation-inhibiting drugs than their normal counterparts (21). While other study also reported that the chemical dichloroacetic acid (DCA), which promotes respiration and the activity of mitochondria, has been shown to kill cancer cells in vitro and in some animal models (27). Therefore, it is important to fully understand the Warburg effect in order to gain insight into the mechanisms of tumor cell energy conversion processes. This will improve the establishment of specific therapies for successful cancer treatment.

1. 3. Metabolic similarities between tumor cells and fermenting yeast

Yeast is a good model for studying Warburg effect in cancer cells. First of all, the fermenting yeast *Saccharomyces cerevisiae* has proven a valuable model organism in which several intracellular processes have been characterized in great detail. And from a metabolic point of view, *Saccharomyces cerevisiae* and tumor cells share several features. In both cell types, there are mechanisms that enhance glycolytic flux concomitantly with the repression of oxidative phosphorylation, and fermentation is preferred even in the presence of oxygen.

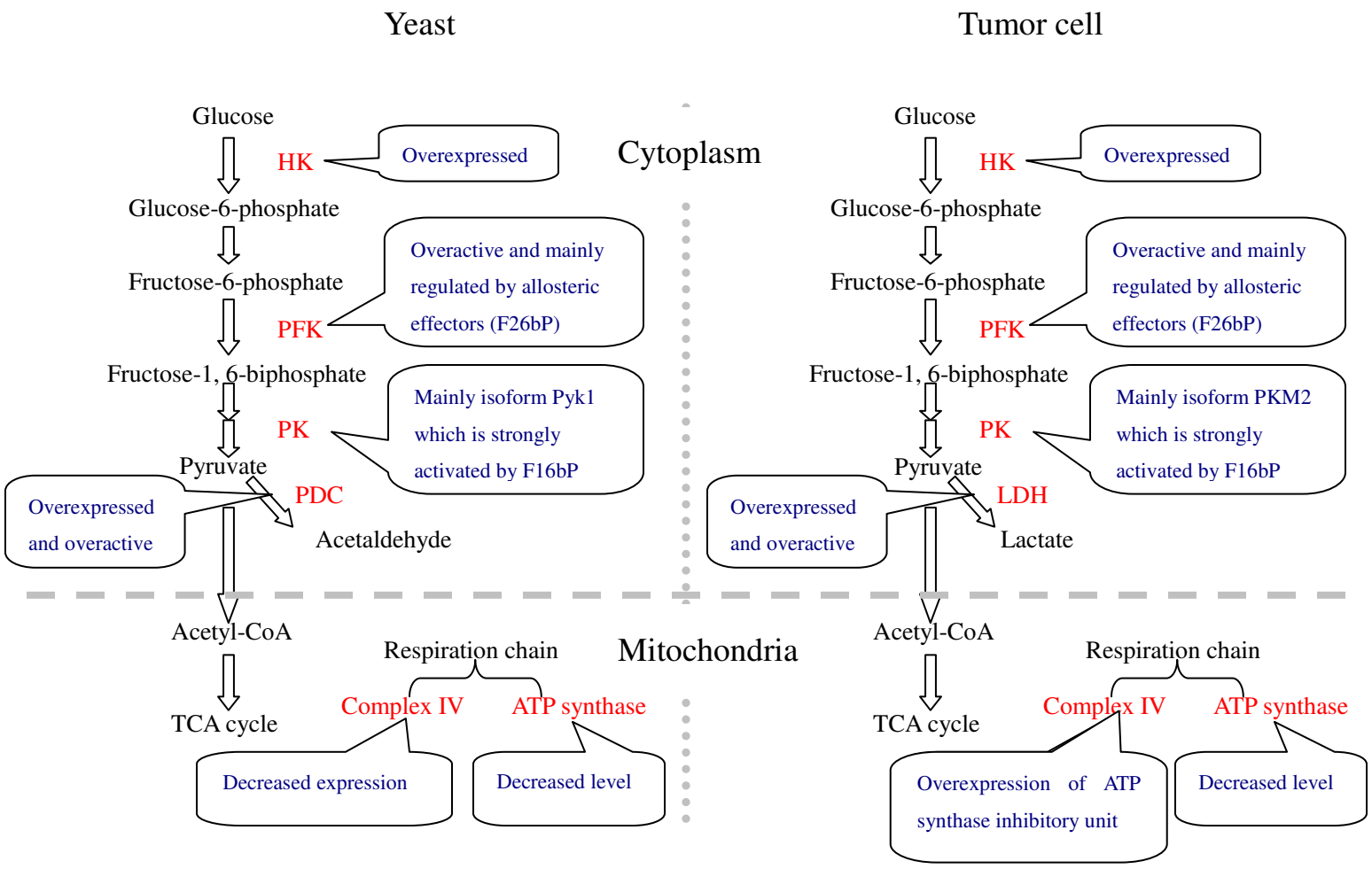
Hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK) are considered the main controlling steps for glycolysis flux modulation as they catalyze the three irreversible reactions in the glycolytic sequence (28). Indeed, an overexpression and/or overactivation of these three enzymes have been described for a number of tumor cells and in yeast (Figure 1.1) (29-31).

Warburg originally hypothesized that in addition to glycolysis acceleration there is an irreversible damage to mitochondria in tumor cells as the origin of “aerobic glycolysis” (10). This seems to be correct for some tumors. For instance, deficiencies in complex I are linked with tumor cell progression and metastasis in lung carcinoma and fibrosarcoma cell lines (32). ComplexII and IV activities decrease in some hepatoma cell lines regarding normal hepatocytes (33). Likewise, tricarboxylic acid (TCA) cycle down-regulation has also been identified in fermenting yeast. In these conditions the enzymatic activities of aconitase, isocitrate dehydrogenase and malate dehydrogenase are decreased (34). The activity of the mitochondrial

respiratory complexes II and IV is also lower in fermentation. This may originate by negative regulation at the transcriptional level (30, 34).

In view of these similarities and given the fact that *Saccharomyces cerevisiae* display a Crabtree effect, thus providing a good model for studying the Warburg effect. Besides that, this yeast is an easy-to-use experimental model; there is possibility of the utilization of yeast as a tool for testing anti-tumor drugs for metabolic therapy (35). Another advantage is the existence of yeasts that are classified as Crabtree-negative (such as *Kluyveromyces lactis* and *Candida albicans*) (1). Hence, the identification of the differences between both kinds of yeast may provide some insight into the mechanism leading to Crabtree effect.

Figure 1.1 Metabolic similarities between yeast and tumor cells. Hexokinase (HK) isoforms insensitive to glucose 6-phosphate-mediated inhibition are overexpressed. Phosphofructokinase (PFK) is activated by higher levels of one of its allosteric activators: fructose 2,6-biphosphate (F26BP). The mainly isoform of Pyruvate kinase (PK) is strongly activate by fructose 1,6-biphosphate (F16BP). The enzymes that metabolize pyruvate in the cell cytoplasm are overexpressed: Lactate dehydrogenase (LDH) in the case of cancer cells and pyruvate decarboxylase in yeast (PDC). In both cases, a decreased expression of mitochondrial complex IV is observed as well as a down-regulation of the ATP synthase.


Yeast
Tumor cell
Glucose
HK
Overexpressed
Cytoplasm
Glucose-6-phosphate
Fructose-6-phosphate
PFK
Overactive and mainly regulated by allosteric effectors (F26bP)
Fructose-1, 6-biphosphate
PK
Mainly isoform Pyk1 which is strongly activated by F16bP
Mainly isoform PKM2 which is strongly activated by F16bP
Pyruvate
Overexpressed and overactive
PDC
LDH
Acetaldehyde
Lactate
Acetyl-CoA
Respiration chain
Mitochondria
TCA cycle
Complex IV
ATP synthase
Decreased expression
Decreased level
Overexpression of ATP synthase inhibitory unit

1.4. Whole genome duplication (WGD) and aerobic fermentation

Genomic duplication has been proposed as an advantageous path to evolutionary innovation, because duplicated genes can supply genetic raw material for the emergence of new functions through the forces of mutation and natural selection. Such duplication can involve individual genes, genomic segments or whole genomes. Direct study of such a cataclysmic genomic event may provide major insights into the dynamics of genome evolution and the emergence of new functions.

Whole-genome duplication (WGD) in yeast, estimated to have occurred in the ancestor of the *Saccharomyces sensu stricto* species complex around 100 million years ago (Mya) (36-39), has been proposed to have led to the evolution of an efficient fermentation system in this lineage (40-42). This hypothesis was supported by various observations. One study indicates that with plentiful glucose, most post-WGD yeast species primarily carry out fermentation to generate energy even under aerobic conditions (Crabtree-positive); while in contrast, pre-WGD yeast species mostly respire under such conditions (1). Several authors have speculated that WGD enhanced *S. cerevisiae*'s ability to metabolize glucose (43-45) and/or to grow anaerobically (40, 41, 46). There is also evidence that the preservation of some duplicate gene pairs created by the WGD was related to their contribution toward high glycolytic flux (47). And more WGD genes than non-WGD genes are found to be dynamically regulated during metabolic oscillation in response to oxygen change (48).

Besides these observations, some studies have recently identified the genetic changes that occurred in ancient yeast progenitors to provide a foundation for the development of efficient fermentation. Ihmels et al. (7) indicates that the loss of a specific cis-regulatory element from dozens of genes following the apparent WGD event rewires the transcriptional network of yeast, thus connected to the emergence of the capacity for rapid fermentation growth. A study on the nuclear-encoded mitochondrial genes between post- and pre-WGD species also suggests that relaxation of yeast mitochondrial functions could occur in the relevant yeast species after whole-genome duplication (49).

All the above studies are inspiring. However, in order to explore in detail the mechanism underlying the evolution of aerobic fermentation, studying individual pair of duplicate genes is necessary to reveal a concrete picture of the mechanism. Thomson et al. (50) proved that several duplicate genes especially Adh1-Adh2 duplication, along with the ability of *S. cerevisiae* and its closest relatives to ferment glucose and accumulate ethanol even in the presence of oxygen, formed the basis for the 'make-accumulate-consume' strategy of ethanol production, which provided the ancestor of *Saccharomyces* yeast with an advantage over its competitors because ethanol is toxic to most other microbes. A recent study provided evidence that hexose transporter (HXT) gene duplication has facilitated the evolution of aerobic fermentation (51), thus emphasize the first step of glucose metabolism of transporting glucose across the plasma membrane in this mechanism. Therefore, in our studies, I focused on two potential duplicates: the gene of yeast pyruvate kinase (PYK) and the gene of target of rapamycin (TOR).

1.5. Aerobic fermentation related WGD genes

1.5.1. Pyruvate kinase

Pyruvate kinase catalyzes the dephosphorylation of phosphoenolpyruvate (PEP) to pyruvate and is responsible for net ATP production within the glycolytic sequence. In contrast to mitochondrial respiration, ATP regeneration by pyruvate kinase is independent of oxygen supply and allows the survival of organs under hypoxic conditions.

In yeast, there are two isoforms of pyruvate kinase (Pyk1 and Pyk2). Pyk1 can be strongly up-regulated by fructose 1,6-biphosphate (F1,6BP) (52), and it seems to be expressed constitutively, although its levels are much higher during fermentation (53). Pyk2 is less sensitive towards F1,6BP and its expression seems to be repressed by glucose (52). So, the presence of only isoform 1 during fermentation could be interpreted as an adaptation that leads to the complete activation of the enzyme and hence allows a maximal glycolytic flux.

In mammals, four isoforms of this enzyme exist as L-, R-, M1- or M2, which are tissue-specific and differ in their kinetic properties. Pyruvate kinase type L (PKL) is the characteristic pyruvate kinase isoenzyme of tissues with gluconeogenesis such as liver and kidney. Erythrocytes express the pyruvate kinase isoenzyme type R (PKR). Pyruvate kinase type M1 (PKM1) is present in tissues in which large amounts of energy have to be rapidly provided such as in muscle and brain. Pyruvate kinase type M2 (PKM2) is characteristic of lung tissues as well as all cells with high

rates of nucleic acid synthesis, including all proliferating cells such as embryonic cells, adult stem cells and especially tumor cells (54-59). Pyruvate kinase isoenzymes type L and R are encoded by the same gene, but are under the control of different promoters. Pyruvate kinase isoenzymes type M1 and M2 are different splicing products of the same mRNA transcript and differ in 21 amino acids (55, 60).

S. Mazurek et al. (61) documented a role for PKM2 in tumor metabolism by demonstrating that the more active tetrameric PKM2 favors glycolysis and lactate production, whereas the less active dimeric form predominantly found in cancer cells favors the diversion of trioses toward synthetic processes such as lipid and amino acid biosynthesis. The tetramer:dimer ratio is not a stationary value, but rather oscillates between the tetrameric and dimeric forms of PKM2 and this oscillation is regulated by the intracellular F1,6BP concentrations. When the F1,6BP levels reach a certain high value, the inactive dimeric form re-associates to form the highly active tetrameric form (62, 63). In contrast, PKM1 is insensitive to F1,6BP regulation (64). Christofk et al. (65) reported that replacement of PKM2 by PKM1 in tumor cell lines rendered them less glycolytically active and diminished tumor xenograft growth, which suggests that PKM2 is responsible for the Warburg effect. In order to further explore the role of PKM2 in tumor cell metabolism, one study found that F1,6BP, the positive allosteric regulator of PKM2, could be released from tetrameric PKM2 by interaction with specific tyrosine-phosphorylated peptides, including that of LDHA, resulting in the less active dimeric form of PKM2 (66). Hence, regulation of PKM2 seems to be pivotal for regulating glycolysis in proliferating cells. Another

recent study showed PKM2 inhibitor that disrupted the regulation of F1,6BP could result in decreased glycolysis and increased cell death following loss of growth factor signaling in cancer cells (67), which in further emphasize the importance of PKM2 regulation in Warburg effect.

1.5.2. Target of rapamycin

The target of rapamycin (TOR) is a large (281kDa) conserved Ser/Thr protein kinase that functions as a central controller of cell growth in response to environmental cues. In 1991, two TOR isoforms named TOR1 and TOR2 which share 67% sequence were identified in budding yeast *Saccharomyces cerevisiae* through analysis of resistant mutants to rapamycin, an immunosuppressive and potential anticancer drug (68, 69). Two TOR orthologs have also been isolated from fission yeast *Schizosaccharomyces pombe* (70). Unlike yeast, which in some cases possess two TOR genes, higher eukaryotes possess only a single TOR gene (mTOR in mammals), highly conserved with yeast TOR1 and TOR2 (40%–60% identity) (71, 72).

In vivo, TOR is associated with two distinct multiprotein complexes: TOR complex 1 (TORC1) and TOR complex 2 (TORC2). Yeast TORC1 comprises either TOR1 or TOR2 bound to KOG1, LST8, and Tco89, whereas yeast TORC2 is formed from TOR2, AVO1, AVO1, AVO3, LST8, and Bit61 (73). KOG1 and AVO3 (the homologs of which are Raptor and Rictor in mammals, respectively) confer distinctive structural and functional features on TORC1 and TORC2.

TORC1 is activated by growth factors, stress, nutrients, and energy signals to regulate cell growth. It mediates the rapamycin-sensitive signaling branch that positively regulates anabolic processes such as transcription, translation, and ribosome biogenesis and negatively regulates catabolic processes such as autophagy and other degradative pathways (72). TORC2 signaling is rapamycin-insensitive and is required for the organization of the actin cytoskeleton (74). In cells of higher eukaryotes, a heterodimer of the tuberous sclerosis proteins TSC1 and TSC2 regulates TOR signaling negatively (75). TSC2 functions as a GTPase activating protein (GAP) for the small GTPase Rheb, which is necessary to elevate the kinase activity of TORC1 (76). Therefore, inactivation of TSC2 could increase TORC1 activity. Curiously, however, budding yeast does not have homologs to the TSC genes, and the loss of RHEB (the homolog of mammalian Rheb) has no obvious effect on the cell cycle progression in this microbe (77).

