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CYTOPLASTIC NAD+ BIOSYNTHESIS AFFECTS GONAD DEVELOPMENT IN *CAENORHABDITIS ELEGANS*

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by

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ABSTRACT

In recent years, nicotinamide adenine dinucleotide (NAD+) has gained renewed attention from scientists. This is because NAD+ serves not only as a cofactor but also as a substrate for numerous enzymes that are widely dispersed in cellular signaling reactions. These signaling pathways affect many aspects of organisms, like aging, development, circadian rhythm and axon pathology. However, the mechanism that NAD+ homeostasis influences development is still elusive.

In my project, I used the *C. elegans pnc-1*(*pk9506*) mutant as a model to understand why blocked NAD+ salvage biosynthesis delays gonad development. Our lab has found that blocking salvage NAD+ biosynthesis affects gonad developmental progression in worms. My project was to investigate the mechanism linking NAD+ production to gonad development in *C. elegans*. Because the block in salvage synthesis had already been linked to a deficit in glycolysis, I hypothesized that disrupted glycolysis caused by NAD+ deficiency leads to the gonad developmental defects. I supplied the *pnc-1(pk9605)* mutants with late glycolytic intermediates 3-phosphoglycerate (3PG) and phosphoenolpyruvate (PEP), and found that these intermediates increased the percentage of normal gonad development in the *pnc-1* population, which supported the hypothesis that disrupted glycolysis causes the gonad developmental defects.

Cells have relatively separated NAD+ pools between mitochondria, nucleus and cytoplasm, and individual NMNAT genes independently regulate these NAD+ pools to meet specific requirements of NAD+ in cells. I would like to use NMNAT function to investigate the compartment-specific requirements for NAD+ biosynthesis in *C. elegans* gonad development. However, it is unclear which of the two *C. elegans* NMNAT genes, *nmat-1* and *nmat-2*, corresponds to which compartment-specific gene. To determine the subcellular localization of the two *C. elegans* NMATs. I fused *nmat-1* and *nmat-2* to CFP under the *myo-3* promoter and examined their subcellular localization in muscle cells. The subcellular localization experiment results showed that NMAT1 is a mitochondrial protein and NMAT2 is a Golgi/cytoplasmic protein. To further explore the function of NMNAT, I applied the new genomic engineering CRISPR/Cas9 system to create an allele of *nmat-1*. Now, I am screening for homozygous *nmat-1* mutants.

This work will provide us the insights on metabolic products function in animal's development and also will shed light on an intimate connection between metabolism and development in human health and disease.

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ACKNOWLEDGEMENTS

To my thesis Advisor Dr. Wendy Hanna-Rose, I would like to give my infinite respect and appreciation to her. She offered me the opportunity to work in her lab and learn from her. She is a fantastic female scientist, and totally changed my impression on female scientists. Before I joined her lab, I thought if a female scientist wanted to get success in her career, she needed to sacrifice her family life. But Dr. Wendy Hanna-Rose balanced both family life and work in a perfect way, which made me plan to be a female scientist in the future. She is not only a scientific advisor to me, but also a life mentor. Whenever I feel depressed, she always cheers me up and let me have the courage to face the troubles in my life. To my beloved husband, I am indebted to him for valuable assistance and unconditional love. Living abroad is like a journey of adventure, because no one knows what will happen in the next second. But with his companionship, I feel that the journey is colorfully filled with numerous joys and happiness, also he understands the role of family in a good life. No matter what happened and is happening, we believe we are on the right track and will go forward. To my parents and friends, I would like to thank them for their kindly supports and their accompanying through the abroad study process. Also, I would like to thank my committee members Dr. Lorraine Santy and Dr. Zhichun Lai for agreeing to be my committee members and for their guidance through my thesis. I thank all the members in Wendy's Lab who help me in many aspects of my experiments and life.

