A Transcript Analysis Reveals the Molecular Basis for Metamorphosis Delay in *RNase Z* $^{\Delta MTS}$ *Drosophila melanogaster*

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Abstract

Mitochondrial dRNaseZ is essential for mitochondrial tRNA processing and *Drosophila* development. The mutant *RNase* $Z^{\Delta MTS}$ ($Z^{\Delta MTS}$) is a knockout of mitochondrial dRNaseZ generated by GAL4/UAS system. The mutant exhibits a developmental delay, especially an elongation of third instar larvae. The transcript levels of three genes that may play important roles in the elongation of third instar larvae were examined in this study. The mRNA level of *broad* does not increase in the mutant synchronously with wild type, but delays for about two weeks. This confirms the elongation of third instar larvae. The increased transcript of L-lactate dehydrogenase *(LDH)* implies a possible metabolic alteration in mutant $Z^{\Delta MTS}$. No significant increase at mRNA level is found in AMP-activated protein kinase (*AMPK)*.

Key word: dRNaseZ, metamorphosis delay, metabolic alteration, broad, LDH

Introduction

tRNAs serve primarily as a part of the protein synthesis machinery and play an important biological role. tRNAs are first transcribed into larger precursors with 5'- and 3'- extension. To generate functional tRNAs, processing of primary tRNA transcripts is required. Both 5'- and 3'- end processing is crucial to yield mature functional tRNAs for aminoacylation (Hartmann *et al.*, 2009).

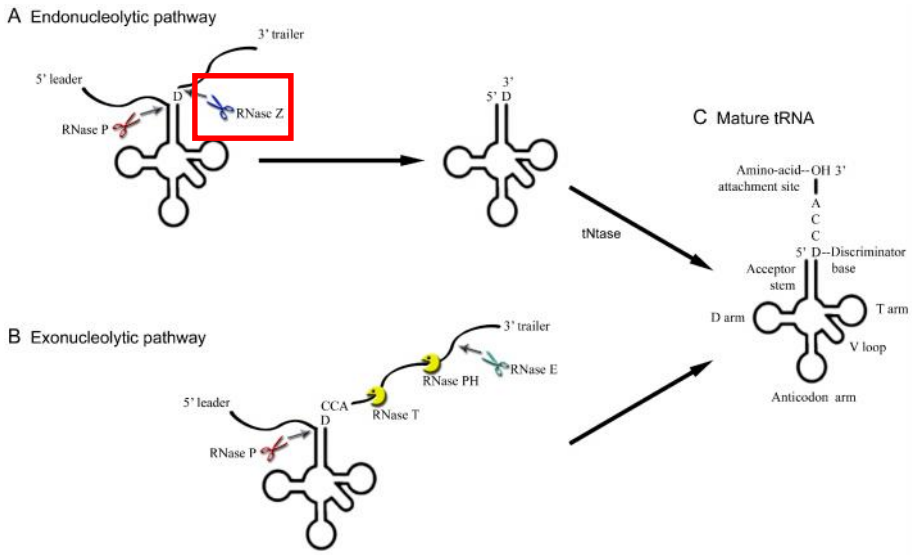

Fig1. Two major pathway of pre-tRNA 3'-end maturation. RNase Z gets involved in the endonucleolytic pathway of tRNA 3'end maturation.

RNase Z belongs to a superfamily of metallo-β-lactamases (MBL). It is an endoribonuclease catalyzing specific cleavage at the 3' end of tRNA precursors (Schiffer et al., 2002). The cleavage site of RNase Z is from the phosphodiester bond quickly 3' to the first unpaired nucleotide following the 7-bp acceptor stem (discriminator base), and a hydroxyl group and a 5' phosphate group on are left on the tRNA 3'-end the 3'-trailer respectively (Fig1, Vogel et al., 2005). Homologous RNase

Z proteins are widely distributed and have been found in every living organism from bacteria to human being. RNase Z protein has two forms: a short form of RNase Z^S and a long form of RNase Z^L. So far, RNase Z^L is only found in the eukarya and in the genome of *Drosophila melanogaster* only the long form exists (Ceballos and Vioque, 2007).