Various alterations of the proto-oncogenes and tumor suppressors along mTOR signaling pathway mark this network as one of the most frequently dysregulated signaling cascades in cancers (78-81). To this end, rapamycin and its analogs which inhibit mTOR are currently being evaluated in clinical trials as cancer treatments (82, 83). mTOR integrates a broad spectrum of input, ranging from growth factor signaling to cellular nutrient status to energy supply, for regulation of protein synthesis and cell growth (84, 85). Even though the downstream events of oncogenic mTOR leading to cancer development are still largely unknown, among numerous mTOR effectors, the proto-oncogene Myc family and hypoxia-inducible factors (HIFs) are often

activated in various cancers and have been considered an “axis of evil” in cancer development (86-89). Although the function of normal c-Myc is inhibited by physiological HIF1α signaling, oncogenic Myc and HIFs collaborate with each other to confer metabolic advantages to cancer cells by induction of the Warburg effect through transcriptional activation of glycolytic enzymes (90). Another protein Akt, an important upstream effector of mTOR, is also proven to stimulate aerobic glycolysis in cancer cells (91). Some recent studies provided evidences to support that mTOR was a major positive regulator of the Warburg effect, as increased mTOR activity is coupled with enhanced lactate production (92, 93). While other studies proposed conflict results that inhibiting mTOR functions in cancer cells enhanced aerobic glycolysis and decreased uncoupled mitochondrial respiration (94, 95). Xu et al. (2005) showed that targeting mTOR pathway in combination with inhibition of glycolysis would synergistically impact the energy metabolism in cancer cells and achieve better therapeutic outcomes to kill malignant cells (96), which may suggest that mTOR pathway impact cancer cell metabolism through a different strategy other than glycolysis. Therefore, it is necessary to further study whether mTOR contributes to aerobic fermentation directly, which can help better understand how mTOR is involved in cancer related metabolisms.

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CHAPTER TWO

Regulation dynamics of WGD genes during yeast metabolic oscillation

2. 1. Summary

Saccharomyces cerevisiae and its close relatives are characterized by their ability to ferment even in the presence of oxygen. It was hypothesized that whole genome duplication (WGD) led to the development of this efficient fermentative life style (WGD-Fermentation hypothesis, Piskur, 2001). In this study, I found that a significantly higher proportion of WGD genes than non-WGD genes are dynamically regulated during metabolic oscillation in response to oxygen change. The same dataset also shows that the WGD genes, as compared to the smaller-scale duplicate (SSD) genes, are enriched with pairs where both copies have cyclic expression during the metabolic oscillation (either with the same or different phases). These results provide new evidences for the WGD-Fermentation hypothesis and new insights into the relationship between genome duplication and the evolution of new life styles in eukaryotic organisms.

2. 2. Introduction

Gene duplication, as a primary source of materials for evolutionary novelties, had long been thought to play an important role in the adaptation of organisms to their environments (1-3). Models that aim to explain the retention of duplicate genes include subfunctionalization (4, 5), neofunctionalization (1) and selection for high dosage (6, 7). Baker's yeast *S. cerevisiae* and its close relatives owe their competitiveness to a combination of several properties including fast growth with or without oxygen, efficient glucose repression of respiration genes, and good ability to produce and consume ethanol (8). It was proposed that the whole genome duplication (WGD) that occurred about 100 million years ago in this lineage (9-12) might have provided the basis for specialization of a number of duplicate genes, enabling their optimal functions in either aerobic or anaerobic conditions, and thus providing a competitive advantage for the "new" *Saccharomyces* group (13).

This "WGD-Fermentation hypothesis" was supported by various observations. A recent study indicates that the ability to grow anaerobically on minimal media and the presence of a Crabtree effect are strongly associated with yeasts possessing the WGD (14). Another study found a general trend for higher rates of ethanol production in post-WGD yeasts than in non-WGD yeasts (15). There are also evidences indicating that many retained WGD genes could contribute to yeast's ability to ferment glucose anaerobically (16), and that glycolytic flux was increased as an outcome of WGD (17). These studies are inspiring; nevertheless, it remains unclear how the WGD genes are regulated between aerobic and anaerobic metabolisms, an issue which should be

the essence of the “WGD-Fermentation hypothesis”.

Based on this hypothesis, I would expect an enrichment of WGD genes underlying the physiological response of *S. cerevisiae* to oxygen change. A recent work investigating genome-wide expression during a robust metabolic oscillation in budding yeast provides an opportunity to test our prediction (18). During the metabolic oscillation, many parameters – such as respiration rate, ethanol production and dissolved oxygen concentration in the media – change periodically, so each cycle could be characterized by a respiratory phase followed by an ethanol fermentative phase (19). The genes that exhibit periodic expression patterns during the metabolic oscillation should be closely related to the physiological regulation between aerobic and anaerobic metabolisms. Using this dataset, I am able to show that WGD genes tend to have more dynamic regulation than non-WGD genes during the oscillation, indicating possible functional specialization of the WGD genes in either respiratory or fermentative stages. Our results provide new evidences for the “WGD-Fermentation hypothesis”.

2. 3. Result

2. 3. 1. Distribution of WGD and non-WGD genes in the metabolic cycle

The genes exhibiting periodic expression patterns during the metabolic oscillation are called metabolic cycling genes (18). I compared 1,108 WGD and 5,101 non-WGD genes to test whether WGD genes were more likely to have cycling expression pattern than the other genes in the genome. As a result, 722 WGD and 2,777 non-WGD genes were identified as metabolic cycling genes (Table 2.1). The proportion of metabolic cycling genes was significantly higher for the WGD genes than that for the non-WGD genes ($P \leq 0.0001$, Fisher's exact test). Ribosomal protein genes (RPs) are commonly retained after WGD (110 out of 137 RPs are WGD genes) and most of them cycle during the metabolic oscillation. Thus, to exclude the possibility that the result above was caused by RPs, I removed them from the data set and redid the analysis; the result remained the same (Table 2.1).

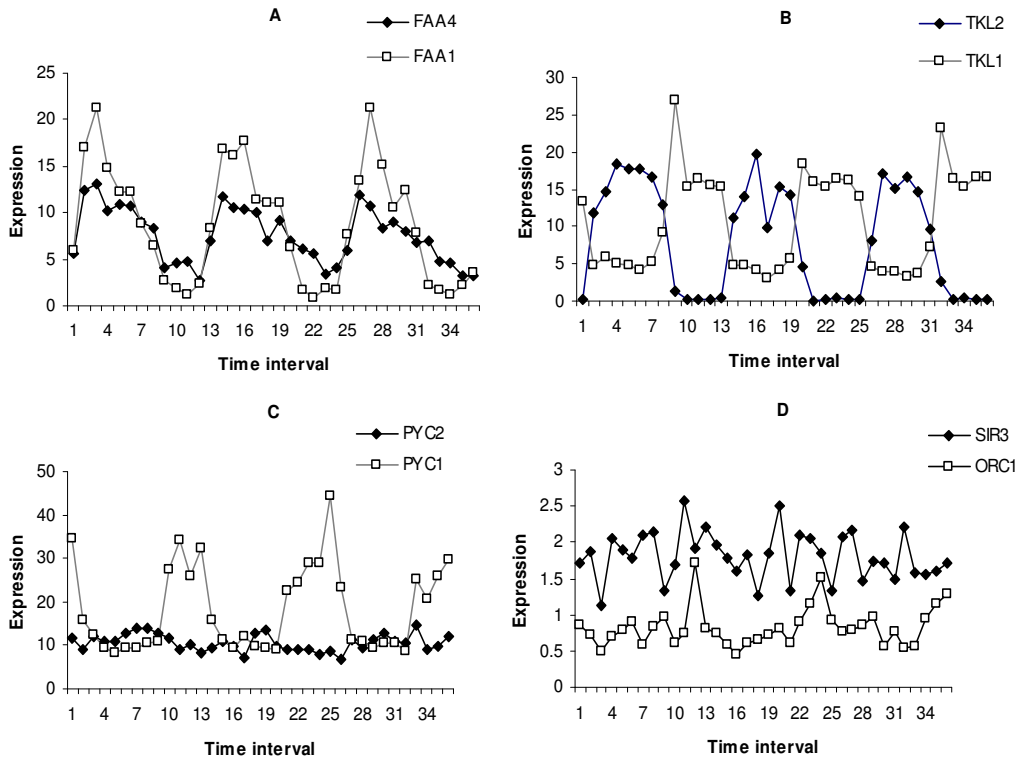
2. 3. 2. Distribution of WGD and non-WGD genes in different expression profiles

There are four possible expression profiles for duplicate gene pairs during the metabolic oscillation (Figure 2.1). The expression profile where two copies have the same cycle (Figure 2.1A) suggests that dosage effect might be important for keeping both genes working together in the same condition. Indeed, most of the RP duplicates have this expression pattern. The expression profile where two copies cycle differently (Figure 2.1B) may indicate subfunctionalization, neofunctionalization, or a combination of the two events (20) in which case

Table 2.1 Distribution of WGD and non-WGD genes in the metabolic cycle.

	Number of cycling genes	Number of non-cycling genes	Ratio
All genes			
WGD genes	722	386	1.87
Non-WGD genes	2,777	2,324	1.19
Excluding ribosomal protein and spurious genes			
WGD genes	595	313	1.90
Non-WGD genes	2,383	1,864	1.28

Figure 2.1. Examples of different expression profiles of duplicate genes during metabolic oscillation. A. Two genes have the same cycle; B. Two genes cycle differently; C. One gene cycles and the other does not; D. Both genes do not cycle.


A
FAA4
FAA1
Expression
Time interval
B
TKL2
TKL1
Expression
Time interval
C
PYC2
PYC1
Expression
Time interval
D
SIR3
ORC1
Expression
Time interval

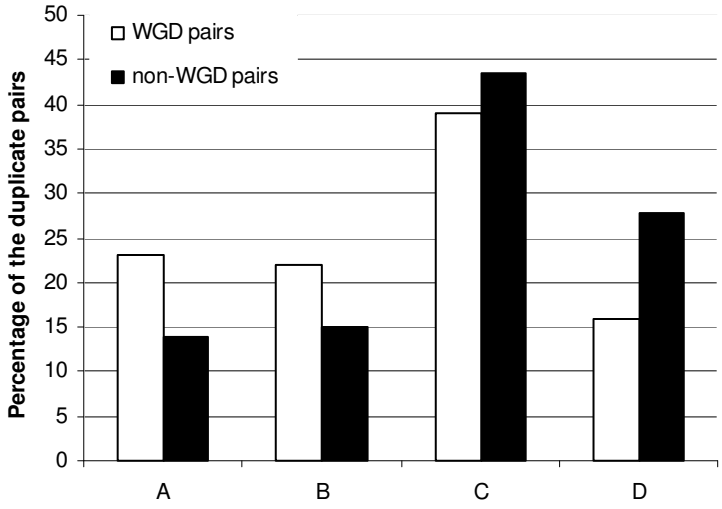
two duplicate genes have divergent functions in aerobic and anaerobic metabolic conditions. The regulation divergence could facilitate the functional optimization of duplicate genes in individual condition. Both expression profiles could provide competitive advantage for organisms during adaptation to the new fermentative lifestyle. On the other hand, the duplicate gene pairs with expression profiles where only one duplicate copy cycles (Figure 2.1C) or where both copies do not cycle (Figure 2.1D) might be functionally less relevant to the evolution of efficient fermentation.

After comparing the expression profiles for 554 WGD duplicate pairs (21) and 1,636 smaller-scale duplicate (SSD) ones (22), I found a significantly higher proportion of the WGD duplicate genes than the SSD ones with pattern A and B ($P \leq 0.0001$ Fisher's exact test), while the gene pairs with the other two expression patterns show opposite trends (Figure 2.2), suggesting dosage effect and functional specialization related with aerobic and anaerobic metabolisms might be important in keeping both duplicate genes after the WGD. The results provide evidence that the WGD event greatly contributed to the fermentative lifestyle, since more WGD genes, rather than SSD genes, are preferentially retained with the selective function of increasing the organism's ability to regulate its metabolism in response to oxygen change.

2. 3. 3. Distribution of WGD genes among functional categories

It has been reported that WGD genes have unique functional distributions when compared with the rest of the genome (22). Using the new dataset, I confirmed the previous results (23) that

Figure 2.2. The proportions of WGD and non-WGD genes with different expression profiles. (A. B. C. D. as indicated in Figure 2.1)


WGD pairs
non-WGD pairs
Percentage of the duplicate pairs
50
45
40
35
30
25
20
15
10
5
0
A
B
C
D

transcriptional factors were preferentially retained after WGD in yeast (6.22% vs. 2.53% for WGD and non-WGD genes, respectively. $P<0.0001$) (Table 2.2). I also found that the WGD metabolic cycling genes are enriched with antioxidant, signal transducer and enzyme regulator function (Table 2.3). This functional bias might be important for the yeast's anaerobic adaptation. To grow efficiently at low oxygen levels, high-level fermentative metabolism is essential, which involves many active enzyme regulators; moreover, it is essential to maintain the redox balance, which may require significant antioxidant activities (8). In addition, diversifying the signal pathways by retaining duplicate genes for many signal transducers would be a powerful way to increase the regulatory complexity of the yeast under anaerobic conditions (24).

Table 2.2. Distribution of transcription factors in WGD and non-WGD genes.

	Number of transcription factors	Number of non-transcription factors
WGD genes	69	1,039
Non-WGD genes	129	4,972

Table 2.3. Distribution of all yeast genes and WGD cycling genes among 13 GO functional categories.

GO functional categories*	Number of yeast genes in category	Number of cycling WGD genes	Percent of cycling WGD genes
Antioxidant activity	28	12	0.43
Signal transducer activity	202	57	0.28
Enzyme regulator activity	253	69	0.27
Structural molecule activity	337	86	0.26
Transcription regulator activity	394	75	0.19
Chaperone regulator activity	32	6	0.19
Translation regulator activity	66	10	0.15
Ase activity	3,790	552	0.15
Catalytic activity	605	83	0.14
Binding	1,295	171	0.13
Transporter activity	713	83	0.12
Protein tag	9	1	0.11
Triplet codon-amino acid adaptor activity	45	0	0.00

* The 13 GO functional categories which contain yeast genes are the first level categories under the Molecular Function GO part, downloaded on 25/Feb/2007.

2. 4. Discussion

In this study, I found a higher proportion of WGD genes than non-WGD genes are regulated in response to oxygen change. In addition, WGD duplicates, as compared to non-WGD pairs, are more likely to have cyclic expression during the metabolic oscillation. These results suggest that WGD event does contribute greatly to the evolution of efficient fermentation in post-WGD yeast species.

I have to emphasize that WGD might not be necessary for all the adaptive properties in a fermentative lifestyle. Some pre-WGD yeast species are also capable of facultative fermentation, yet none of them are comparable to *S. cerevisiae*. The fission yeast, *S. pombe*, which diverged from the ancestor of *S. cerevisiae* much earlier than the WGD, can conduct efficient aerobic fermentation. It can also consume ethanol but not as its sole carbon source (25) owing to the absence of a complete glyoxylate cycle. *K. lactis*, which is a poor producer of ethanol, can efficiently consume ethanol as its only carbon source. Therefore, it seems these adaptive properties are unevenly developed among different yeast lineages.

On the other hand, the WGD event is also not sufficient for fermentative lifestyle development. Soil yeast, *K. polysporus,* cannot carry out efficient fermentation although the organism experience WGD during its evolution (26). It was recently reported that *K. polysporus* and *S. cerevisiae* diverged very soon after the WGD, and that the following gene loss in these two clades proceeded completely independently (27). Increased availability of fruit sugars at the end

of the Cretaceous period might have provided a selective pressure for the development of fermentative ability (9), and the WGD happened to coincide with that event, thus providing a possible cause for the evolution of the efficient fermentation in *Saccharomyces* lineage. However, soil yeast *K. polysporus*, due to its unique living environment, might not be influenced much by the abundant fruit sugars, thus evolving in a totally different direction.

It remains unclear whether there were other molecular events that initiated the evolution of efficient fermentation in the ancestor of *S. cerevisiae* after its divergence from *K. polysporus*. Nevertheless, in accordance with previous observations, our results indicate that the WGD event could have led to a unique combination of adaptive properties related with efficient fermentation in *S. cerevisiae* and its closest relatives, thus providing new insights into the relationship between whole genome duplication and the evolution of new life styles in eukaryotic organisms.