Chapter 1

Introduction

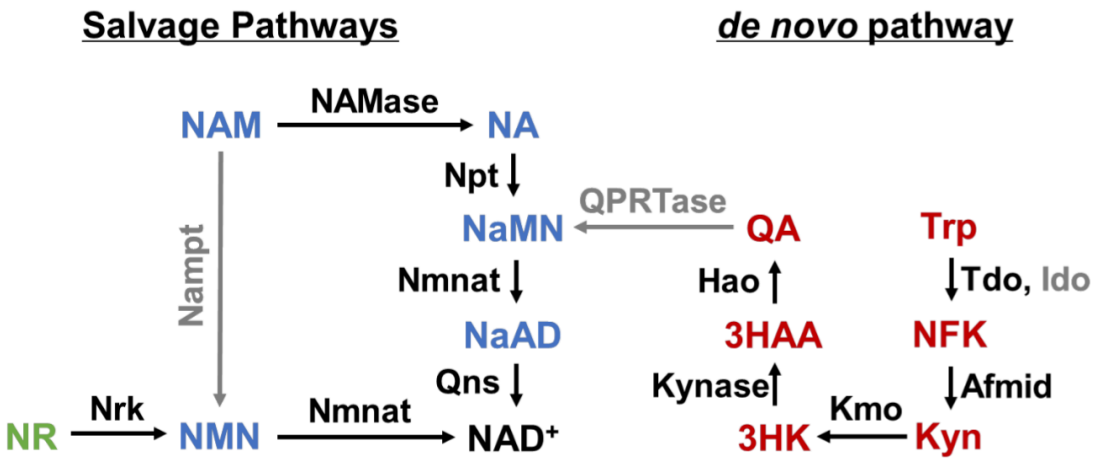
1.1 NAD+ Overview

Nicotinamide adenine dinucleotide (NAD+) was discovered by William John Young and Arthur Harden in the beginning of the 20th century (Harden，A. and W.J., 1906). The important small molecule first obtained attentions for its electron transporting function. It transfers electrons from one compound to another and becomes the reducing form NADH. Also, NADH can donate electrons to form the oxidized NAD+ (Sauve, 2008). In the past, NAD+ was only considered as a metabolite involved in various redox reactions. Recently, NAD+ has gained renewed attention from scientists. This is because NAD+ serves not only as cofactor but also as substrate for numerous enzymes in cellular signaling reactions (Ziegler, 2000). These signaling pathways affect many aspects of organisms, like aging, development, circadian rhythm and axon pathology (Verdin, 2015; Vrablik et al., 2011; Wang and He, 2009).

1.2 NAD+ Biosynthesis in eukaryotes

There are three metabolic pathways that synthesize NAD+ (Figure 1-1). The first pathway is the de novo synthesis pathway, which uses quinolinic acid (QA) as the precursor for NAD+. Different organisms generate QA from distinct resources. For most animals and some bacteria, QA is derived from tryptophan (Trp) through 5 reactions (Foster and Moat, 1980). However, in plants and some other bacteria, QA is derived from aspartic acid (Katoh et al., 2006). QA is converted into nicotinic acid mononucleotide (NaMN) by quinolinic acid phosphoribosyl transferase (QPRTase) and then nicotinamide/nicotinate mononucleotide adenine transferase (Nmnat) transfers a moiety adenylate to NaMN and forms nicotinic acid adenine dinucleotide (NaAD). QPRTase

does not exist in all of organisms, for instance, QPRTase could not be found in *C. elegans*. Finally, NaAD is converted to NAD+ by NAD synthetase.



adapted from Wang (2015) PhD thesis

Figure 1-1 NAD+ biosynthesis pathways.

This figure shows three NAD+ biosynthesis pathways in eukaryotes. In vertebrates, NAD+ is salvaged from NAM via converting to NMN by enzyme Nampt and then to NAD+ by enzyme Nmnat. In invertebrates, the salvage pathway is longer. NAM is convert to NA by nicotinamidase and then flows into Preiss-Handler pathway to form NAD+. de novo NAD+ biosynthesis occurs in various of animals, but the QPRTase homologue has not been discovered in *C. elegans* or *Drosophila melanogaster* genomes yet. Grey color, Nampt, QPRTase and Ido, means they are not present in *C. elegans*.
Abbreviations: NAM, nicotinamide; NA, nicotinic acid; NaMN, nicotinic acid mononucleotide; NaAD, nicotinic acid adenine dinucleotide; NR, nicotinamide riboside; NMN, nicotinamide mononucleotide; Trp, tryptophan; NFK, N-formayl kynurenine; Kyn, kynurenine; 3HK, 3-hydroxy kynurenine; 3HAA, 3-hydroxy anthranilic acid; QA, quinolinic acid; NAMase, nicotinamidase; Npt, nicotinic acid phosphoribosyl transferase; Nmnat, nicotinamide/nicotinate mononucleotide adenine transferase; Qns, glutamine-dependant NAD+ synthetase; Nampt, nicotinamide phosphoribosyl transferase; Nrk, nicotinamide riboside kinase; Tdo, tryptophan 2,3-dioxygenase; Ido, idoleamine 2,3- dioxygenase; Afmid, arylformamidase; Kmo, kynurenine 3-monooxygenase; Kynase, kynureninase; Hao, hydroxyanthranilate oxygenase; QPRTase, quinolinic acid phosphoribosyl transferase.

The second pathway to synthesize NAD+ is the salvage synthesis pathway. This pathway differs from de novo synthesis pathway, which uses amino acid as precursor to

form NAD+. Salvage pathway recycles compounds containing nicotinamide, such as nicotinic acid (NA), nicotinamide (NAM) and nicotinamide riboside (NR), as building blocks to assemble NAD+. NA flows into the conserved Preiss-Handler pathway to reform NAD+ (Preiss and Handler, 1958). The Preiss-Handler pathway refers to the steps that synthesize NAD+ from NA. Vertebrates and invertebrates use distinct pathways to convert NAM to NAD+. In vertebrates, nicotinamide phosphoribosyltransferase (Nampt) catalyzes NAM into nicotinamide mononucleotide (NMN), and then NMN will convert to NAD+. However, invertebrates take more steps to generate NAD+ for salvage pathway. Nicotinamidase catalyzes NAM into NA, and then NA enters into the Preiss-Handler pathway (Belenky et al., 2007; Vrablik et al., 2009).