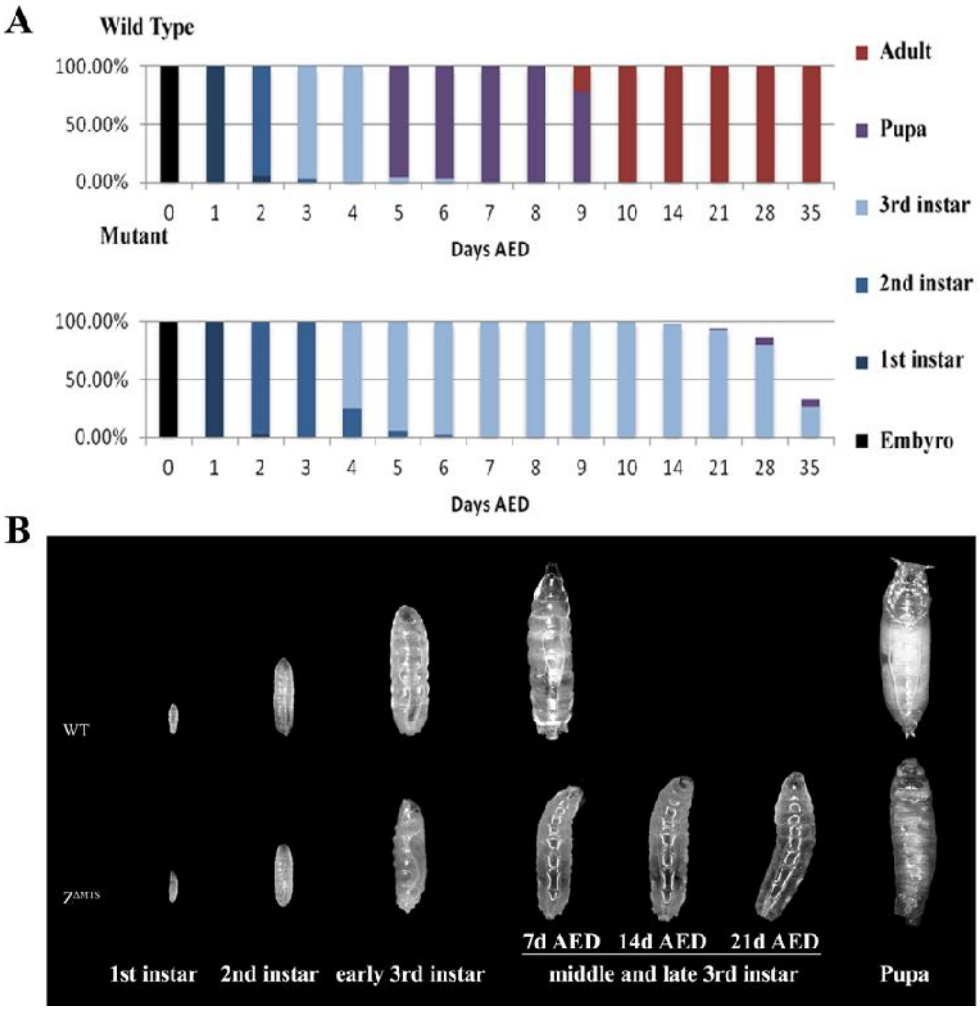

Fig2. Developmental delay is a hallmark in the *RNase* $Z^{\Delta MTS}$. A. the statistical analysis of developmental profile between wild type (WT) and mutant. Different colors present different developmental stages. Y-axis presents the percentage of individual in each developmental stage. X-axis presents days after egg deposition (AED). In 10 days AED, wild type develops from embryo to pupa to adult. However, the developmental delay occurs in the mutant primarily through the elongation at third instar larvae. At 14 days AED, mutant begins to die. At 21 days AED, only a few of them enter pupa stage. None of mutant enters adult stage and dies very quickly after pupation. B. the developmental profile between wild type and mutant.

RNase Z^L could be involved in and affect cell growth and proliferation through tRNA processing intermediates. In the *C. elegans*, knockdown of hoe-1, the *RNase* Z^L homolog, leads to a worm developmental delay and producing sterile adults (Smith and Levtian, 2004). Absence of the *RNase* Z^L function could cause fission yeast to stop growing (Zhao et al., 2009). In budding yeast, accumulation of pre-tRNAs in nucleus with unprocessed 5'- or 3'-ends could trigger a translational depression of Gcn4 transcription factor (Qiu et al., 2000) which is required for the general amino acid control depression response in yeast (K.Arndt and G.R.Fink, 1986)

Drosophila RNase Z^L *(dRNaseZ)* is involved in both nuclear and mitochondrial tRNA 3'-end maturation. The mutant *RNase* $Z^{\Delta MTS}$ ($Z^{\Delta MTS}$) in this study is generated by rescuing *dRNaseZ* knockout flies (Xie et al., 2010) with a construct lacking mitochondrial targeting sequence (MTS). Absence of mitochondrial dRNaseZ affects mitochondrial function. Knockout of mitochondrial dRNaseZ increases mitochondrial DNA level but affects mitochondrial transcript maturation.