2. 5. Experimental Procedures and Methods

Expression data during the metabolic oscillation were taken from Tu et al. (2005) (18). There are three super gene-clusters based on the expression data: Ox (oxidative), R/B (reductive/building) and R/C (reductive/charging). Each of these superclusters comprises genes that are periodically expressed and peak within a certain window of the yeast metabolic cycle. The 554 WGD pairs were from Byrne and Wolfe 2005. The 1,636 SSD pairs were derived from all the yeast's duplicate genes that are not associated with WGD (22). The re-annotated gene list from Wood et al (2001) (28) was also used in our analysis.

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CHAPTER THREE

F1,6BP regulation of pyruvate kinase is involved in mediating yeast aerobic fermentation

3. 1. Summary

The embryonic M2 isoform of pyruvate kinase (PKM2) which express exclusively in tumor cells is necessary for the shift in cellular metabolism towards aerobic glycolysis and this promotes tumorigenesis. *S. cerevisiae* pyruvate kinase isoform 1 (PYK1) shares similar features with PKM2 as it is also important in yeast fermentation growth when oxygen is present, and both genes can be well regulated by D-Fructose-1,6-Bisphosphate (F1,6BP). Here I measure the F1,6BP regulation abilities on PYK in 6 yeast species with different fermentation abilities. The result indicates that yeasts with higher capabilities on the allosteric regulation of PYK tend to have higher fermentation abilities, which indicates the function of F1,6BP regulation on PYK might be important in the development of fermentation lifestyle in yeast. A mutant called T403E which is defect in F1,6BP regulation shows raised oxidative phosphorylation and decreased fermentation rate, as well as an increased lifespan. There results provide new evidences to support the critical function of F1,6BP regulation on PYK in aerobic fermentation, which may shed lights on the mechanism of PKM2 in Warburg effect.

3. 2. Introduction

Glycolysis and oxidative phosphorylation are primary sources of cellular energy. Pyruvate, the end product of glycolysis, is either metabolized through the citrate cycle and respiratory chain or fermented to lactate or ethanol. In the first half of the twentieth century, Otto Warburg and his coworkers observed that cells switch from oxidative to fermentative metabolism during tumorigenesis. Despite the presence of oxygen, most cancer tissue respires with low efficiency but has increased glucose consumption and lactate secretion (1-3). Recently, it has become clear that this metabolic transition is not specific to cancer cells but rather a common metabolic feature of cells that rapidly proliferate. Warburg-like effects have been described in yeast (4), during T cell proliferation (5), and upon reprogramming fibroblasts into induced pluripotent stem cells (6).

The glycolytic enzyme pyruvate kinase (PYK) which catalyzes the conversion of phosphoenolpyruvate (PEP) to pyruvate has been implicated in the regulation of the Warburg effect. The low-active splice form of the PKM-type pyruvate kinase (PKM2) is present at higher concentration in cancers compared to matched control tissue (7, 8). And it's regulation by D-Fructose-1,6-Bisphosphate (F1,6BP) is confirmed to be important in cancer metabolism and tumor growth (8-10). A change to expression of the higher active PKM1 which is insensitive to F1,6BP increased respiration and slowed cancer progression in xenograft models (8). Studying the problem why PKM2 rather than PKM1 increases aerobic fermentation is critical in understanding the Warburg effect, thus improving the establishment of specific therapies for

cancer treatment.

A possible explanation is that PKM1 preferentially shuttles pyruvate to the mitochondria or PKM2 preferentially shuttles pyruvate to lactate dehydrogenase, as tyrosine phosphorylation of lactate dehydrogenase might facilitate its binding to PKM2 (8). Another study indicates there could be an alternative glycolytic pathway avoiding PYK step in PKM2-expressing cells, which is corresponding mostly to the high lactate production (11). Furthermore, it is reported that PKM2 hydroxylation on proline 403/408 stimulates binding and activation of hypoxia-induced factor HIF1α, increasing the expression of metabolic enzymes under hypoxia in a PYK activity-independent manner (12). However, the mechanism why F1,6BP regulation on PKM2 is important in Warburg effect is still unclear.

Some yeasts, like *S. cerevisiae,* display a Crabtree effect (aerobic fermentation), thus providing a good model for studying the phenomenon of Warburg effect. There is also existence of yeasts that are classified as Crabtree-negative such as *K. lactis*. Therefore, the identification of the differences between these two kinds of yeast may shed lights on the mechanism leading to Crabtree effect. The metabolic features shared between *S. cerevisiae* and tumors have been previously identified (13). *S. cerevisiae* has two copies of PYK genes which showed similar characteristics compared to their homologs in mammal. Like PKM2, *S. cerevisiae* PYK1 which can be well regulated by F1,6BP is dominantly expressed when yeast performs aerobic fermentation; while *S. cerevisiae* PYK2 which is insensitive to F1,6BP has expression when

glucose concentration is low and cells start doing respiration, a feature comparable to PKM1 (14, 15). Here I measured the F1,6BP regulation capabilities on PYK in some representative Crabtree-negative and positive yeasts, and showed that this regulation ability might be important for crabtree effect. Experiments on a T403E mutant strain which is defect in F1,6BP regulation suggested possible mechanisms and advantages for the allosteric regulation of PYK in cells which can perform aerobic fermentation.

3. 3. Result

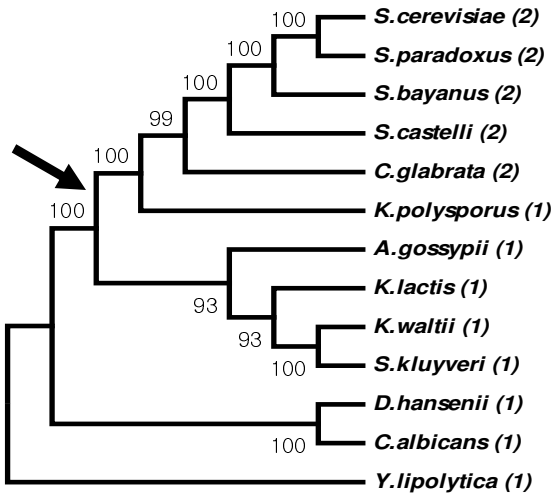
3. 3. 1. Distribution of copy number of pyruvate kinase genes in yeast phylogenetic tree

Many studies supported that the WGD event has led to the evolution of an efficient fermentation system in *Saccharomyces sensu stricto* lineage (16-19). Given that fact that PYK also belongs to WGD genes (20) and plays an essential role in glycolysis, I was interested in investigating the distribution of PYK gene number in representative fungi species and match these data with corresponding fermentation abilities. An interesting association between the copy number of PYK genes and the fermentation ability in yeast species was found as shown in Figure 3.1. Most post-WGD yeast species having two PYK genes are better fermenters (21) than those pre-WGD species with only one PYK gene. One post-WGD yeast *K. polysporus* which diverged very soon after the WGD with *S. cerevisiae,* cannot carry out efficient fermentation (22) and amazingly just kept one PYK gene in its genome, while the other one copy was lost during evolution. Therefore, comparing the differences of the characteristics of PYK genes between both kinds of yeast may provide some insights into the mechanism by which PYK duplication improved fermentation ability in yeast, thus shed light on the role of pyruvate kinase in Warburg effect in tumor cells.

3. 3. 2. F1,6BP regulation of pyruvate kinase in pre- and post-WGD yeast species

In *S. cerevisiae*, there are two PYK genes which are WGD duplicates. PYK1 can be positively up-regulated by F1,6BP and it seems to have dominant function during fermentation (23, 24);

Figure 3.1. Number of PYK genes in clade *Saccharomycotina*. Arrow indicates the whole genome duplication events. Numbers in parenthesis are number of PYK genes in each species.


100
100
100
99
100
100
93
93
100
100
S.cerevisiae (2)
S.paradoxus (2)
S.bayanus (2)
S.castelli (2)
C.glabrata (2)
K.polysporus (1)
A.gossypii (1)
K.lactis (1)
K.waltii (1)
S.kluyveri (1)
D.hansenii (1)
C.albicans (1)
Y.lipolytica (1)

while PYK2 is insensitive towards F1,6BP and it may only work when glucose concentration is low and cells switch energy metabolism towards respiration (23). This pattern is quite similar to PKM2 and PKM1 in mammals. I therefore studied F1,6BP regulation function of pyruvate kinase among three post-WGD yeast species (*S. cerevisiae, S. baynus, K. polysporus*) and three pre-WGD species (*K. lactis, S. kluyveri, C. albicans*), in order to test the hypothesis that F1,6BP regulation of pyruvate kinase contributed greatly to the evolution of aerobic fermentation.

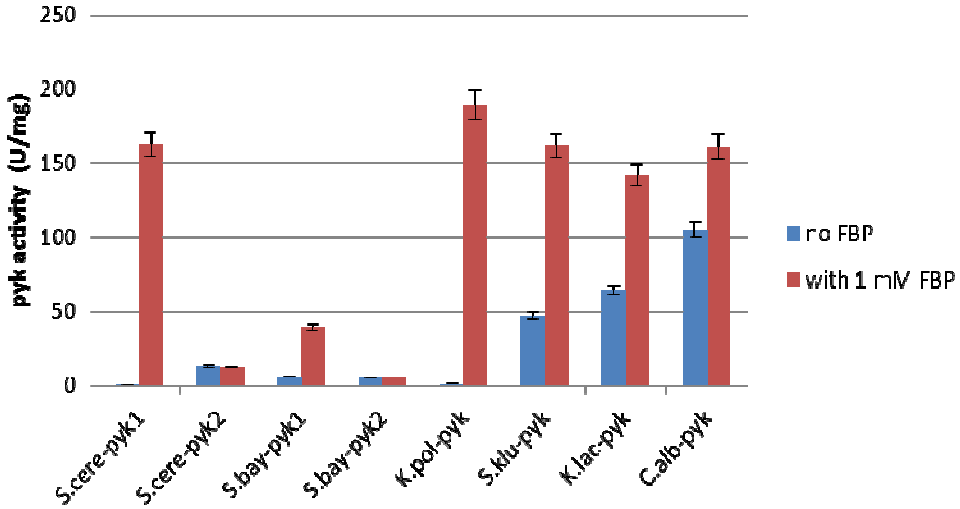
The in vitro pyruvate kinase activity study confirmed previous results that in *S. cerevisiae* PYK1 is strongly regulated by F1,6BP; while PYK2 has lower activity and is insensitive to F1,6BP regulation. The pyruvate kinase activity in other yeast species showed similar value to *S. cerevisiae* PYK1 when F1,6BP is added, with an exception of *S. baynus* in which even the activity of PYK1 is relatively low. This may result from different intracellular environment conditions, such as PEP concentrations. In addition, *S. baynus* PYK2 shares the same pattern with *S. cerevisiae* PYK2, as having no regulation of F1,6BP. However, the situation is quite different in all yeast species when F1,6BP is absent. As all three post-WGD yeast species nearly have no pyruvate kinase activity detected in isoform 1, all three pre-WGD species show significant pyruvate kinase activity, even without F1,6BP addition. Interestingly, the pyruvate kinase activities without F1,6BP in the three pre-WGD species seem to coordinate with their fermentation abilities, as *S. kluyveri,* the best fermenter among three (21) has the lowest pyruvate kinase activity, while *C. albicans,* which dominantly perform respiration (25), has the highest (Figure 3.2A). In order to better understand the extent of F1,6BP regulation of pyruvate kinase in

different yeast species, I calculated pyruvate kinase activity ratio (activity with F1,6BP divided by w/o F1,6BP) for PYK genes in all yeast species (Figure 3.2B). I can see a clear trend that post-WGD species have better F1,6BP regulation ability on PYK (for isoform 1) than pre-WGD yeast species.

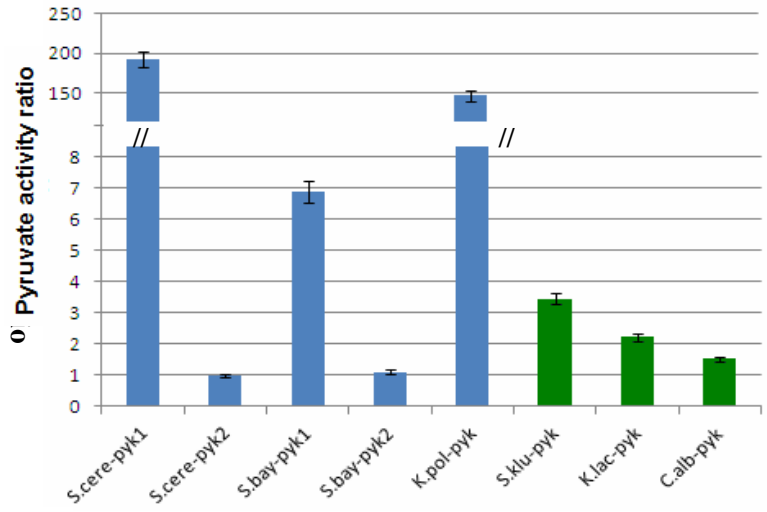
I then measured the fermentation ratios of all 6 yeast species (Figure 3.2C), and compared them with their corresponding pyruvate kinase activity ratio (Figure 3.2B). Fermentation ratio refers to ethanol production divided by glucose consumption. For both respiration and fermentation could cost glucose while only fermentation would yield ethanol, thus the fermentation ratio could represent the fermentation ability of yeast cells. I could see an approximately positive correlation between their pyruvate kinase activity ratios and their fermentation abilities, especially for the three pre-WGD yeast species, among which this correlation is quite clear. The incoordinated parts may have explanations. The fermentation ratio of *K. polysporus* is even lower than some pre-WGD species at beginning. This is consistent with the study that *K. polysporus* cannot carry out efficient fermentation due to a lot of gene loss after WGD (7). In addition, *S. kluyveri* has comparable fermentation ability with some post-WGD species, since although *S. kluyveri* is a petite-negative yeast, it still can grow anaerobically, a big difference from other pre-WGD species (26). Therefore, my results may suggest that the F1,6BP regulation on PYK plays an importance role on aerobic fermentation in yeast. The duplication of PYK after WGD event may experience subfunctionalization as keeping one copy insensitive to F1,6BP and strengthening the F1,6BP regulation on the other copy, thus helping most post-WGD species evolve into good

Figure 3.2 The ability of F1,6BP regulation on pyruvate kinase is related to corresponding yeast's fermentation ability. (**A**) The pyruvate kinase activity (U/mg) for 6 yeast species with or without 1mM F1,6BP treatment. (**B**) Pyruvate kinase activity activity ratio derived from Figure 3.3A. For each PYK gene, this ratio is calculated as activity w/o F1,6BP divided by that with 1mM F1,6BP. (**C**) Fermentation ratio at different time points for all 6 yeast species.

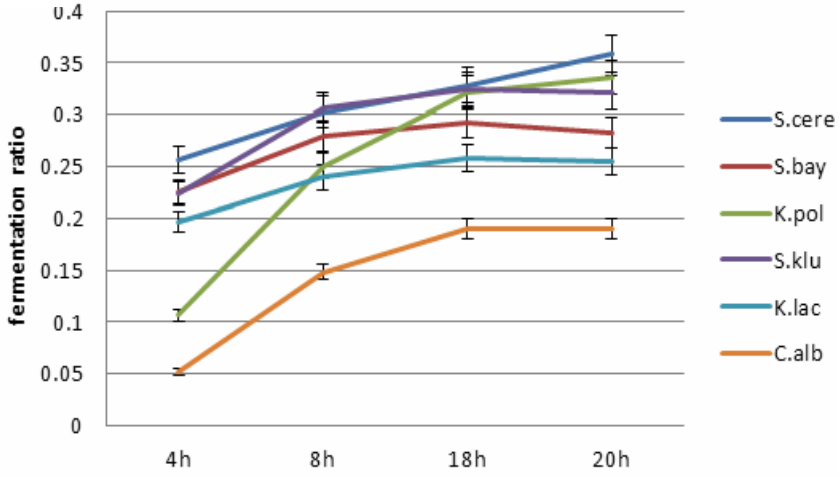
A


250
200
150
100
50
0
pyk activity (U/mg)
S.cere-pyk1
S.cere-pyk2
S.bay-pyk1
S.bay-pyk2
K.pol-pyk
S.klu-pyk
K.lac-pyk
C.alb-pyk
no FBP
with 1 mM FBP

B


250
200
150
8
7
6
5
4
3
2
1
0
Pyruvate activity ratio
S.cere-pyk1
S.cere-pyk2
S.bay-pyk1
S.bay-pyk2
K.pol-pyk
S.klu-pyk
K.lac-pyk
C.alb-pyk

C


0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
fermentation ratio
4h
8h
18h
20h
S.cere
S.bay
K.pol
S.klu
K.lac
C.alb

fermenters.