Nicotinamide riboside kinase (Nrk) pathway is the third pathway to generate NAD+, which was discovered by Bieganowski and Brenner in 2004 (Bieganowski and Brenner, 2004). NR or nicotinic acid riboside (NaR) is phosphorylated by nicotinamide riboside kinase (Nrk) to form NMN or NaMN, respectively. These products flow into salvage pathway to yield NAD+. Nrk pathway is highly conserved among eukaryotes (Bieganowski and Brenner, 2004).

1.3 Role of NAD+ in Cellular Function

In general, NAD+ has dual functions in cells. It plays the cofactor role to transport electrons, also it servers as a substrate for some enzymes. As a cofactor, NAD+ is involved in many redox reactions, such as specific reactions in glycolysis, the Kreb's cycle, alcohol metabolism and β-oxidation (Fouquerel and Sobol, 2014). The NAD+ phosphate form NADP+, which has an additional phosphate group on the ribose ring (Figure 1-2), is essential in anabolic reactions, such as fat, sugar, and nucleotide biosynthesis pathways.

In addition to acting as a cofactor in redox reactions, NAD+ works as a substrate for its consumers. One group of NAD+ consumers is sirtuins. Sirtuins act as nutrient sensors attracting more and more attentions in scientific research, because many the literatures have reported that dietary restriction boosts sirtuins activities, and this leads to increased life-span in many organisms, including yeast (Kaeberlein et al., 2004), worms (Berdichevsky et al., 2006; Tissenbaum and Guarente, 2001; Viswanathan et al., 2005),

flies (Rogina and Helfand, 2004), and mice (Cohen et al., 2004; Satoh et al., 2013). The data indicates that the function of sirtuins in mediation of longevity is conserved among eukaryotes. In mammals, there are seven sirtuins presenting in various subcellular localization. SIRT1, SIRT6, and SIRT7 are nuclear; SIRT2 is cytoplasmic; SIRT3, SIRT4, and SIRT5 contain mitochondrial targeting sequences, so they are mitochondrial. (Guarente, 2013).

Another group of NAD+ consumer is mono(ADP-ribose) transferases (ARTs) and poly(ADP-ribose) polymerases (PARP) (Okayama et al., 1977; Ueda and Hayaishi, 1985). These enzymes remove nicotinamide from NAD+ and generate ADP-ribose monomers. Then the generated ADP-ribose is added onto the target protein. PARP, the nuclear enzyme is highly conserved in eukaryotes (Jayaram et al., 2011). PARP-1 is the best-investigated enzyme in this group. This enzyme manipulates many biological processes, like recombination, gene expression, DNA repair, apoptosis, autophagy and long-term memory (Burkle and Virag, 2013; D'Silva et al., 1999; Masutani and Fujimori, 2013; Rosado et al., 2013; Virag et al., 2013; Ying, 2008; Ying, 2013).

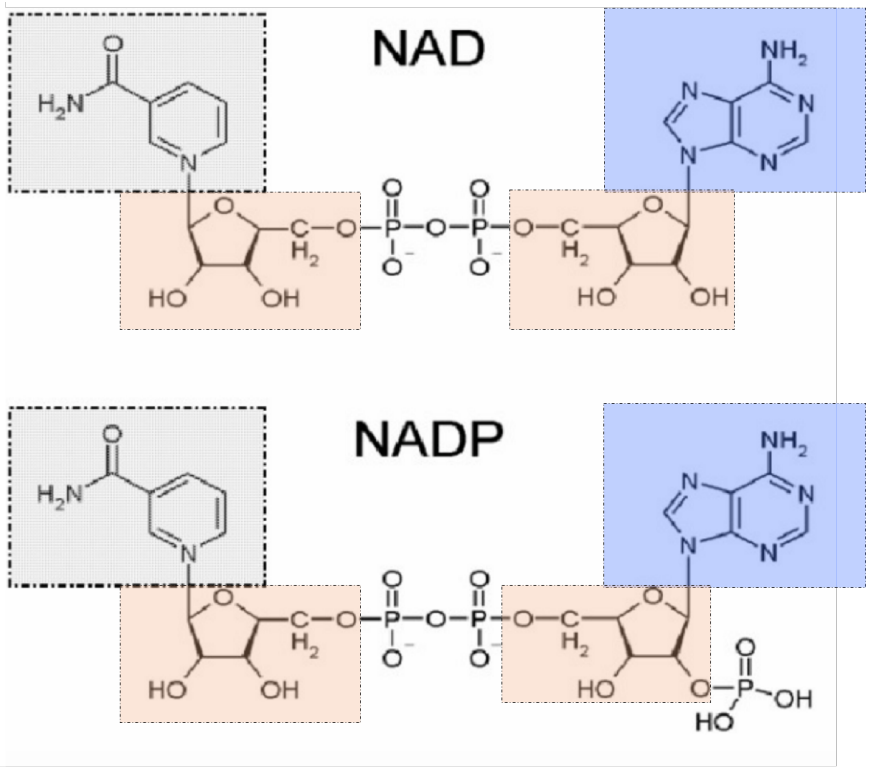


Figure 1-2 Structure of NAD & NADP.