More interestingly, the mutant shows development delay, especially the elongation of third instar larvae and survives for at least 2 weeks; additionally, almost all the larvae die at the end of the third instar larvae without metamorphosis (Fig2).

The goal of this study is to analyze the developmental profile of *RNase* $Z^{\Delta MTS}$ at the transcript level. We hypothesize that transcript of some gene could reveal the molecular basis for metamorphosis delay in the *RNase* $Z^{\Delta MTS}$ larvae and there may be a metabolic alteration in the mutant. Three genes were selected: *broad*, *LDH* and *AMPK*.

Material and methods

Fly stocks

The wild type (Z^{24}/*GCyO; tubGal4/*$Z^{\Delta MTS}$) and mutant (Z^{24}/ Z^{24}; *tubGal4/*$Z^{\Delta MTS}$) *Drosophila* larvae were all kindly provided by Dr. Dubrovsky's lab.

RNA extraction and RNA sample

Total RNA from whole *Drosophila* larvae was extracted and purified from homogenate using the RNeasy® Plus Mini Kit (QIAGEN), following the manufacturer's instructions. The RNAs were eluded with 30μl of dH_2O. The RNA samples of 2nd instar larvae and 3rd instar larvae were extracted and purified using TRIZOL (Invitrogen®) and were kindly provided by Dr. Dubrovsky's lab. The concentration of each RNA extract was tested by spectrophotometer. A 10ng/μl dilute was made from each RNA extract and used as a template in the following RT-PCR.

RT-PCR

RT-PCR was performed using QIAGEN® One-Step RT-PCR Kit following the manufacturer's instructions. 20ng of RNA were amplified in a 20μl RT-PCR reaction system: 4μl 5×QIAGEN® One-Step RT-PCR buffer, 0.8μl dNTPs (10mM each), 0.8μl QIAGEN® One-Step RT-PCR Enzyme Mix, 0.8μl 10pmol/μl forward primer, 0.8μl 10pmol/μl reverse primer, 2μl 10ng/μl RNA and 10.8μl dH2O. RT-PCR was performed as the following condition: one cycle of 50°C for 30min and 95°C for 15min, 30 or 35 cycles of 94°C for 30 sec, 57°C for 30 sec, and 72°C for 30 sec, and a

final extension of 72°C for 7min followed by a final hold at 4°C. *Rp49* and *LDH* were amplified for 30 cycles. *Broad* and *AMPK* were amplified for 35 cycles.

Electrophoresis

4µl of loading dye was added to each 20µl RT-PCR product and mixed well. 6µl of the product with loading dye was separated on a 1% agarose gel (2.0g of agarose was dissolved in a 200ml 1×TBE by boiling. 20µl ethidium bromide was added when the temperature of melting agarose is around 50-60°C and gel was poured into a mould). *Rp49* served as a loading control. The electrophoresis was performed at 180V for about 30 min. Gels were visualized and pictures were taken in Bio-Rad® UV trans-illuminator.

RT-PCR product purification and sequencing

RT-PCR products were purified using QIAquick® PCR Purification Kit following the manufactures instructions. The purified products were sequenced by GENEWIZ. Sequencing results were analyzed using BLAST to confirm the identity of each RT-PCR product.

Primers

Primers used in RT-PCR and Sequencing were shown in the Table 1.

Table 1 Primers used in RT-PCR and Sequencing

Gene	Primer	GC %	Length	Sequence (5'→3')	Expected Size (bp)
broad	mBr-1b	50	22	CTTCAGTACGATCAAGCACCAC	479
	mBr-2b	55	20	TGAGACCTAGCAACGCTGAG	
LDH	ImpL3-1	55	20	TGTTCACTCCAGACCAAACG	247
	ImpL3-2	41.67	24	CTGGTAGAGTACAGTCCCGATACC	
AMPK	SNF1A-1b	45.45	22	GCACATCATCAAGTTGTACCAG	531
	SNF1A-2b	50	20	ACTTGCAGCATCTGACAGAC	
rp49	mRp49-1	50	20	GACAATCTCCTTGCGCTTCT	251
	mRp49-2	50	20	CTAAGCTGTCGCACAAATGG	

qRT-PCR

Real-time PCR was performed using QuantiTect® SYBR Green RT-PCR Kits following the manufactures instructions. Primers used in qRT-PCR are the same with those in RT-PCR shown in the Table 1.