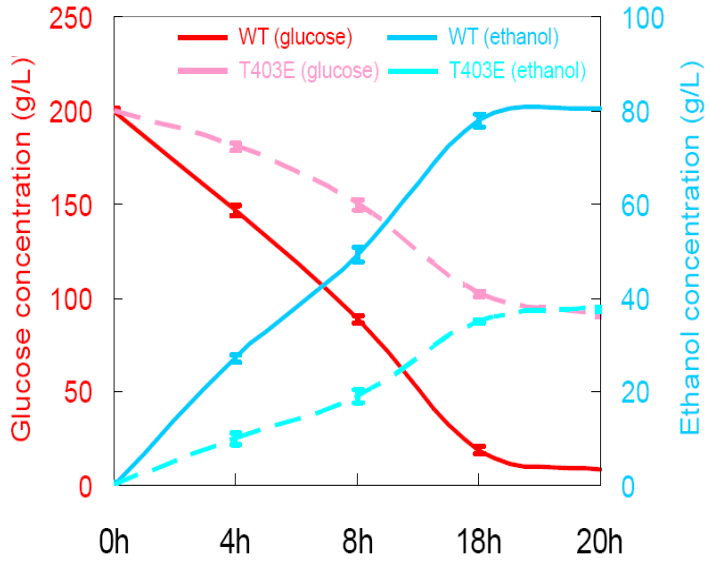
3. 3. 3. The fermentation ability of a mutant (T403E) which disrupt in F1,6BP regulation of pyruvate kinase

As I observed a positive correlation between the capability of F1,6BP regulation on pyruvate kinase and the corresponding yeast's fermentation ability, I want to further confirm this hypothesis within molecular mechanism. A mutant (T403E) with a point mutation in *S. cerevisiae* PYK1, which could block the binding of F1,6BP thereby preventing allosteric activation (27), may provide us a good opportunity to study this problem.

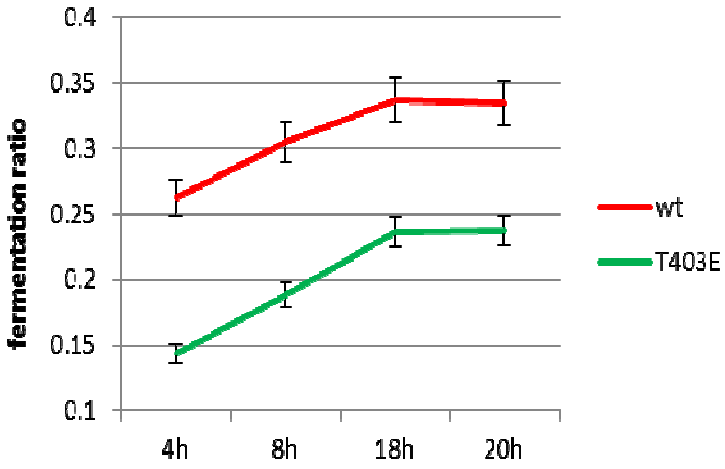
In order to get rid of the possible influence of PYK2, I constructed this mutant strain on a PYK2 deletion background and measured its fermentation ratio along with its wild-type control strain. I found that the wild-type strain consumed glucose much quicker and produced more ethanol as compared to T403E mutant (Figure 3.3A). This is consistent with the fact that the wild-type grows much faster than T403E mutant (data not shown). The fermentation ratio of the wild-type strain is also showed to be significantly higher than that of T403E mutant (Figure 3.3B), which proves our hypothesis that F1,6BP regulation on pyruvate kinase is important on aerobic fermentation, as disruption of this regulation could result in lower fermentation capability. I also measured the oxygen consumption of these two strains, and found that the T403E mutant consumed more oxygen than wild-type (Figure 3.3C). This result further supports our hypothesis and indicates that the disruption of F1,6BP regulation could change the metabolic switch from

Figure 3.3. The mutation T403E lowers fermentation and increases respiration. (**A**) Shown are glucose, ethanol concentrations during aerobic batch cultivation of wild-type strain and T403E mutant. (**B**) Fermentation ratio of wild-type strain and T403E mutant during aerobic batch culture. (**C**) The oxygen consumption (mg/ml/10^7) of wild-type strain and T403E mutant.

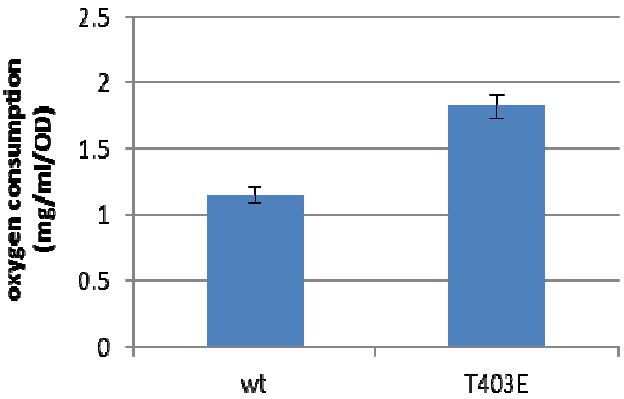
A


WT (glucose)
WT (ethanol)
T403E (glucose)
T403E (ethanol)
Glucose concentration (g/L)
Ethanol concentration (g/L)
250
200
150
100
50
0
100
80
60
40
20
0
0h
4h
8h
18h
20h

B


fermentation ratio
0.4
0.35
0.3
0.25
0.2
0.15
0.1
4h
8h
18h
20h
wt
T403E

C


oxygen consumption (mg/ml/OD)
2.5
2
1.5
1
0.5
0
wt
T403E

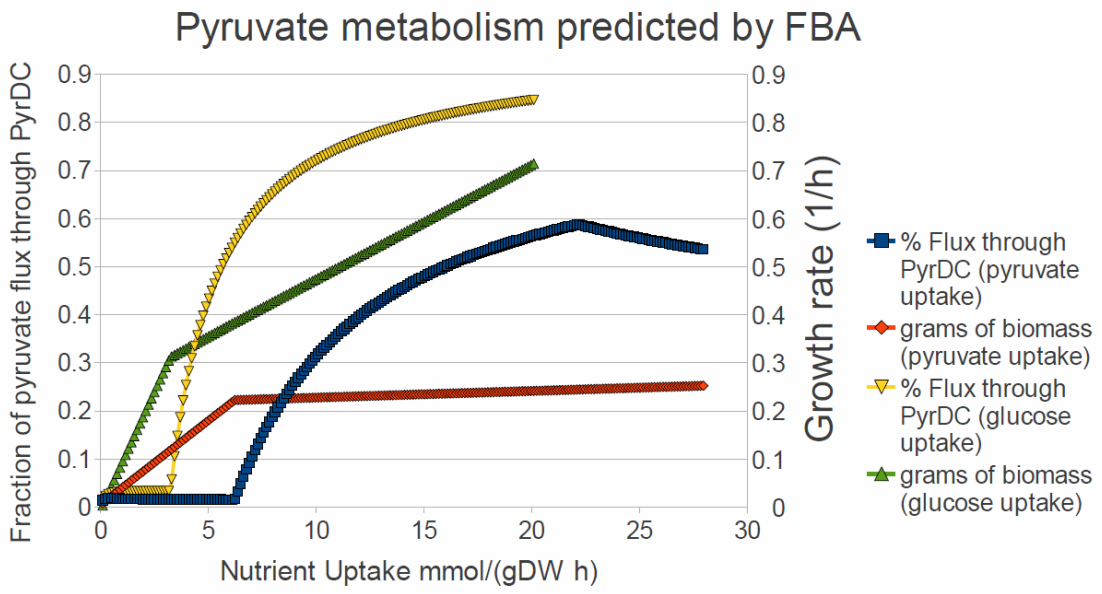
fermentation to respiration.

3. 3. 4. FBA simulation of pyruvate flux through fermentation

Flux Balance Analysis (FBA) is a metabolic modeling framework which employs the known stoichiometry of a system to simulate metabolism without requiring kinetic parameters or assumptions regarding specific fluxes (28-32). Microbes such as *S. cerevisiae* have evolved to maximize their growth rate; FBA uses a pseudo-reaction with biomass constituents that have been experimentally measured as reactants, scaled to the amount need for one gram of biomass (dry) so that it represents the growth rate. The growth rate is maximized, allowing us to see the activity of pathways used for growth in specific nutritive conditions.

The FBA simulation shows the fraction of pyruvate flux going through pyruvate decarboxylase (PyrDC) and the amount of biomass produced as a function of glucose uptake or pyruvate uptake when maximizing biomass production (Figure 3.4). PyrDC catalyzes the nonoxidative decarboxylation of pyruvate to acetaldehyde, thus leading the pyruvate flux towards fermentation (33). I observed that the flux through C rose along with the increase of glucose or pyruvate uptake, thus indicating that F1,6BP regulation on pyruvate kinase may influence the fermentation flux by controlling pyruvate production.

Figure 3.4. The fraction of pyruvate flux going through pyruvate decarboxylase (PyrDC) and the amount of biomass produced as a function of glucose uptake or pyruvate uptake when maximizing biomass.


Pyruvate metabolism predicted by FBA
Fraction of pyruvate flux through PyrDC
Growth rate (1/h)
Nutrient Uptake mmol/(gDW h)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
0
5
10
15
20
25
30
% Flux through PyrDC (pyruvate uptake)
grams of biomass (pyruvate uptake)
% Flux through PyrDC (glucose uptake)
grams of biomass (glucose uptake)

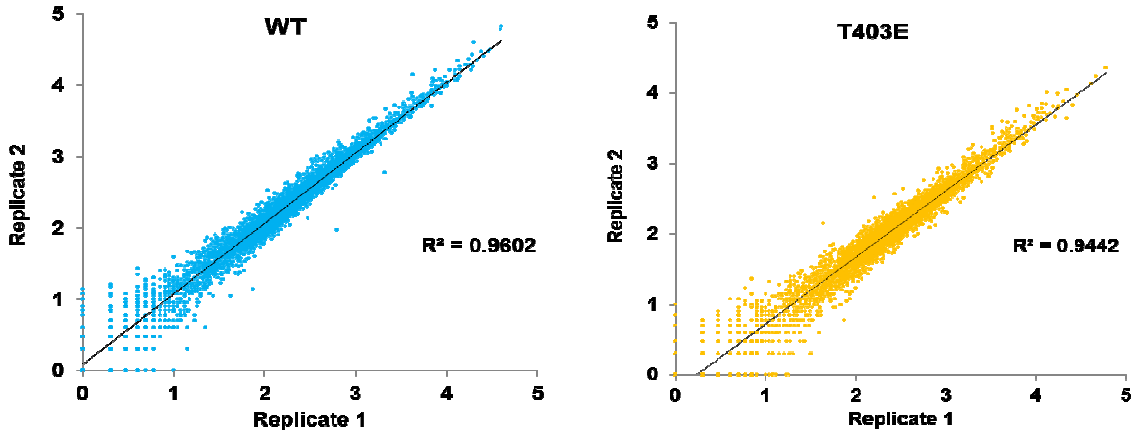
The simulation also indicates a moderate decrease in pyruvate through PyrDC when growing on pyruvate later on. This could be due to an increase in pyruvate flux through alanine aminotransferase (ALT1) to synthesize alanine, not other energy metabolic pathways (34).

3. 3. 5. Gene expression profiling of T403E by RNA-seq

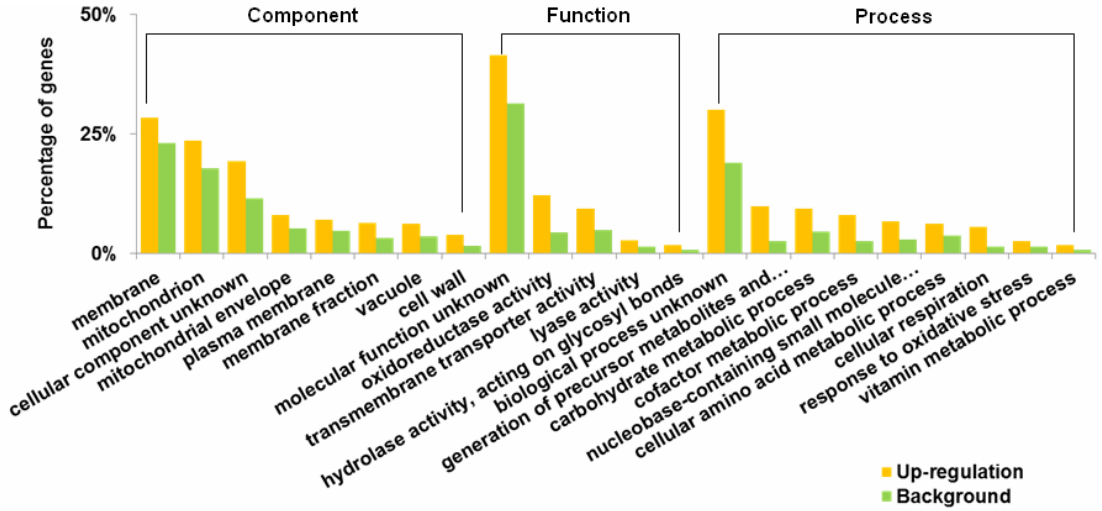
Previous results indicated that the T403E mutant, which is disable in F1,6BP regulation of PYK1, may make energy metabolism switch towards respiration by shutting down pyruvate flux. In order to prove this assumption and better understand the molecular mechanism change in T403E mutant, I employ RNA-seq to compare the transcriptome profiling of this mutant with that of wild-type. The Pearson's correlation between two replicates is quite high in both cases (Figure 3.5A), which makes the RNA-seq results more convincing. All the up-regulated and down-regulated genes in T403E are grouped into different GO function categories respectively. The up-regulated genes in T403E are significantly enriched in 22 second level GO terms, among which I found several GO terms inspiring: for example, mitochondrion, mitochondrial envelope under component category; oxidoreductase activity under function category; cellular respiration and response to oxidative stress under process category (Figure 3.5B). This result is consistent with previous data that the T403E mutant consumes more oxygen, and provides strong evidence to support that the dysfunctional F1,6BP regulation could affect energy metabolism by improving respiration. In the meanwhile, I also notice that the down-regulated genes in T403E mutant are enriched in ribosome and translation related function categories (Figure 3.5C), which explains the slower growth of the T403E mutant (data not shown).

Figure 3.5. The functional enrichment of up- and down-regulated genes in T403E strains. (**A**) Pearson's correlation of two expression profiling replicates for wild-type and T403E mutant respectively. The Y axis and X axis are $\log_{10}$ of total reads for each gene in corresponding replicate. (**B**) The X axis denotes the GO terms which showed significant enrichment of up-regulated genes in T403E (P-value < 0.01). The Y axis represents the percentage of genes in a certain functional category. The orange bars are up-regulated genes in T403E strains and the green bars are the percentage of genes at genomic background. (**C**) Significant enrichment of down-regulated genes in T403E (P-value < 0.01). The X and Y axis are similar to Figure 3.5B.