NADP+ is the phosphate form of NAD+, which attaches an additional phosphate group to the ribose rings. Grey rectangle: nicotinamide; purple rectangle: adenine; orange rectangle: ribose.

Recently, cyclic ADP-ribose synthases are cataloged as a third group of NAD+ consumers. These enzyme use NAD+ is used to form cyclic ADP-ribose (cADPR) and is not are reversible. cADPR, its derivatives cADPRP and NAADP+, and massagers inositol-1,4,5-triphosphate (InsP3) serve as second regulating calcium signaling (Barone et al., 2002; Ziegler, 2000). cADPR and InsP3 control intracellular calcium pool, but the receptor activated by cADPR is different from InsP3. InsP3 require inositol trisphosphate receptor (InsP3R) to conduct calcium signals, however, cADPR activates ryanodine receptor and the calcium signal triggers calcium-induced calcium release (CICR) (Galione et al., 1991).

1.4 Biological Functions of NAD+

1.4.1 NAD+ Interaction in Diabetes

Pancreatic β-cell and neurons are two types of cells specifically susceptible to changes in NAD+ metabolism because these cells contain low levels of intracellular Nampt (iNampt), which is the rate-limiting enzyme in NAD+ synthesis pathway in vertebrates (Verdin, 2015). To maintain their optimal functions and NAD+ level, they take in extracellular NMN which is synthesized by adipose tissue derived from extracellular Nampt (eNampt) (Imai and Yoshino, 2013). iNampt and eNampt are generated through different post-translational modifications and have different molecular weights (Revollo et al., 2004; Revollo et al., 2007), so it seems that iNampt and eNampt have distinct roles in regulating cell functions. Some research reported that boosted eNampt affects many reactions and pathways in cells, such as oxidative stress response, apoptosis, lipid and glucose metabolism, inflammation and insulin resistance, which ultimately result in obesity and type 2 diabetes (Garten et al., 2009; Garten et al., 2015). For obesity occurrence, it's found that eNampt level influences food intake. Injection of eNampt into rats and chicks increased their feed intake (Brunetti et al., 2012; Cline et al., 2008). There is a close association between obesity and type II diabetes (Neurohr et al.,

2011). Among type II diabetes patients, over 80% of them are also diagnosed with obesity. Increased adiposity causes chronic low-grade inflammation, disturbed metabolism, abnormal hormone secretion in pancreatic β-cells, which lead to insulin resistance (Al-Goblan et al., 2014; Newman et al., 2006). Increased eNampt has been reported to contribute to type 2 diabetes (Chen et al., 2006; El-Mesallamy et al., 2011; Lopez-Bermejo et al., 2006; Motawi et al., 2014; Yilmaz et al., 2008). The mechanism is still unclear, but Revollo et al. strongly suggested that eNampt mediates insulin secretion in pancreatic β-cells via its NAD+ biosynthetic activity (Revollo et al., 2007). iNAMPT is also related to the occurrence of type II diabetes. The mechanism for iNAMPT contributing to type II diabetes appears due to decreasing abundance of iNAMPT with age or/and in response to the inflammatory cytokine tumor necrosis factor α (Wan et al., 2011).

1.4.2 NAD+ Relationship with Aging

NAD+ levels are related to aging in animals. In aged animals, NAD+ levels are reduced (Mouchiroud et al., 2013). Further, boosting NAD+ levels increases mitochondrial activity, prevents age-related disease and extends lifespan (Houtkooper and Auwerx, 2012). Sirtuin, the consumer of NAD+, is associated with the longevity (Guarente, 2013). Among these sirtuins, Sir2 is the first one that has been characterized that plays an important role in regulating longevity. *sir-2.1* in worms is well studied. Lots of papers from different aspects showed that *sir-2.1* mediates longevity. First, *C. elegans* with higher level of SIR-2 has an increased longevity (Tissenbaum and Guarente, 2001; Viswanathan and Guarente, 2011). Second, increasing NAD+ levels in worms promotes longevity through *sir-2.1* deacetylase, which activates mitochondrial unfolded protein response and the FOXO signaling pathway (Mouchiroud et al., 2013). In addition, the dauer-inducing small molecules ascarosides control lifespan in a *sir-2.1* dependent manner (Ludewig et al., 2013). This phenomenon was also found in other organisms, like flies and yeast (Kaeberlein et al., 1999; Rogina and Helfand, 2004). In these animals, Sir2 regulates lifespan extension through the pathways that are related to caloric restriction

(Anderson et al., 2003; Lin et al., 2000; Rogina and Helfand, 2004; Wang and Tissenbaum, 2006).

The mammalian Sir2 ortholog Sirt1 is also very important in mammalian aging. Overexpression of SIRT1 in mice not only expands their lifespan, but also maintains the mice in youthful physiology, like enhanced physical activity, quality of sleep and oxygen consumption (Mouchiroud et al., 2013). Conversely, mice with downregulation of Sirt1 demonstrated accelerated aging phenotypes, such as reduced dermal and subcutaneous thickness and decreased hair regrowth and increased wound healing time (Sommer et al., 2006). However, Herranz D et al. (Herranz et al., 2010) overexpressed Sirt1 in mice under its own regulatory elements, but they failed to increase lifespan in these mice. Although these transgenic mice had normal lifespan, they displayed healthier metabolic conditions and enhanced cancer protection. These studies demonstrated that sirtuin benefits metabolic health regardless of its anti-aging activity.