Results

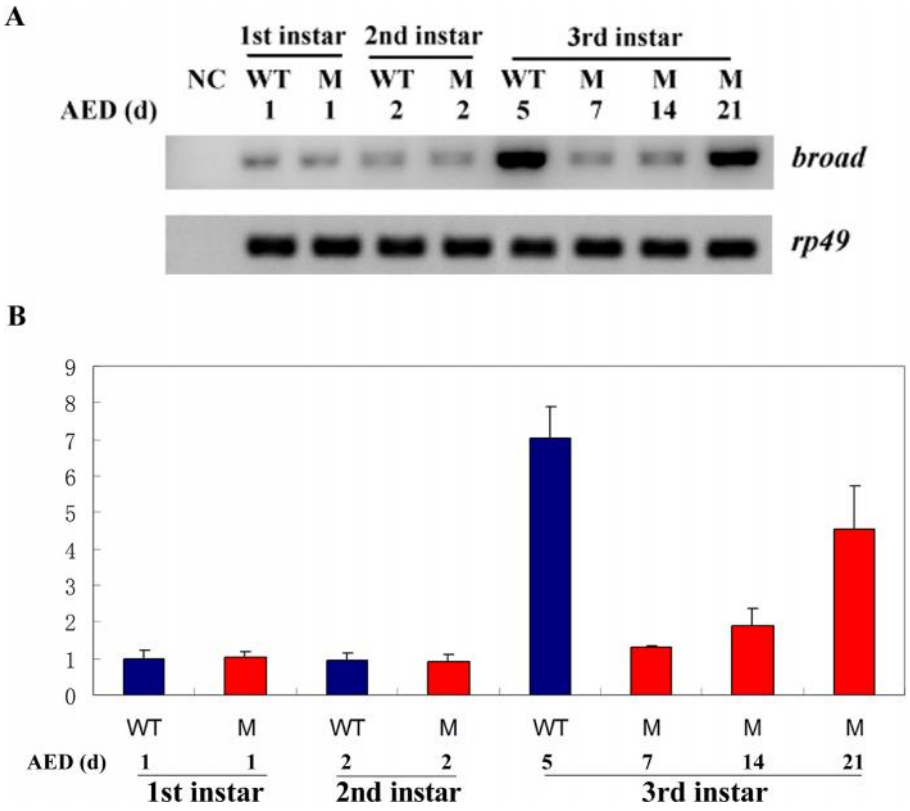

Fig3. *Broad* transcript pattern indicates developmental delay in *RNase Z*$^{\Delta MTS}$ *Drosophila.* A. RT-PCR result of *broad* transcripts of wild type and *RNase Z*$^{\Delta MTS}$ in different developmental stages. Total RNA was isolated from wild type and mutant larvae in different stages and subjected to RT-PCR. The result was shown in a 1% agarose gel. *Rp49* is used as a loading control. B. semi-quantitative results of *broad* transcript in each larval stage. The amount of RT-PCT products were quantified after gel electrophoresis by densitometric analysis using IMAGE J. The results were normalized to the amount of *rp49*. The others were compared with wild type 1d AED larvae. Results are presented as mean ± SD.

Broad transcript pattern provides a molecular evidence for metamorphosis delay in the mutant RNase Z$^{\Delta MTS}$ *Drosophila.*

To examine the molecular basis for the metamorphosis delay in the mutant *RNase Z*$^{\Delta MTS}$ *Drosophila*, the transcript of *broad*, a metamorphosis determinant, was studied. The mRNA level of *broad* increases dramatically at third instar larvae of wild type right before metamorphosis (Fig3A). However, in the mutant, the transcript of *broad* does not increase synchronously with wild type but delays for over two weeks.

Even in 7 days AED, the transcript of broad is still at a low level. Semi-quantitative result shows no significant differences in 1st and 2nd instar larvae but an over 7-fold enhance in wild type of 5d AED. Also, we could see the transcript level of *broad* increases significantly in the mutant of 21 days AED (Fig3B). A small percentage of larvae begin to enter pupa stage just at 21 days AED.