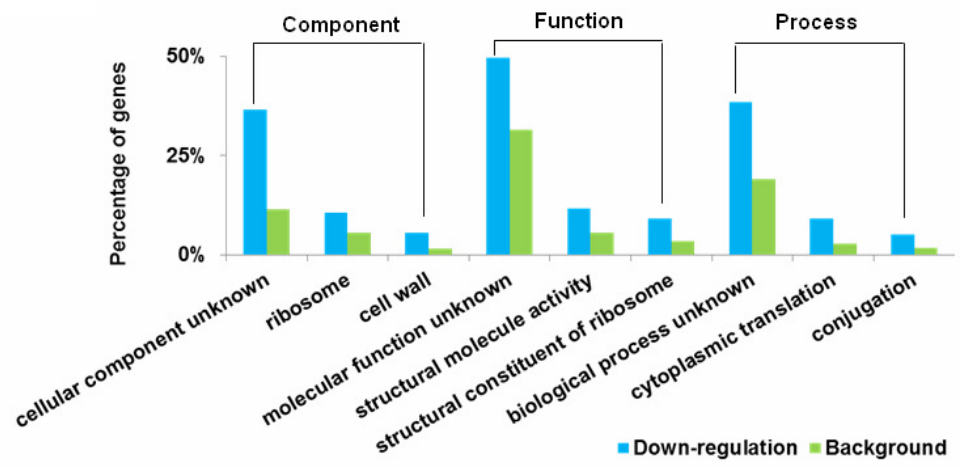
A


WT
Replicate 2
Replicate 1
R² = 0.9602
T403E
Replicate 2
Replicate 1
R² = 0.9442

B


Component
Function
Process
Percentage of genes
50%
25%
0%
membrane
mitochondrion
cellular component unknown
mitochondrial envelope
plasma membrane
membrane fraction
vacuole
cell wall
molecular function unknown
oxidoreductase activity
transmembrane transporter activity
hydrolase activity, acting on glycosyl bonds
lyase activity
biological process unknown
generation of precursor metabolites and...
carbohydrate metabolic process
cofactor metabolic process
nucleobase-containing small molecule...
cellular amino acid metabolic process
cellular respiration
response to oxidative stress
vitamin metabolic process
Up-regulation
Background

C

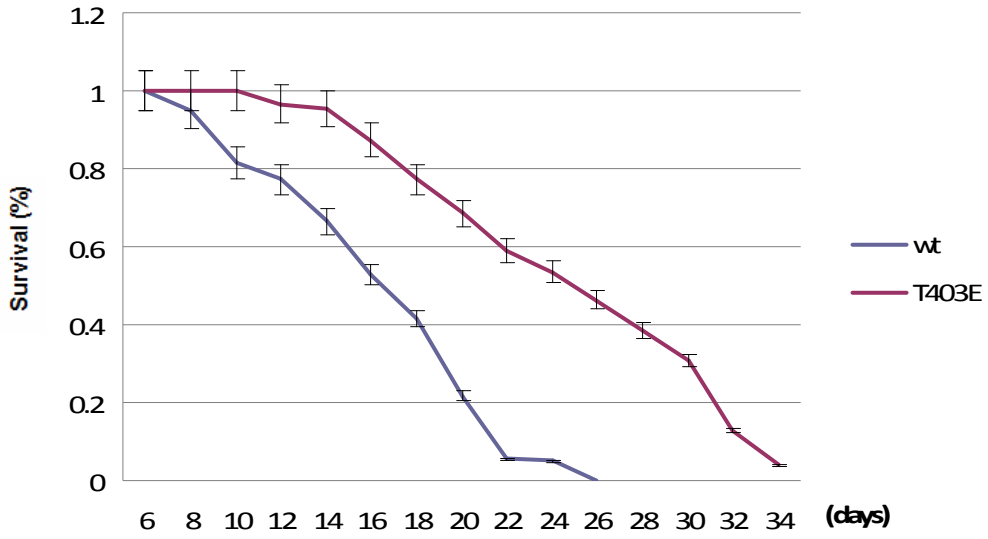

Component
Function
Process
Percentage of genes
50%
25%
0%
cellular component unknown
ribosome
cell wall
molecular function unknown
structural molecule activity
structural constituent of ribosome
biological process unknown
cytoplasmic translation
conjugation
Down-regulation
Background

3. 3. 6. The life span of T403E is increased compared to wild-type

It is reported that the shunting of carbon metabolism toward the mitochondrial tricarboxylic acid cycle and the concomitant increase in respiration plays a central part in life span extension in *Saccharomyces cerevisiae* during calorie restriction (35, 36). Though there is also contradictory study which claimed that calorie restriction in yeast does not require respiration to extend life span of mother cells (37), Su-Ju Lin et al. explained that this contradiction was just caused by different experimental protocols (38). So far the mechanism by which mitochondrial respiration affects yeast life span has not, to my knowledge, been determined to date. Since I uncovered increased respiration in T403E mutant, I am interested to further test the chronological life span of this mutant and wild-type strains.

As expected, the T403E mutant with higher respiration does have longer life span compared to wild-type (Figure 3.6). Therefore, I found new evidence to support that higher respiratory activity increases lifespan in yeast. As yeasts evolve aerobic fermentation to be better competitive by fast growing and ethanol production, they seem to have a trade-off for these advantages by shorting lifespan due to decreased respiration. Therefore, a good regulation ability of F1,6BP on PYK1 could be necessary to evolve along with aerobic fermentation, as at high glucose concentration, high level of F1,6BP could stimulate PYK1 to maximize pyruvate flux, thus facilitating aerobic fermentation to beat other competitors; while glucose is about to depleted, PYK1 will be sensitively inactivated due to lack of enough F1,6BP, therefore the diminishing pyruvate flux would make energy metabolism switch from fermentation to

Figure 3.6. Chronological life span analysis of wild-type and T403E mutant. Average lifespans: wild-type, 25.6 days; T403E, 34.3 days.


1.2
1
0.8
0.6
0.4
0.2
0
Survival (%)
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
(days)
wt
T403E

respiration, which is not only more economic at energy production but also keeps the yeasts at good status under starving conditions.

3. 4. Discussion

In the present study, I have shown that the duplication of pyruvate kinase gene after WGD event provides opportunities to develop a much more sensitive F1,6BP regulation function, which facilitating the evolution of aerobic fermentation. The observations in Figure 3.2B are construed to suggest that this allosteric regulation function might be largely improved right after the WGD, since the PYK of *K. polysporus* which diverged very soon after the WGD with *S. cerevisiae,* also possess as good F1,6BP regulation ability as *S. cerevisiae* PYK1, even if *K. polysporus* cannot carry out efficient fermentation (7). This may suggest that the well regulated F1,6BP regulation function on PYK is not sufficient for fermentative lifestyle development. However, it is reported that although the acquisition by an enzyme of some new regulatory feature may not cause a large increase in fitness, it would be retained in evolution whenever it provides a selective advantage (39). This explanation may apply to our case.

On the other hand, a sensitive F1,6BP regulation function on pyruvate kinase seems to be necessary for efficient aerobic fermentation, which is supported by many recent studies on PKM2 in cancer cells (8-10). Christofk et al (8) found that switching pyruvate kinase expression to PKM1, which is insensitive to F1,6BP yet more active than PKM2, could lead to reversal of the Warburg effect, as judged by reduced lactate production and increased oxygen consumption. Another research group showed that a PKM2 mutant Y105F, which elevated PKM2 activity by preventing the release of F1,6BP from PKM2, had increased oxygen consumption and decreased lactate production relative to H1299 lung cancer cells expressing wild-type PKM2 (9). Our

results also indicate that this assumption could be true in yeast since T403E mutant which cannot be activated by F1,6BP on PYK1 increases oxidative phosphorylation while decreases its fermentation ratio.

It has been reported that low PYK activity could activate yeast respiration (40). In cancer cells, knockdown of PKM2 expression also results in reduced glycolysis and decreased cell proliferation (10). Our FBA simulation predicted a positive correlation between the amount of pyruvate uptake and the flux towards fermentation. Therefore, decreased pyruvate flux caused by dysregulation of F1,6BP on PYK1 could be regarded as a possible mechanism leading to decreased fermentation ability in T403E mutant. However, studies in cancer cells on PKM2 (8, 9) indicate that pyruvate kinase activity is not the determinant reason for the energy metabolism change, as pyruvate kinase with elevated activity yet cannot be regulated by F1,6BP still decrease lactate production in cells. Our results also show that pre-WGD species like *C. albicans* which cannot conduct efficient fermentation have PYK with high activity even in the lack of F1,6BP (Figure 3.2A).

All these results together suggest that F1,6BP regulation on pyruvate kinase is necessary for aerobic fermentation. Pyruvate flux changed by dysfunction of F1,6BP regulation might be a potential mechanism to influence the switch of energy metabolism but not the determinant reason. Other mechanisms caused by F1,6BP regulation on PYK may play more important roles on facilitating efficient fermentation, but still need to be explored. One possible explanation why

allosteric regulation of PKM2 is important in aerobic fermentation and tumor growth is that the less active PKM2 dimer form results in partial inhibition of glycolysis, which accumulates all upstream glycolytic intermediates as an anabolic feed for synthesis of lipids and nucleic acids, whereas reassociation of PKM2 into active tetramer replenishes the normal catabolism as a feedback after cell division (41). Therefore, the regulation of PKM2 activity may allow for a balance between ATP production and fatty acid/nucleic acid production. Alternatively, F1,6BP regulation of PKM2 enzymatic activity may provide a direct link between cell growth signals and control of glycolytic metabolism (42). Our result on T403E life span also suggests that the less active form of pyruvate kinase may provide cells with healthier environments which are necessary for the fitness of the cells that can perform aerobic fermentation.

3. 5. Experimental Procedures and Methods

Phylogenetic tree of yeast

This Phylogenetic tree was constructed using the Maximum-Likelihood (ML) method with 1000 bootstrap replicates by Phyml 2.4 (43) using the ribosome protein gene sequences from 13 fungal species.

Cloning of pyruvate kinase genes

We independently cloned 8 PYK genes onto ppSUMO Kan plasmid. These genes and the corresponding cloning primers are *S. cerevisiae* PYK1 (5' CAGATTGGTGGATCCATGTCTAGATTAGAAAGATT3' & 5' GTGGTGGTGCTCGAGTTAAACGGTAGAGACTTGCA3') / PYK2 (5'CAGATTGGTGGATCCATGCCAGAGTCCAGATTGCA3' & 5' GTGGTGGTGCTCGAGCTAGAATTCTTGACCAACA 3'), *S. baynus* PYK1 (5' CAGATTGGTGGATCCATGTCTAGATTAGAAAGAT3' & 5' GTGGTGGTGCTCGAGTTAAACAGTAGAGACTTGCA3') / PYK2 (5' CAGATTGGTGGATCCATGCCAGAATCAAGACTGC3' & 5' GTGGTGGTGCTCGAGCTAAAACTCTTCACCAA3'), *K. polysporus* PYK (5' CAGATTGGTGGATCCATGATGCAATCTAGATTAGG3' & 5' GTGGTGGTGCTCGAGTTAAACAATGGTAACTCTCA3'), *K. lactis* PYK (5' CAGATTGGTGGATCCATGGAATCTAGATTAGGCTG 3' & 5' GTGGTGGTGCTCGAGTTAAACGGTCAAGACACGTA 3'), *S. kluyveri* PYK (5'

CAGATTGGTGGATCCATGGAATCTAGACTAGGTTG3’ & 5’ GTGGTGGTGCTCGAGTTAGACGGTCAAGACACGCA 3’), and *C. albicans* PYK (5’CAGATTGGTGGATCCATGTCTCACTCATCTTTATC3’ & 5’ GTGGTGGTGCTCGAGTTAAGCTTGGACGATTCTAA3’). Each PYK cloned as BamHI-XhoI fragment (sites are underlined in the sequence of the primers) into the same plasmid.

Expression and purification

Pyruvate kinase was purified from *E.coli* DH5a containing ppSUMO-PYK vector. Overnight culture was transferred to fresh LB+Kan media and grown up to ~0.6 OD under 37°C. After lower the template to 25°C and continue grow for 0.5 hr, 1M IPTG was added to stimulate protein expression for 4 hr at 25°C. Cells were then collected and stored at -80°C.

I largely simplified the purification of the enzyme. After breaking the cells in cold PBS solution by supersonic, the suspension were mixed with washed His-sumo tag beads (GLODBIO, CAT #H-310-5) at 4°C for 1 hr. The beads were then washed with PBS buffer for several times. A protein assay dye (Bio-Rad, CAT #500-0006) was used for quick protein detection. After no proteins were detected at the washed elution, the beads were transferred into a 2ml tube with addition of fresh PBS and restriction enzyme UlpI, and then incubated with mild shaking at 4°C overnight. The suspension will contain the PYK I wanted and can be store at -80°C.

Pyruvate kinase assay

Pyruvate kinase activity was measured by a continuous assay coupled to lactate dehydrogenase (LDH) as previously described (44, 45). Activity was measured by the change in absorbance at 340 due to oxidation of NADH by lactate dehydrogenase. Spectrophotometric measurements were made on either a Biotek machine or on a Cary 100 UV/VIS spectrophotometer with a cuvette holder thermocoupled at RT. Assay conditions were 0.05 M Imidazole-HCl buffer, pH 7.6, containing 0.12 M potassium chloride and 0.062 M magnesium sulfate; 4.5 mM Adenosine diphosphate; 6.6mM NADH and ~10 unit/ml Lactate dehydrogenase (LDH). F1,6BP and PEP concentrations were varied in these assays. The reaction volume was 3 mL.

Reactions were initiated by addition of between 0.01 and 0.04 ug of PYK. Steady-state kinetic rates were determined by measuring the slope of the tangent to the reaction progress curve. Stock substrate concentrations were verified and adjusted using an end point PYK assay by adding an excess of PYK and then measuring the total decrease in absorbance at 340 nm after 5 min. Pyruvate contamination in the PEP stock was measured by adding LDH to the assay buffer and measuring the total decrease in absorbance at 340 nm. The extinction coefficient of NADH used was 6.22 mM^{-1} cm^{-1}. PYK concentrations were measured by a protein assay dye: Bradford (Bio-Rad, CAT #500-0006) with a spectrometer at 595nm. 1mg/ml BSA were used for Standards.

Fermentation ratio measurement

Three independent clones for each strain are picked. The overnight cultures are transferred into 2% YPD media and grow for 2 days. Collected cells are washed with distilled water and then grow in 10ml SDC medium of 20% glucose at a 150ml flask under 250 rpm at 30°C. The cell density starts as high as OD_{600}=15 at the beginning. In these experiments, the cell concentration was prepared at a high level in order to rapidly evaluate the effects of the carbon materials on fermentation, and don't have to consider about the effect of different growth rates for each species. Sample media are collected every 4hr for HPLC analysis. The OD_{600} is also monitored during the whole procedure.

A Waters Associates liquid chromatograph, model ALC/GPC 244 equipped with a RID detector and an H-column (3.2 mm i.d. x 20 cm) are used to measure the concentration of glucose and ethanil. The mobile phase is 5% H_2SO_4. Operating conditions are flowrate: 0.6 ml/min, detector sensitivity: 8X, and chart speed: 0.4 in/min. 5 standards containing both glucose and ethanol have glucose concentrations ranging from 100, 50, 10, 1 to 0.5 g/L, and ethanol concentrations ranging from 50, 20, 10, 1 to 0.5 g/L.

The fermentation ratio is measured by ethanol yield per glucose consumption, defined as (EtOH[t]-EtOH[0])/ (Glucose[0]-Glucose[t]), where t indicates the time points when glucose and ethanol are estimated. The higher the ratio is, the stronger the aerobic fermentation is.

Construction of T403E mutant strain

The vector carrying T403E are kindly provided by B.L. Stoddard (46). One PCR fragment was amplified by using this plasmid as template and with two long primers: 5'CTTGTTTCTATTTACAAGACACCAATCAAAACAAATAAAACATCATCACAATGTCTA GATTAGAAAGATT3' and 5'TGGCTAGAAGAATAGGACGGAGTAGCACTTATCGTTTCTGATAAGAGGATCAATCCT TTGAAATCTTGAA3'. This fragment which contains the latter part of *S. cerevisiae* PYK1 with T403E mutation and a following kanMX marker was then transferred into a PYK2 deleted BY4742 strain (MATa his3Δ leu2Δ lys2Δ ura3Δ pyk2Δ). The transformed clones were picked and sequenced for the PYK1 gene. The ones with T403E mutation on PYK1 are the wanted mutants, and the ones with wild-type PYK1 are used as control.