Chapter 2

NAD+ homeostasis affects gonad development in *C. elegans* by affecting glycolysis pathway

2.1 Introduction of *C. elegans*

Caenorhabditis elegans (*C. elegans*), a small nematode, has several advantages for scientists to choose it as a model organism in laboratory. *C. elegans* use *Escherichia coli* (*E. coli*) as food, and the size of them are tiny, about 1 mm length, so maintenance of the worms is affordable. The worm is transparent; because of this feature it is easy to trace nearly every cell's fate during development. The life cycle of *C. elegans* is short—about 3 days at 20˚C (Byerly et al., 1976), and hermaphrodites can produce more than 200 progeny. This model is a great tool in aging and genetic research. By comparing *C. elegans* and human genomes, Lai et al (Lai et al., 2000) reported that more than 80% of *C. elegans* proteome has human homology, so in recent decades *C. elegans* has been used as a research tool for studying several human diseases, like aging, neuron degeneration and muscle dystrophy (Chakraborty et al., 2013; Chartier and Simonelig, 2013; Martins et al., 2015).

However, there is little research focused on how NAD+ homeostasis affects animal development. Based on the beneficial aspects from *C. elegans*, we will use *C. elegans* as a model to study the role of NAD+ in animal development.

2.2 Glycolysis pathway

Glycolysis is a ten-step metabolic pathway which converts glucose into pyruvate and yields NADH and adenosine triphosphate (ATP) (Figure 2-1). All organisms have this pathway and it takes place in the cytoplasm. This ten-step pathway is considered the first stage of the glucose oxidation pathway, which releases chemical energy in glucose and forms biological energy ATP. The first step of glycolysis is the phosphorylation of glucose by hexokinase and it forms glucose 6-phosphate (G6P), during this step an ATP

is consumed. Then G6P is converted to fructose 6-phosphate (F6P) by glucose phosphate isomerase. Phosphofructokinase-1 phosphates F6P and generate β-D-fructose 1,6-bisphosphate (F1,6BP). This step is another ATP consuming step. F1,6BP will be split into D-glyceraldehyde 3-phosphate (GADP) and dihydroxyacetone phosphate (DHAP) by aldolase. Next, triosephosphate isomerase (TPI) quickly isomerizes DHAP to GADP, so a molecule of F1,6BP is converted to 2 molecules of GADP. The 2 molecules of GADP use 2 molecules of NAD+ and form 2 molecules of D-1,3-bisphosphoglycerate (1,3BPG). Phosphoglycerate kinase (PGK) excises a phosphate group away from 1,3BPG and attaches it to ADP, yielding ATP and 3-phosphoglycerate (3PG). 3PG would be changed to 2-phosphoglycerate (2PG) by phosphoglycerate mutase (PGM). Then enolase dehydrates 2PG and forms phosphoenolpyruvate (PEP). In the final step of glycolysis, the enzyme of pyruvate kinase (PK) transfers a phosphate group from PEP to ADP, and generates pyruvate and ATP. During the glycolysis pathway, 1 molecule of glucose will produce 2 molecules of ATP and consumes 2 molecules of NAD+.

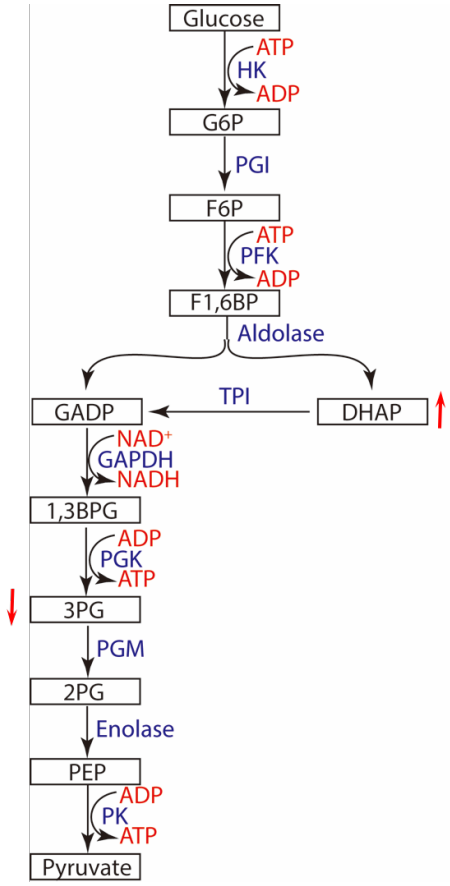


Figure 2-1 Glycolysis pathway.