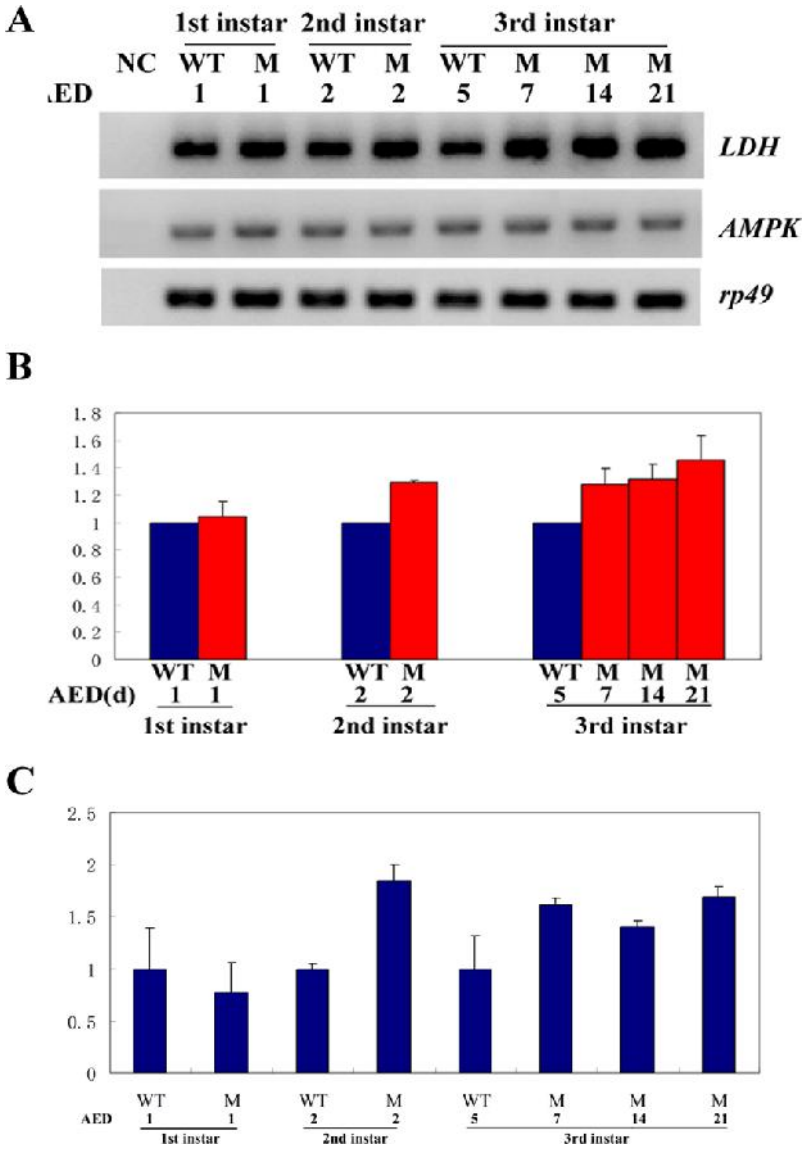

Fig4. The transcripts of *LDH* indicates metabolic alteration in RNase $Z^{\Delta MTS}$ Drosophila. A. RT-PCR result of *LDH* transcripts of wild type and mutant in different developmental stages. B. semi-quantitative results of *LDH* transcript in each larval stage. Semi-quantitative analysis was performed as described in Fig.3. C. qRT-PCR result of *LDH* transcripts. The CT values of *LDH* were normalized by the CT of *rp49*. Results are presented as mean ± SD.

The transcripts of LDH indicates a metabolic alteration in RNase $Z^{\Delta MTS}$ Drosophila.

To examine whether an energy-related metabolic change occurs in the mutant, the transcripts of *LDH* and *AMPK*, an energy sensory gene, were examined. We found the transcripts of *LDH* increase in the mutant of both second and third instar larvae. Also, mRNA level of *LDH* increases in the mutant of third instar larvae during the development. Unexpectedly, no significant augment is found in *AMPK* (Fig. 4).

Discussion

The transcript patterns of *broad* provide the molecular evidence for the metamorphosis delay in the mutant. It has been reported that *Drosophila* broad-complex plays a key role in controlling ecdysone-regulated gene expression at the onset of metamorphosis (Felix D. Karim, 1993).