Oxygen consumption measurement

Cells were grown to an OD_{600} of 1 in 2% YPD, spun down, and kept on ice. One hour before the measurements, cells were resuspended in SD–2% glucose and put on a shaker at 30°C for 1 hour. OD_{600} measurements and cell counts were performed immediately before putting cells in the oxygen sensing setup. The oxygen depletion rate was monitored by using a lab-built respirometer comprising a polarographic dissolved oxygen probe of Orion Star™ Series DO Meter (Thermo Scientific) in a glass syringe with constant agitation (47). Oxygen consumption rates were expressed as mg/ml O_2 per minute per 1 X 10^7 cells.

FBA simulation

I employ the iMM904 model for *S. cerevisiae*, which includes 904 genes, 1412 reactions, and 1212 metabolites to see how pyruvate consumption in different pathways varies depending on glucose or pyruvate uptake. A minimum of 0.1 mmol/(gDW h) glucose is consumed with increasing amounts of either pyruvate or glucose uptake, extending to a maximum experimental uptake for each metabolite.

Library Construction and Data Analysis for Illumina-Based Strand-Specific Multiplex RNA-Seq

Two independent clones of each strain are cultured in 2% YPD until OD_{600} reached 0.5. RNA exaction with TRIzol was then performed (48) and the pellets were re-suspended in 0.05–0.3 mL RNA-secure^{TM} solution (Ambion, CA) based on the pellet size and heated at 60°C for 10 minutes before stored at -80°C in a freezer.

I used a low-cost and robust protocol to produce Illumina-compatible (HiSeq2000 platform) RNA-seq libraries (49). The starting total RNA concentration was measured using NanoDrop (Thermal Scientific, DE) and 15 ug of total RNA was used for mRNA purification. After heated on 65°C for 2 minutes, the RNA samples were mixed with 30 uL of Dynabeads (Invitrogen, CA) in binding buffer (Tris-HCl 20 mM, LiCl 1 M, EDTA 2 mM) and incubated at room temperature with mild agitation for 10 minutes. The beads were then washed twice with 150 uL of washing buffer (Tris-HCl 10 mM, LiCl 0.15 M, EDTA 2 mM), and eluted with 50 uL elution buffer

(Tris-HCl 10 mM) at 80°C on an incubator/shaker for 2 min with mild agitation. The concentration of mRNA was immediately measured with 1 uL of sample in a QubitH Fluorometer (Invitrogen, CA). 30 ng of mRNA was used for cDNA synthesis. 4 uL of spiking RNA mix and 4uL of 5 x first strand buffer (Invitrogen, CA) were then added to each mRNA sample. The mixture was incubated at 94°C for 4.5 minutes to fragment RNA and then immediately chilled on ice before the next step.

The 12ul fragmented mRNA, 0.5 uL of Random primer (Invitrogen, CA), 0.75 uL of SupeRase-In (Ambion, CA) and 1 uL of DTT (100 mM) were heated at 65°C for 3 minutes. Then 4 uL of water, 1 uL of DTT (100 mM), 0.1 uL of dNTPs (25 mM), 0.5 uL of SupeRAse-In and 0.5 uL of Superscript II (Invitrogen, CA) were added and incubated in a PCR machine using the following conditions: 25°C for 10 minutes, 42°C for 50 minutes, 70°C for 15 minutes and a 4°C hold. The resulting RNA/cDNA double-stranded hybrid was then purified with AMPure XP beads and added to 2 uL of 10x NEB buffer-2 (NEB, MA), 1 uL of dUTP mix (10 mM dATP, dCTP, dGTP and 20 mM dUTP), 0.5 uL of RNAse H (2 U/mL), 1 uL of DNA polymerase I and 0.5 uL of DTT(100 mM). The mixture was incubated at 16°C for 2.5 hours. The resulting dUTP-marked dsDNA was again purified with AMPure XP beads.

The purified dsDNA (16 uL) was mixed with 2 uL of 10xEnd Repair Buffer (Enzymatics, MA), 1 uL of dNTP mix (10 mM each) and 1 uL of End Repair enzyme mix (Enzymatics, MA). The mixture was incubated in a PCR machine for 30 minutes at 20°C and purified with AMPure XP

beads. It was then added to 2 uL of 10x NEB buffer-2 (NEB, MA), 1 uL of 10 mM dATP mix, and 0.5 uL of Klenow 3'–5' exo$^-$ (Enzymatics, MA). The mixture was incubated in a PCR machine at 37°C for 30 minutes and then purified. The resulting end-repaired and dA-tailed product was then added to a mixture of 1 uL of indexed adaptor with 1ul T4 DNA ligase (Enzymatics, MA) and 2 x ligation buffer. Half of the ligation solution was mixed with 12 uL of "12p XP" beads and incubated at RT for 6 minutes. The supernatant was then mixed with 12 uL of AMPure XP beads and 5 uL of 40% of PEG8000 and eluted with 10 uL of nuclease-free water.

The size-selected dsDNA product was mixed with 1 uL of uracil DNA glycosylase (Enzymatics, MA) and incubated at 37°C for 30 minutes. The mixture was then added to 2 uL of Illumina PE primers (5 mM each), 6 uL of 5xPhusion HF buffer, 1 uL of 10 mM dNTP, 1 uL of Phusion Hot Start 2 High-Fidelity DNA Polymerase (NEB, MA) and 4.5 uL of water. The PCR mix was incubated with a programmed cycle as following: 94°C for 30 sec, 8-16 cycles (depend on final concentration) of 98°C, for 10 sec, 65°C for 30 sec, 72°C for 30 sec; 72°C for 5 min followed by a hold at 4°C. The final product was purified with 43 uL of AMPure XP beads and eluted with 12 uL of EB buffer. The concentration of PCR products was measured using the dsDNA-HS protocol on the Qubit Fluorometer. Equal quantities of libraries (approximately 5 ng per sample) with different indices were mixed and stored in -80°C freezer. The illumine sequencing was down in genomics resources core facility at WCMC with the platform of HiSeq2000 58 cycles.

Life span measurement

Streak yeast onto YPD plates and incubate for 2 days at 30°C. Pick a few colonies (four to five) into 1–2 mL of 2% SDC medium and grow overnight. Dilute the overnight culture to an initial density as OD_{600} of 0.1–0.2 in 10–50 mL of SDC. Incubate at 30°C in flasks with shaking at 220 rpm. Each strain has three replicates.

After 3 days, start monitoring survival by measuring the ability of an individual yeast cell/organism to form a colony. Cultures are serially diluted to reach a $1{:}10^5$ dilution in sterile distilled water and 100 uL of the same dilution are plated in duplicate onto two YPD plates. The forming clones are counted after 2–3 days. The maximum number of clones usually at day 3 is considered to be the initial survival (100% survival) and is used to determine the age-dependent percent survival. Monitor the number of clones every 48 hr by adjusting the dilution factor and the volume plated according to the mortality rate. Continue until survival reaches 1% to record a time value that approximates the maximum survival time (50).

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CHAPTER FOUR

Duplication of the target of rapamycin (TOR) gene contributes to the fermentation life style in yeast

4. 1. Summary

The budding yeast *Saccharomyces cerevisiae* and its relatives predominantly ferment glucose into ethanol even in the presence of oxygen. The underlying genetic basis of the evolution of aerobic fermentation remains unclear. There are growing evidences to support that the target of rapamycin (TOR) might be involved in the regulation of energy metabolism, the role of TOR gene in the evolution of aerobic fermentation has not been fully explored. In this study, I revealed a positive correlation between the copy number of TOR genes and the strength of aerobic fermentation, suggesting that TOR gene duplication has facilitated the evolution of aerobic fermentation. I further doubled the TOR gene in a Crabtree-negative yeast *K.lactis* to mimic the TOR gene duplication during yeast history, and observed that the fermentation ratio in this mutant is significantly increased. My results not only provide evidence to support that TOR gene duplication contributes greatly to the evolution of fermentation life style in yeast, but also shed light on the causal correlation between dysregulation of mTOR signaling and Warburg effect in cancer cells.

4. 2. Introduction

Aerobic fermentation is a glucose metabolic pathway that predominantly ferments glucose to ethanol even in the presence of oxygen. The hemiascomycete yeasts that prefer aerobic fermentation are called Crabtree-positive yeasts (1); these include *Saccharomyces cerevisiae* and its relatives. In contrast, the Crabtree-negative yeasts, such as the dairy yeast *Kluyveromyces lactis*, prefer to completely oxidize glucose to CO_2 through the mitochondrial respiration pathway for maximum energy and biomass gain (2). The fission yeast *Schizosaccharomyces pombe*, which diverged from the hemiascomycete lineage around 330–420 Ma (3), also prefers aerobic fermentation (4). Therefore, it is believed that aerobic fermentation has evolved independently in the *S. cerevisiae* lineage and in the *S. pombe* lineage.

Many studies have shed light on the underlying genetic basis for the origin and evolution of aerobic fermentation, but different explanations were proposed (5-10). Several studies have shown that the target of rapamycin (TOR) might be involved in the regulation of energy metabolism (11-15). TOR is an essential ser/thr protein kinase that functions in two distinct multiprotein complexes, TOR complex 1 and 2. The structure and functions of these complexes have been conserved from yeast to man. TOR complex 1 is inhibited by rapamycin and is thought to couple growth cues to cellular metabolism; TOR complex 2 is not inhibited by rapamycin and appears to regulate spatial aspects of growth such as cell polarity (16-18).

Tumor cell energy metabolism shares some common features with yeast metabolism, as tumors

display an enhanced glycolytic activity and oxidative phosphorylation downregulation (Warburg effect) (19-22). It is also widely accepted that hyperactivation of mTOR signaling is one of the most frequent occurrences in human cancer (23). However, the regulatory mechanism of Warburg effect is still obscure. On the other hand, the underlying mechanisms that cause tumorigenesis as a result of activated mTOR remain largely unknown. Several recent studies indicate a role of mTOR signaling on direct control of energy metabolism in cancer cell, yet meet in huge conflicts. While some researchers found that inhibiting the rapamycin-sensitive subset of mTOR functions in cancer cells enhanced aerobic glycolysis and decreased uncoupled mitochondrial respiration (24, 25), other studies provided evidences to support that mTOR was a major positive regulator of the Warburg effect (26-29). Since metabolism regulations are complex in different kinds of cancer cells, yeasts may serve as a simple model to clarify this mechanism given the common features and conserved pathways with cancer cells.

Studies in *S. cerevisiae* showed that reduced TOR signaling was accompanied by increased respiration and upregulation of mitochondrial gene expression (11), which suggests a possible role of TOR signaling on the regulation of energy metabolism. However, in-depth investigation of the evolutionary history of TOR genes and their role in the evolution of aerobic fermentation in yeasts has not been reported so far. In our study, I firstly identified an interesting association between the copy number of TOR genes and the fermentation ability in yeast species. In view of this fact, mimicking duplication event of TOR gene in pre-WGD yeast species such as *K.latics* will likely provide new insights into the evolution of yeast aerobic fermentation, which may also

shed lights on the question whether and how the change of TOR signaling lead to Warburg effect in cancer cells.

4. 3. Results

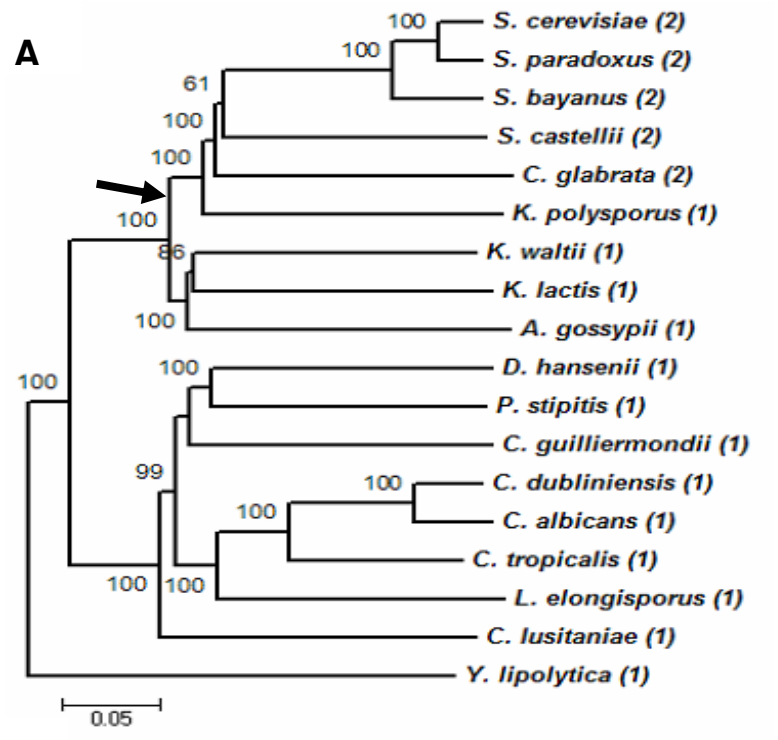
4.3.1. Distribution of copy number of TOR genes in yeast phylogenetic tree

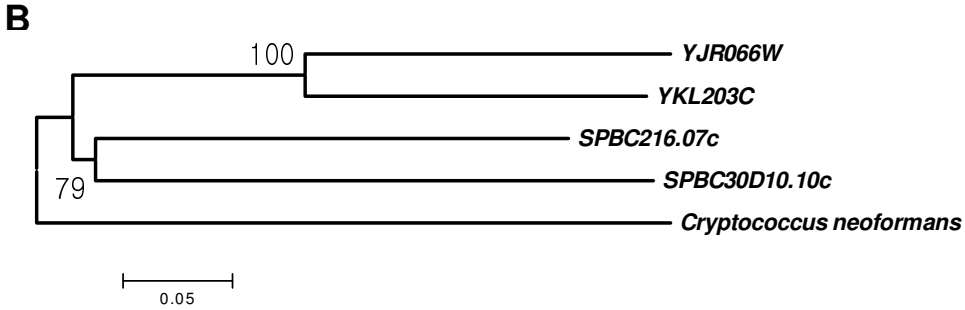
To study the evolution of the TOR gene, I constructed a phylogenetic tree using the homologous sequences from 18 yeast species. I found that almost all the post-WGD yeast species possess two copies of TOR gene while the pre-WGD yeast species only have one (Figure 4.1A). The WGD event has been proposed to have led to the evolution of fermentation lifestyle in yeast (30-33) which makes the post-WGD species better fermenters compared to those pre-WGD ones in most cases (2), however, it remains unclear how many and which WGD genes have the most contribution. Given the evidences that TOR gene is involved in controlling energy metabolism (11-15), I want to ask if TOR gene duplication from WGD plays an important role in the development of fermentation ability.

I found one post-WGD yeast *K. polysporus* that cannot carry out efficient fermentation (34) just retains one TOR gene in its genome, with the other one lost during the evolution (Figure 4.1A). On the other hand, the fission yeast, *S. pombe*, which diverged from the ancestor of *S. cerevisiae* much earlier than the WGD, can conduct efficient aerobic fermentation, and as well has two TOR genes that are derived from an independent gene duplication event (Figure 4.1B). These correlations suggest that the TOR gene duplication may have facilitated the evolution of aerobic fermentation.

I also found three large-scale independent duplications of ribosomal protein (RP) gene occurred

Figure 4.1. A. Number of *tor* genes in clade *Saccharomycotina*. Arrow indicates when WGD occurred. Numbers in parenthesis are number of *tor* genes in each species. **B.** Phylogenetic tree of two *tor* genes in *S. cerevisiae* and *S. pombe* shows independent *tor* gene duplication. *S. cerevisiae tor* genes: YJR066W and YKL203C; *S. pombe tor* genes: SPBC216.07c and SPBC30D10.10c.