Glycolysis is a ten-step metabolic pathway which converts glucose into pyruvate and yields NADH and ATP. During glycolysis pathway, 1 molecule of glucose will produce 2 molecules of ATP and consumes 2 molecules of NAD+. The red arrows indicate the metabolites that are changed in *pnc-1(pk9605)* mutants.
Abbreviations: HK, hexokinase; G6P, glucose 6-phosphate; PGI, glucose phosphate isomerase; F6P, fructose 6-phosphate; PFK, Phosphofructokinase-1; F1,6BP, β-D-fructose 1,6-bisphosphate; GADP, D-glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; TPI, triosephosphate isomerase; 1,3BPG, D-1,3-bisphosphoglycerate; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; PGK, Phosphoglycerate kinase; 3PG, 3-phosphoglycerate; PGM, 2-phosphoglycerate; PEP, phosphoenolpyruvate. PK, pyruvate kinase.

Cancer cells have several obvious metabolic changes; a change in glycolysis is one of the hallmarks (Pavlova and Thompson, 2016). The proliferation of tumor cells requires increasing the amount of nutrients, so the uptake of glucose and glutamine is dramatically boosted, which is used to maintain cell survival and biosynthesis in tumor cells (Almuhaideb et al., 2011; Som et al., 1980; Warburg et al., 1924). Glucose has several functions in cells: building blocks are derived from its metabolites for building various macromolecules. In addition, its oxidation drives electron transport to generate ATP. Also, during its oxidation, NADH, FADH2 and NADPH are formed, which helps maintain cellular redox capacity (Pavlova and Thompson, 2016).

Our lab is interested in the relationship between NAD+ homeostasis and organisms' development. We use *C. elegans* as a model to explore the relationship between metabolism and development. Previous studies in our lab already found that NAD+ homeostasis is essential for organisms' development, and disturbed NAD+ homeostasis have impacts on organisms' development (Vrablik et al., 2009; Vrablik et al., 2011; Wang et al., 2015). Our lab's previous data showed that in the absence of the nicotinamidase *pnc-1*(*pk9605*), *C. elegans* shows a gonad developmental delay phenotype (Vrablik et al., 2009; Vrablik et al., 2011). We measured NAD+ level by using LC-MS. It showed that NAD+ level in *pnc-1*(*pk9605*) is 30% less than wild-type worms (Wang et al., 2015). We supplemented *pnc-1*(*pk9605*) mutant worms with precursors for forming NAD+ that don't require PNC-1 for use, such as NA, NMN or NAD+ itself, and

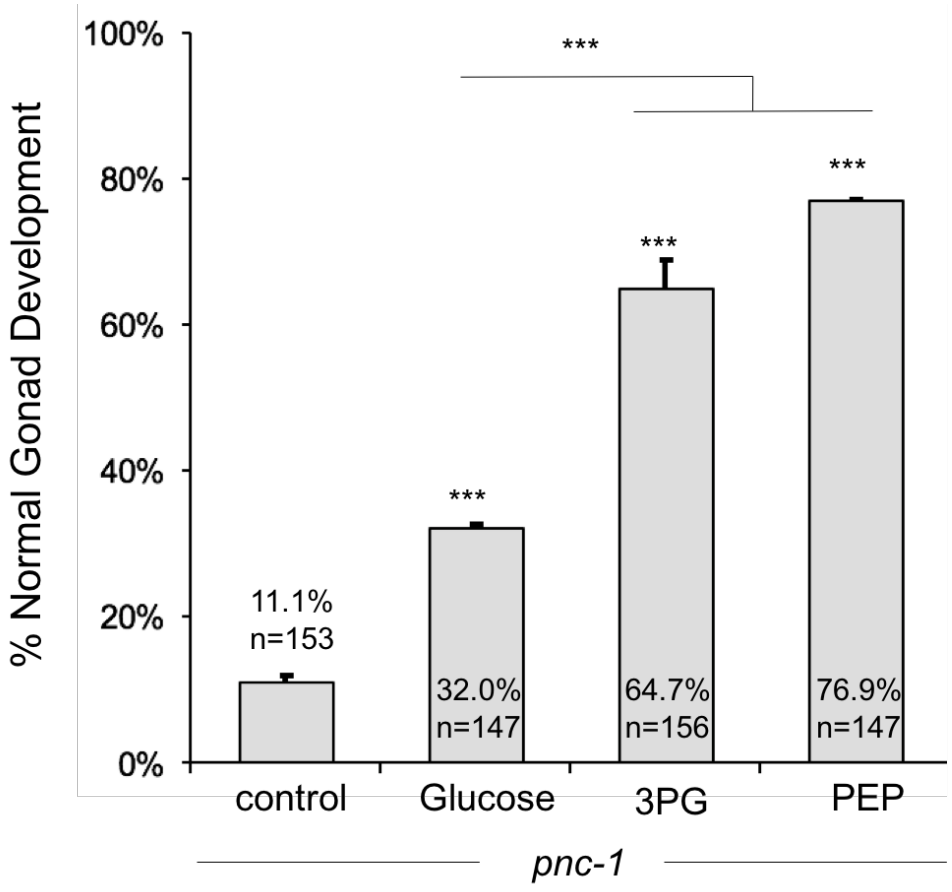
they rescue the gonad delay phenotype. We also expressed human NAMPT in the *pnc-1*(*pk9605*) mutants to introduce the vertebrate NAD+ salvage biosynthesis pathways for salvage of NAM. In these transgenic worms, gonad developmental delay phenotype is rescued (Vrablik et al., 2009; Vrablik et al., 2011). Alternatively, we prevented the consumption of NAD+ in *pnc-1*(*pk9605*) by inactivating the NAD+ consumer, PME-1/ PARP, and gonad delay is rescued in these double mutants (Wang et al., 2015). These data suggested that NAD+ homeostasis contributes to gonad development in *C. elegans*.