A large amount of energy is required during metamorphosis. Due to the defect in mitochondria, efficiency of ATP generating is expected to decrease significantly in mitochondria. Two ATPs are generated by consuming one glucose via glycolysis pathway (Fig5). Because of the increased requirement of ATP during metamorphosis, more pyruvate is expected to generate. The mRNA of *LDH* increases in the mutant, which indicates a metabolic alternation in RNase $Z^{\Delta MTS}$ larvae. The increasing pattern of *LDH* transcript in mutant of third instar larvae seems similar to that of *broad* transcript in mutant of third instar larvae. It may indicate a correlation between the metamorphosis delay and metabolic alteration.

Moreover, the increased AMP/ATP ratio resulting from the mitochondrial damage may cause a higher mRNA level of AMPK, the energy sensory gene. No significant change of *AMPK* is found at transcript level. It is possible that there is an augment of Ampk at protein level or of phosphorylated Ampk, an active form, at protein activity level (Michelle L, 2010). Further study on Ampk protein level/activity is required.

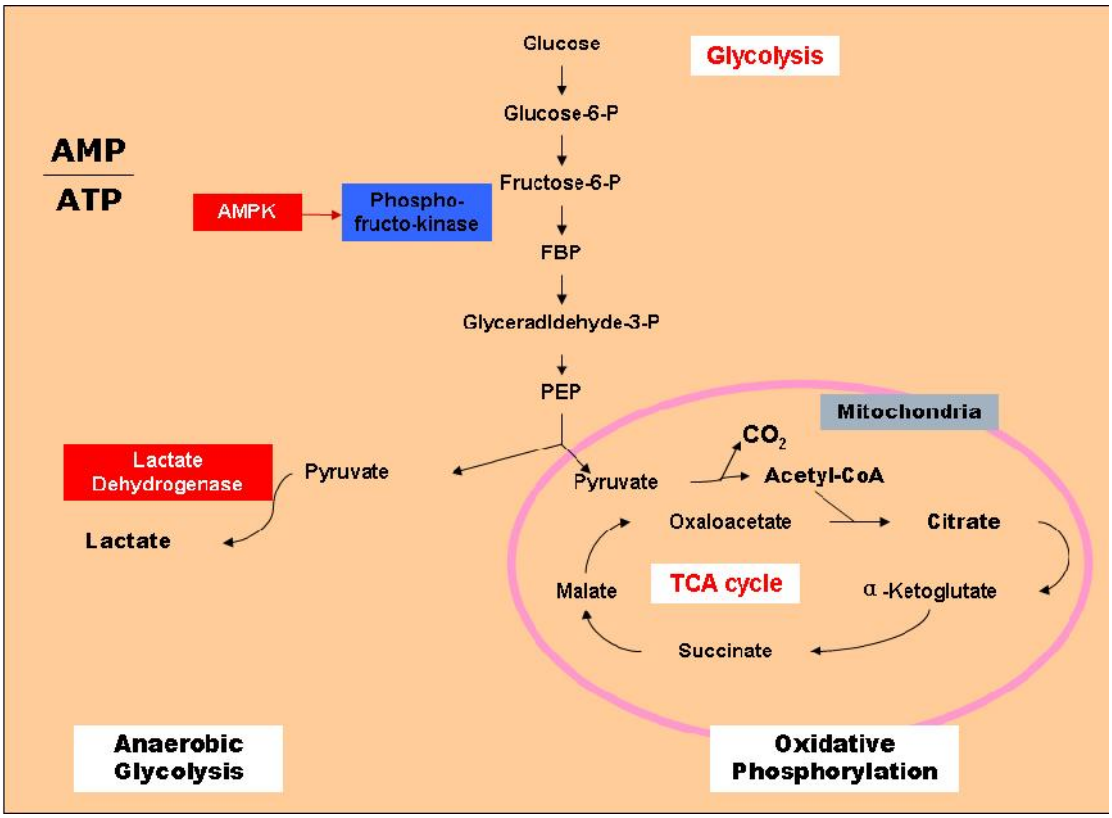

Fig 5. Anaerobic glycolysis and oxidative phophorlyation. Pyruvate usually has two metabolic pathway: converting into lactate in anaerobic glycolysis or TCA cycle in oxidative phosphorylation

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