A
100
100
61
100
100
100
86
100
100
100
99
100
100
100
100
S. cerevisiae (2)
S. paradoxus (2)
S. bayanus (2)
S. castellii (2)
C. glabrata (2)
K. polysporus (1)
K. waltii (1)
K. lactis (1)
A. gossypii (1)
D. hansenii (1)
P. stipitis (1)
C. guilliermondii (1)
C. dubliniensis (1)
C. albicans (1)
C. tropicalis (1)
L. elongisporus (1)
C. lusitaniae (1)
Y. lipolytica (1)
0.05


B
100
79
YJR066W
YKL203C
SPBC216.07c
SPBC30D10.10c
Cryptococcus neoformans
0.05

during fungi evolution. One is in the filamentous fungus *Rhizopus oryzae*, which is an obligate aerobe as well as a good fermenter that is often used for industrial production of L-(+)-lactic acid (35). Another two RP dupications events happened in the common ancestor of *S. pombe* and *S. japonicus,* and in the common ancestor of *Saccharomyces sensu stricto* species complex after the WGD (Figure 4.2). This duplication pattern of RP genes is quite related to aerobic fermentation and also consistent to that of TOR gene duplication. Since TOR regulates ribosome biosynthesis (16), this result indicates that the TOR signaling is enhanced coupling with TOR gene duplication, which further contributes to the development of aerobic fermentation.

4.3.2. Double TOR gene in the genome of *K.lactis*

In order to test our hypothesis that TOR gene duplication may contribute to the evolution of aerobic fermentation, I choose *K.lactis* as a good experimental model since *K.lactis* is a pre-WGD species with only one TOR gene in its genome and cannot perform aerobic fermentation as well as budding yeast. Therefore, Doubling TOR gene in its genome would mimic the TOR gene duplication event in the yeast ancestor of budding yeast.

As TOR gene is involved in the control of cellular growth and proliferation (16), I do observe that the doubled TOR mutant grows faster than its control (Figure 4.3). The average doubling time for the doubled TOR mutant is 4.87 hours in YPD media, compared to 5.52 hours for the control ($P = 0.00089$). In addition, I also find that the doubled TOR mutant is more resistant to

Figure 4.2. Duplication pattern of ribosomal protein genes in all sequenced fungi species. Each row represents a RP gene and each column represents a fungi species. Green indicates one copy of RP gene, while red indicates more than one copy. Black indicates that no orthologous gene was found in that species. Three large scale independent RP gene duplications occurred during fungi evolution in *Rhizopus oryzae* (left red column), in the common ancestor of *S. pombe* and *S. japonicus* (middle red columns) and the common ancestor of *Saccharomyces sensu stricto* species complex (right red columns) from WGD. The phylogenetic relationship for all fungi species is shown at the top of the figure.

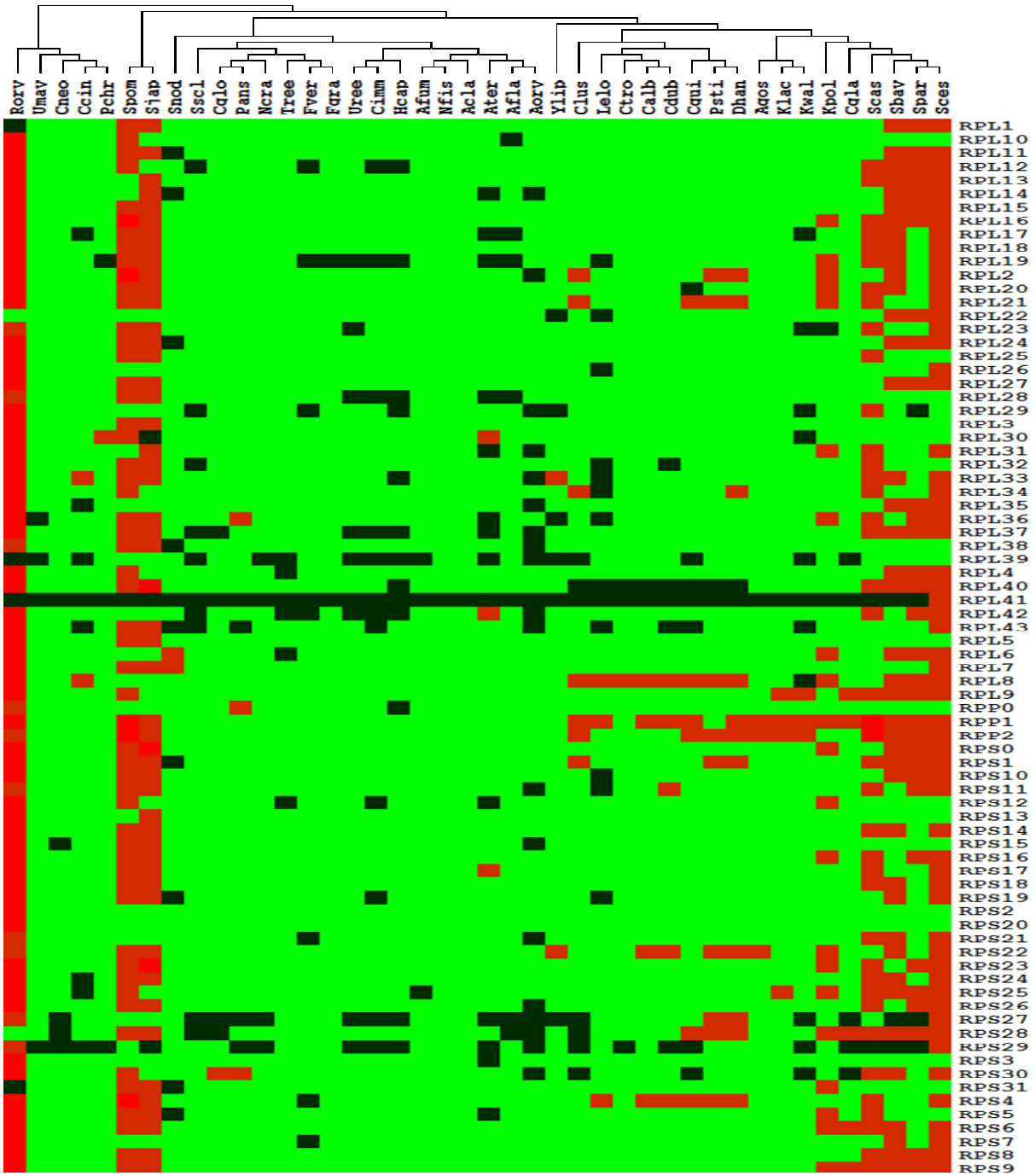
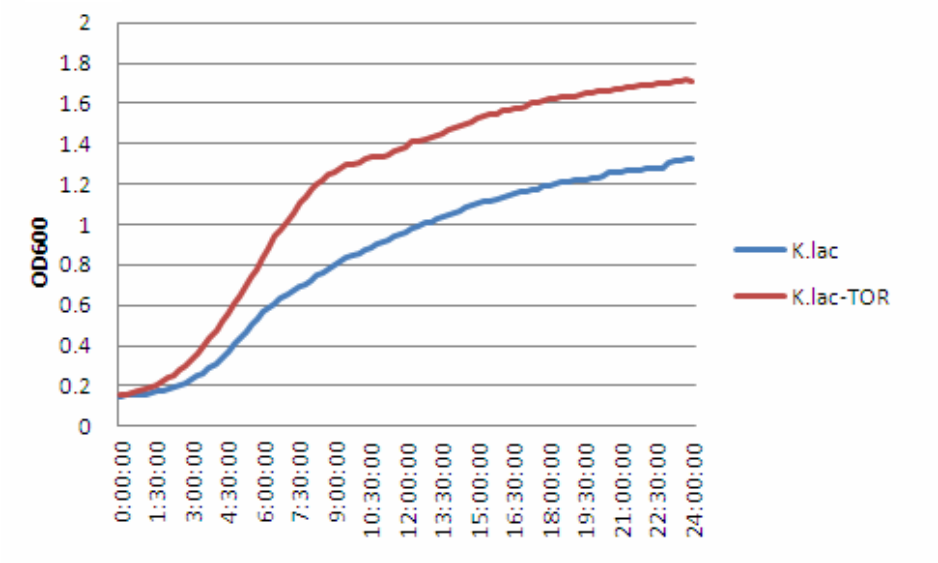
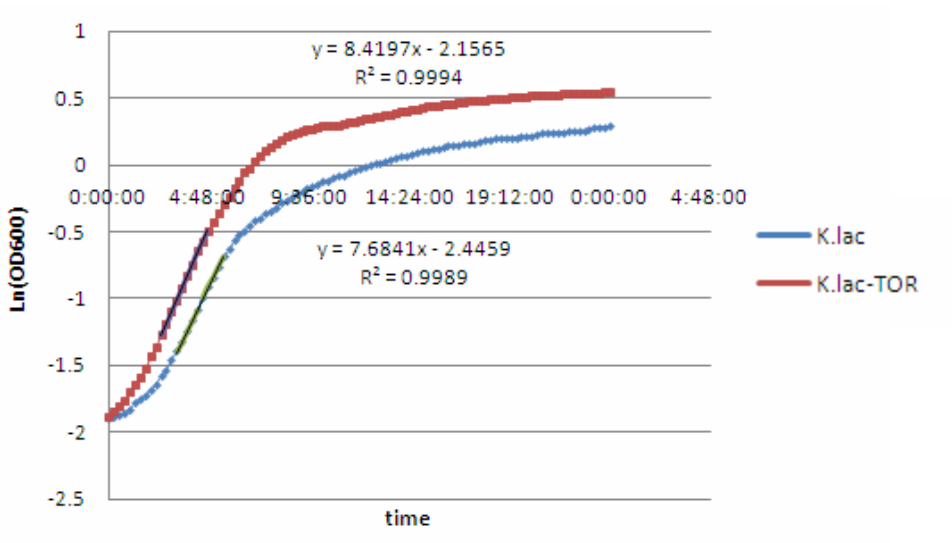

Rorv
Umav
Cneo
Ccin
Pchr
Spom
Siap
Snod
Sscl
Cglo
Pans
Ncra
Tree
Fver
Fgra
Uree
Cimm
Hcap
Afum
Nfis
Acla
Ater
Afla
Aory
Ylip
Clus
Lelo
Ctro
Calb
Cdub
Cqui
Psti
Dhan
Aqos
Klac
Kwal
Kpol
Cgla
Scas
Sbay
Spar
Sces
RPL1
RPL10
RPL11
RPL12
RPL13
RPL14
RPL15
RPL16
RPL17
RPL18
RPL19
RPL2
RPL20
RPL21
RPL22
RPL23
RPL24
RPL25
RPL26
RPL27
RPL28
RPL29
RPL3
RPL30
RPL31
RPL32
RPL33
RPL34
RPL35
RPL36
RPL37
RPL38
RPL39
RPL4
RPL40
RPL41
RPL42
RPL43
RPL5
RPL6
RPL7
RPL8
RPL9
RPP0
RPP1
RPP2
RPS0
RPS1
RPS10
RPS11
RPS12
RPS13
RPS14
RPS15
RPS16
RPS17
RPS18
RPS19
RPS2
RPS20
RPS21
RPS22
RPS23
RPS24
RPS25
RPS26
RPS27
RPS28
RPS29
RPS3
RPS30
RPS31
RPS4
RPS5
RPS6
RPS7
RPS8
RPS9

Figure 4.3. The growth curves of the doubled TOR mutant and its control in YPD media. **A**. OD_{600} VS time. **B.** Natural log of OD_{600} VS time. The maximum growth rate (Max V) is derived from Figure 4.3 B. The doubling time is calculated by [ln (2) / Max V] * 60. Each data has three replicates. Here shows the representative growth curve. The mean doubling time for *K. Lactic* control is 5.52 hr, and for *K. Lactic* doubled TOR mutant is 4.87 hr, the P value is 0.00089.

A


OD600
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0:00:00
1:30:00
3:00:00
4:30:00
6:00:00
7:30:00
9:00:00
10:30:00
12:00:00
13:30:00
15:00:00
16:30:00
18:00:00
19:30:00
21:00:00
22:30:00
24:00:00
K.lac
K.lac-TOR

B


Ln(OD600)
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
y = 8.4197x - 2.1565
R² = 0.9994
y = 7.6841x - 2.4459
R² = 0.9989
0:00:00
4:48:00
9:36:00
14:24:00
19:12:00
0:00:00
4:48:00
time
K.lac
K.lac-TOR

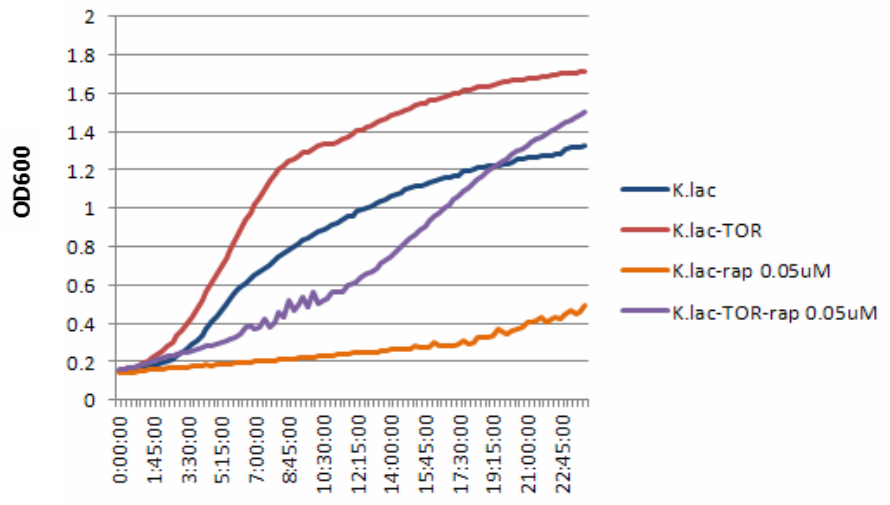
rapamycin than control (Figure 4.4), as the normalized doubling time of rapamycin treatment is 3.35 hours for the doubled TOR mutant, compared to 4.34 hours for the control ($P = 2.24 \times 10^{-6}$). Therefore, both results above suggested that the doubled TOR mutant has increased TOR activity. The gene expression level change of TOR gene in the doubled TOR mutant will be examined in RNA-seq data.

4.3.3. The fermentation ability of the doubled TOR mutant and wild-type

After investigating the metabolites by HPLC in growing media, we found that the doubled TOR mutant consumed much more glucose and had a significantly higher production of ethanol as compared to wild-type (Figure 4.5A). This result is consistent with the fact that the doubled TOR mutant grows faster (Figure 4.3). The Fermentation ratio of the doubled TOR mutant is also shown to be significantly higher than that of wild-type (Figure 4.5B), which proves our hypothesis that TOR gene duplication plays an important role in contributing to the evolution of aerobic fermentation. However, we didn't find significant difference in oxygen consumption between the doubled TOR mutant and its control (Figure 4.5C), which may suggest that increased TOR activity affects cellular energy metabolism not by repressing respiration but rather by enhancing fermentation.

Figure 4.4. The sensitivity to rapamycin of the doubled TOR mutant and its control. (**A**) Growth curve of the doubled TOR mutant and its control treated with or without 0.05uM rapamycin. (OD_{600} VS time) (**B)** Converted from Figure 4.4 A. Natural log of OD_{600} VS time. The maximum growth rate (Max V) is derived from Figure 4.4 B. The doubling time is calculated by [ln (2) / Max V] * 60. Each data has three replicates. Here shows the representative growth curve. The mean doubling time (DT) for *K. Lactic* control is 5.52 hr without rapamycin, and is 23.99 hr with 0.05uM rapamycin; the mean DT for *K. Lactic* doubled TOR mutant is 4.87 hr without rapamycin, and is 16.31 hr with 0.05uM rapamycin. The normalized DT of rapamycin treatment is calculated by DT(rap)/DT(w.o. rap). For *K. Lactic* control, this value is 4.34 hr, and for *K. Lactic* doubled TOR mutant, it's 3.35 hr, $P = 2.24 \times 10^{-6}$.