My project is to investigate a specific question about the mechanism of NAD+ in the gonad development in *C. elegans:* why disrupted NAD+ biosynthesis affects gonad development? This project will provide us the insights on metabolic products function in animal's development and also shed light on intimate connection between metabolism and development.

Because NAD+ participates in numerous cellular pathways, we compared the metabolic profiles between *pnc-1* mutants and wild-type worms to find out what changes are due to the disturbed NAD+ homeostasis. We set up three groups of experiments to examine the hypothesis. There are 123 named metabolites that differ significantly between *pnc-1* mutants and wild-type worms (Wang et al., 2015). We also compared metabolic profiles between *pnc-1* mutants and *pnc-1* mutants with NA. The combination of LC-MS and GC-MS assays results showed that 145 metabolites have been changed. Among these metabolites, 62 metabolites are also changed in *pnc-1* mutants vs. wild-type metabolic profiles. In the third group, the relative levels of metabolites are examined in *pnc-1* mutants and *pnc-1* mutants fed with UV-killed OP50. *pnc-1* mutants fed with dead OP50 exacerbated the gonad developmental delay phenotype. 14 of the changed metabolites are overlapping with the changed 62 metabolites. After we focused on the 14 changed metabolites, we found that these changed metabolites are tightly related to glycolysis: *pnc-1* mutant accumulate glycolytic intermediates prior of the step using NAD+ (DHAP, etc) and are depleted in metabolite immediately after the step using NAD+ (3PG). Supplementation with 2.5 mM NA could rescue perturbed glycolysis in *pnc-1* mutant, suggesting insufficient NAD+ disrupted glycolysis (Wang et al., 2015). Based on the data, I hypothesize that perturbed glycolysis contributes to gonad developmental delay phenotype in *pnc-1* mutant.

Results

In order to investigate whether perturbed glycolysis caused gonad delay developmental delay phenotype in *pnc-1* mutant worms, I restored the products of glycolysis in *pnc-1* mutants by supplementing *pnc-1* mutants with the metabolites that are depleted in the mutants and then tested if it could rescue the *pnc-1* phenotype. I fed *pnc-1* mutant worms with 2.5 mM PEP, 3PG and glucose by adding these metabolites on OP50 plates. Compared to glucose, PEP and 3PG significantly rescued the gonad developmental delay phenotype in *pnc-1* mutant worms (Figure 2-2), which in consistent with our hypothesis that a deficit in glycolysis is causative of gonad development delay in *pnc-1* mutants.



(Wang et al., 2015)

Figure 2-2 Effects of supplementation with glycolytic intermediates on gonad development in *pnc-1* mutants.

PEP and 3PG significantly rescued the gonad developmental delay phenotype in *pnc-1* mutant worms. These metabolites show significantly higher efficiency in the rescue of gonad development than glucose. pnc-1 mutant worms were fed with UV-killed OP50. Error bars are S.E. *, $0.01 < p < 0.05$; **, $0.001 < p < 0.01$; ***, $p < 0.001$; calculated using Fisher's exact test. (published in Comparative Metabolomic Profiling Reveals That Dysregulated Glycolysis Stemming from Lack of Salvage NAD+ Biosynthesis Impairs Reproductive Development in *Caenorhabditis elegans*. *The Journal of biological chemistry* 290, 26163-26179)

Discussion

Because I was able to rescue *pnc-1* gonad development phenotype by supply with late glycolytic intermediates PEP and 3PG (Figure 2-2), we conclude that disrupted glycolysis is the reason behind the gonad delay phenotype. Our model is: after the synthesis of NAD+ is disrupted, then glycolysis will be disrupted, and ultimately cause gonad developmental deficiency in *C. elegans*.

I also tried a second strategy to investigate my hypothesis. I tried to block glycolysis in wild type and then examine if it could mimic the *pnc-1* phenotype. I attempted to block glycolysis using RNAi of glycolytic enzymes. Glyceraldehyde-3-phosphate dehydrogensase (G3PDH) in glycolysis is an ideal target for RNAi approach in my project. I fed the wild-type worm with G3PDH RNAi culture, but the RNAi plasmids have no effect on gonad developmental phenotype. It is possible, but not yet investigated experimentally, that the RNAi plasmids didn't knock down expression levels. Our lab colleague knocked down glycolysis genes *pfk-1.1*, *pfk-1.2*, and *tpi-1* by RNAi (Wang et al., 2015), and found that RNAi to these genes in wild type worms cannot cause gonad developmental delay phenotype, however, in *pnc-1* mutants, RNAi in these genes exacerbated the gonad developmental defects. These data indicate that besides the glycolysis pathway, another pathway affected by lack of NAD+ contributes to the phenotype as well.