A


OD600
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0:00:00
1:45:00
3:30:00
5:15:00
7:00:00
8:45:00
10:30:00
12:15:00
14:00:00
15:45:00
17:30:00
19:15:00
21:00:00
22:45:00
K.lac
K.lac-TOR
K.lac-rap 0.05uM
K.lac-TOR-rap 0.05uM

B

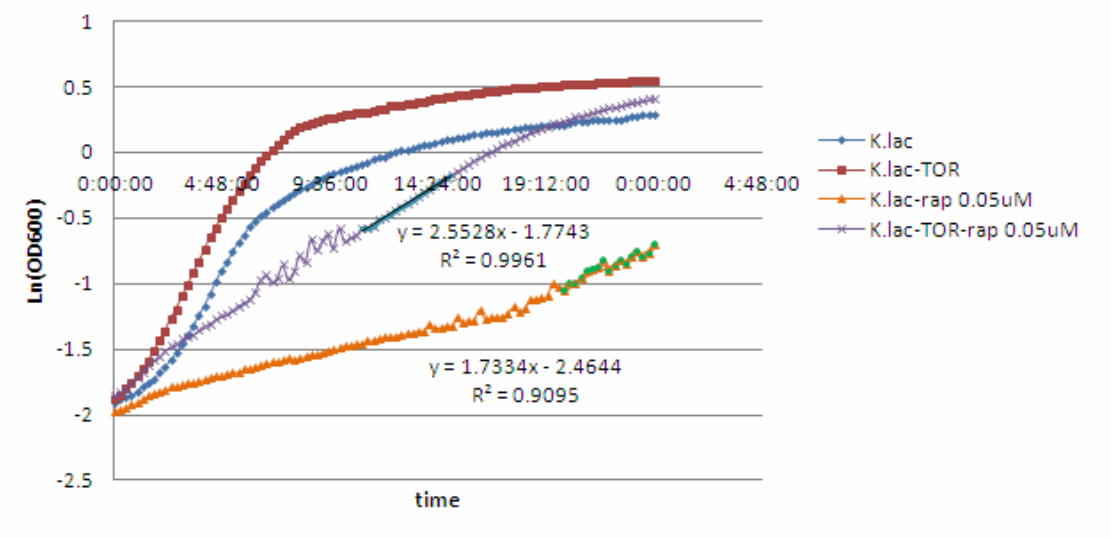
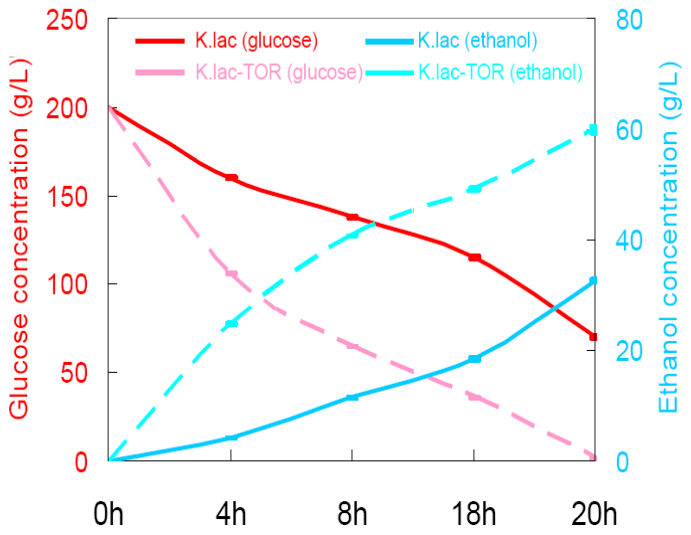
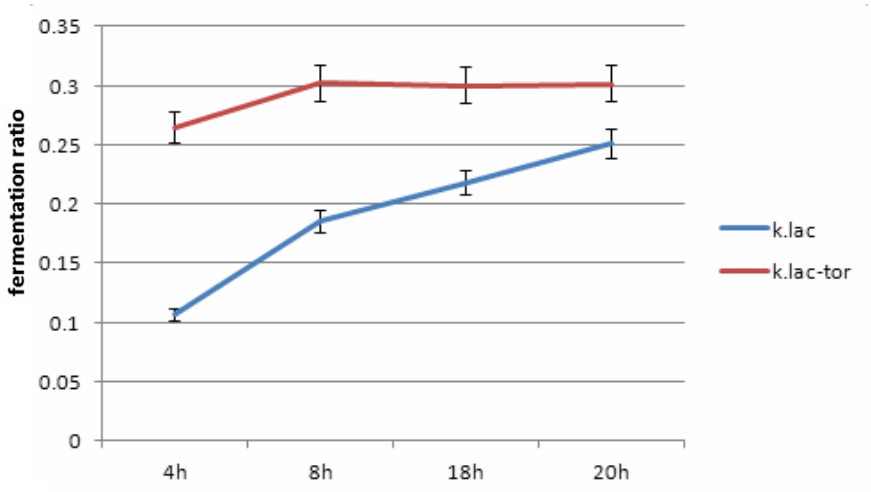

Ln(OD600)
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
0:00:00
4:48:00
9:36:00
14:24:00
19:12:00
0:00:00
4:48:00
time
y = 2.5528x - 1.7743
R² = 0.9961
y = 1.7334x - 2.4644
R² = 0.9095
K.lac
K.lac-TOR
K.lac-rap 0.05uM
K.lac-TOR-rap 0.05uM

Figure 4.5. The doubled TOR mutant has higher fermentation ability compared with the control. (**A**) The glucose, ethanol concentrations during aerobic batch cultivation of wild-type strain and doubled TOR mutant. (**B**) Fermentation ratio of wild-type strain and doubled TOR mutant during aerobic batch culture. (**C**) The oxygen consumption (mg/ml/10^7) of wild-type strain and doubled TOR mutant.

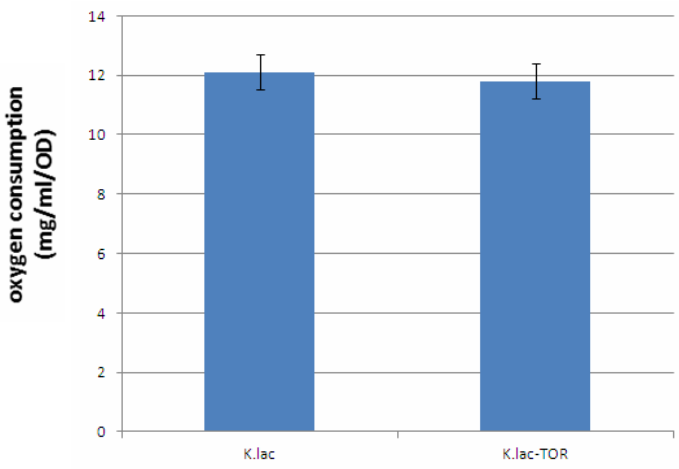
A


K.lac (glucose)
K.lac (ethanol)
K.lac-TOR (glucose)
K.lac-TOR (ethanol)
Glucose concentration (g/L)
Ethanol concentration (g/L)
250
200
150
100
50
0
80
60
40
20
0
0h
4h
8h
18h
20h

B


fermentation ratio
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
4h
8h
18h
20h
k.lac
k.lac-tor

C


oxygen consumption (mg/ml/OD)
14
12
10
8
6
4
2
0
K.lac
K.lac-TOR

4.3.4. Expression profiling of doubled TOR mutant by RNA-seq

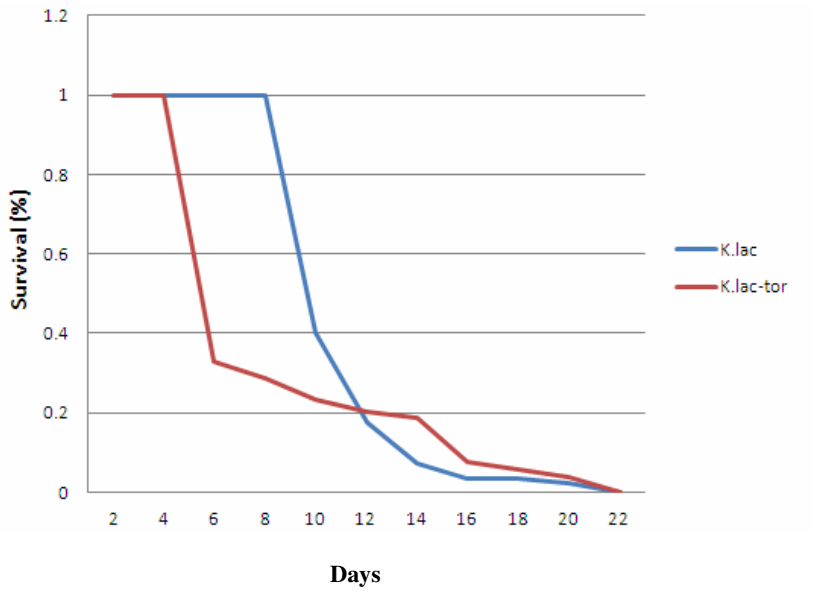
In order to better confirm and understand the mechanism in doubled TOR mutant how the fermentation ability is enhanced, we plan to employ RNA-seq technique to compare the transcriptome profiling of this mutant with its control strain. The mRNA libraries are already constructed and waiting for HiSeq2000 sequencing.

4.3.5. The life span of the doubled TOR mutant and wild-type

The TOR pathway appears to have a conserved role in regulating life span. Although it remains unclear precisely which aspects of TOR contribute most significantly to aging and life span regulation, some studies have documented that there might be complex relationships between TOR signaling, mitochondrial function and yeast chronological life span (CLS), as reduced TOR signaling may extend CLS via increased respiration and up-regulation of mitochondrial gene expression (11, 36-38). Since we uncovered energy metabolism change in doubled TOR mutant, we are interested to further test whether the CLS of this mutant is also affected.

Our result showed that the doubled TOR mutant didn't have significant difference in life span compared with control (Figure 4.6). Although we observed increased fermentation in the doubled TOR mutant (Figure 4.5B), its oxygen consumption was similar with that in wild-type (Figure 4.5C) which suggested that respiration might not be affected in this mutant. Previous studies indicate that CLS extension in yeast with reduced TOR signaling requires increased mitochondrial respiration (11, 37), which may explain our data. However, we still found an

Figure 4.6 Chronological life span analysis of doubled TOR mutant and the control. Each data has two replicates. Here shows the representative growth curve. Average lifespans: K.lac wt, 21.6 days; K.lac doubled TOR mutant, 22.5 days. P-value is 0.85.


1.2
1
0.8
0.6
0.4
0.2
0
Survival (%)
2
4
6
8
10
12
14
16
18
20
22
Days
K.lac
K.lac-tor

interesting difference in life span patterns of these two strains (Figure 4.6), as a sudden drop of survival ratio occurred much earlier in the doubled TOR mutant than in the wild-type strain. A possible explanation could be the faster consumption of glucose in the doubled TOR strain (Figure 4.5A), which makes this mutant confront starvation and other stressful conditions earlier.

4. 4. Discussion

In the present study, we have shown that doubling TOR gene in *K.lactis* could improve its fermentation ability, which provides strong evidence to support that the duplication of TOR gene contributes greatly to the evolution of aerobic fermentation in yeast history. *S. cerevisiae* and *S. pombe*, the two aerobic fermentation lineages, use TOR gene duplication as a common strategy to develop their fermentation life style, and the subsequent functional and regulatory divergence of TOR gene after gene duplication may help better adaptation to the new life style. In contrast, yeasts which cannot ferment very well only keep one TOR gene in the genome, even by gene loss such as *K. polysporus.* However, it seems that these yeasts still hold the potential to increase fermentation, as doubling TOR gene in *K.lactis* can clearly show the effect (Figure 4.5B). Yet this may lead to negative consequences on affecting the yeast's fitness under certain conditions, since the *K.lactis* doubled TOR mutant has a much earlier sharp decrease on survival rate compared with control (Figure 4.6). One possible reason could be the non-ferment yeasts, like *K.lactis*, don't have a good adaption mechanism for increased fermentation. Therefore, we proposed that TOR gene duplication might be one of the most important factors to initiate the evolution of aerobic fermentation by dosage effect, yet the well developed fermentation life style may need further subfunctionalization of TOR duplicates and coordination with other genes on a global way.

In addition, our study also indicates a direct role of TOR signaling on regulating aerobic fermentation. This result is supported by several recent works in mammalian cells. Qian Sun et al.

(26) found that mTOR was a major positive regulator of the Warburg effect, and identified PKM2 as a critical glycolytic enzyme in oncogenic mTOR-induced Warburg effect. Another study reported that lactate dehydrogenase B (LDHB), a critical enzymatic activator of glycolysis, was a downstream target of mTOR that was critical for oncogenic mTOR-mediated tumorigenesis (27). Moreover, another research group proposed that elevated mTOR signaling in cancer cells could increase phospholipase D (PLD) activity, which is critical for the metabolic shift to aerobic glycolysis (28). All these studies are in good agreement with our results, which suggests our doubled TOR mutant could be served as a good model to study the detail mechanism how TOR signaling controls the highly glycolytic phenotype, thus shed lights on developing new therapeutic targets in cancers.

4. 5. Experimental Procedures and Methods

Phylogenetic tree of two TOR genes in *S. cerevisiae* and *S. pombe*

The sequences of two TOR genes in *S. cerevisiae* (YJR066W and YKL203C) and in *S. pombe* (SPBC216.07c and SPBC30D10.10c) are downloaded from NCBI. The ortholog of TOR gene in *Cryptococcus neoformans* is used as outgroup. Tree was constructed using neighbor-joining method in software package MEGA.

Phylogenetic tree of all sequenced fungi species

Orthologs among fungi species were downloaded from InParanoid and then checked manually. A random set of 10 one-to-one orthologs in all species were used to reconstruct the tree. Muscle was used to conduct multiple sequence alignment and ClustalW was used to reconstruct the tree based on protein distances. The bootstrap values for all nodes are larger than 50%.

Cloning of *K.lactic* TOR gene

K. lactis wild type strain (PM6-7A) was kindly provided by X. J. Chen (The Australian National University, Australia). One ~9 Kbp fragment containing the whole coding region of the *K. lactis* TOR gene as well as its 1Kbp 5' end UTR and ~300 bp 3' end UTR was cloned into PRS406 vector with URA3 marker by a three fragment amplification method. Three short fragments (~3000bp each) were amplified by PrimeStar PCR system (Takara Bio), using *K. lactis* genomic DNA as the template. Each fragment was digested by corresponding digestion enzymes and then sub-cloned into PRS406 in sequence. Each sub-clone will be confirmed by DNA sequencing

before moving on to the next sub-cloning. The primers for the first short fragment were 5'CCCGGGCTCGAGCGTGGCAATGGATTATGAAA3' and 5'GTTTCATAGGGGTAGGGG3', and the fragment would be digested by XhoI and BamHI. The second short fragment was amplified by primers 5'TGTACTATCTGGTGAGAAG3' and 5' TTTCATATCTTGTTTAGGCT 3', with restriction sites BamHI and SpeI. The third fragment has the primers of 5'TTCTAAAGGTACGGTCACTA3' and 5' AATTGCGGCCGCGGTATCACCAGAACGAACA 3', the corresponding restriction enzymes are SpeI and NotI.

Constructing doubled TOR mutant

SpeI was used to cut in the middle of TOR gene, which would result in a linear PRS406-TOR. This linear DNA was transformed into *K. lactis* by Lithium Acetate method. Stable ura3+ strains were selected and confirmed as doubled TOR mutant. Two sets of confirmation primers are 5'TGTGAGAACGTAATGATGGT3', 5' ATTGGTGTAATTGAGCATTT3' and 5' AAATCGGCAAAATCCCTTAT3', 5' ACGCCTGGTATCTTTATAGT3', respectively. In order to make a perfect control for the doubled TOR mutant, we inserted PRS406 after TOR gene into the genome, with a similar strategy above, just by replacing the ~9 Kbp long DNA with a fragment of 3' UTR of TOR gene in PRS406 cloning.

Growth curve

Three independent clones for either control or doubled TOR strains were picked and grew in YPD media with a start $OD_{600} = 0.1$. The growth curve was measured by Bioteck machine.

Fermentation ratio measurement

Detail procedures described in 3.5.

Oxygen consumption measurement

Detail procedures described in 3.5.

Library Construction for Illumina-Based Strand-Specific Multiplex RNA-Seq

Detail procedures described in 3.5.

Life span measurement

Detail procedures described in 3.5.

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