Among the 14 changed metabolites we identified, some of them are also participating in the pentose phosphate pathway (PPP). The pattern in the PPP is similar to the metabolite change trends in glycolysis, in which *pnc-1* mutants showed accumulation glycolytic intermediates prior of the step using NAD+ and depletion in metabolite immediately after the step using NAD+. The level of intermediate that is a product of the enzyme using NAD+ as a cofactor (6-PG), is increased and the levels of the metabolites

that are directly downstream of the enzymatic reactions requiring NAD+ as a cofactor, (ribulose-5-phospate and ribose-5-phosphate (R-5-P)), are decreased. This metabolomics data suggests that there are likely other pathways, perhaps PPP, that are also affected in *pnc-1* mutant worms.

Material and Methods

Maintenance of *C. elegans* and Supplements treatments

C. elegans are cultured under 20˚C environment feeding with *E. coli* strain OP50 as standard condition (Brenner, 1974). The strain N2 used as the wild-type strain. To prepare NGM plates seeded with OP50 lawn. Single colony of OP50 was picked and cultured in LB broth (10 g Bacto-tryptone, 5 g Bacto-yeast, 5 g NaCl, add H_2O to 1 liter) under 37˚C incubator for 2-4 days, and then applied 300-400ul *E. coli* OP50 liquid culture on Nematode Growth Medium (NGM) plates (6 g NaCl, 5 g Bacto-peptone, 34 g agar, 2 ml 1 M $CaCl_2$, 2 ml 1 M $MgSO_4$, 50 ml 1 M KPO_4 buffer, 2 ml 5 mg/ml cholesterol in ethanol, H_2O to 2 liter) by sterile pipets.

Phosphoenolpyruvate (PEP) (Sigma), 3-phosophoglycerate (3PGA) (Sigma), dextrose (Mallinckrodt Baker), pyruvate (Sigma) are dissolved in H_2O and filtered to sterilize. Add appropriate volume of these solution OP50 plates. Then place the plated at room temperature for 1-2 day. The purpose of this step is to make final concentration of these solutions is 2.5 mM evenly on the whole plates.

To identify the gonad delay phenotype on OP50 food lawn, I transferred 5 *pnc-1* or N2 adult worms on a seeded NGM plates with metabolites supplements and cultured them at 20 ˚C until the next generation of worms reached mid-L4 stage.

Gonad delay Phenotype assessment

Gonad delay phenotype was screened as described by Vrablik (Vrablik et al., 2009). Normal gonad has an open uterine lumen above ‘Christmas tree’ shape like vulva at mid-L4 stage, and most of *pnc-1* worms have delayed developmental gonad, which shows

closed uterine lumen at mid-L4 stage. The phenotypes are characterized under 10X eyepieces and 40X to 100X objectives (total magnification is 400 X to 1000 X).

Statistic Analysis

The *p* values of gonad delay phenotype assessment are calculated by using Fisher's exact test. Error bars are S.E.; ***, $p < 0.001$.

Chapter 3

Characterization of NMNAT subcellular localization in *C. elegans*

3.1 Introduction of NMNATs

The enzyme NMNAT participates in a single reaction but two different substrates. It catalyzes the reaction yielding NaAD from NAMN and the reaction yielding NAD+ from NMN. NMNAT is the rate-limiting enzyme in NAD+ synthesis pathway, so it is the essential enzyme to regulate NAD+ metabolism. Several decades ago, NMNAT was considered as a nuclear enzymatic activity, but with the development of sequencing, it has been found that, in humans, there are three NMNAT genes and they encode NMNAT1, NMNAT2 and NMNAT3, respectively. The three human NMNATs have different subcellular localizations.

The human NMNAT1 is a homohexamer, consisting of 279 amino acids (31.9KD) (Jayaram et al., 2011) and expressed in all tissues (Garwood et al., 2010). The sequence contains a nuclear localization signal motif, which leads to NMNAT1 localization in the nucleus. The NAD+ consumer PARP is located in nucleus, and NMNAT1 produces NAD+ in nuclei; therefore, it is not a surprise to find that NMNAT1 is tightly associated with PARP activity (Ruggieri et al., 1990). Also, NMNAT1 is associated with nuclear matrix proteins (Balducci et al., 1992) and is sensitive to DNA damage (Song et al., 2013). DNA damage triggers the expression of NMNAT1, and NMNAT1 has the function of preventing cell death after damage. NMNAT1 plays an essential role in axon growth and maintenance as well (Feng et al., 2010).

NMNAT2 is a homodimer protein and has 307 amino acid residues (34.4 KD). It docks on the Golgi (Jayaram et al., 2011). Axon survival is affected by NMNAT2 activity. Loss of NMNAT2 promotes the degeneration of axon (Di Stefano et al., 2015; Gilley et al., 2013). Although NMNAT1 also has the protective function in neuron cells, NMNAT2 is relatively more important than NMNAT1 in protecting the nervous system (Conforti et al., 2011). Recently, it's been found that the tumor suppressor p53 can bind to NMNAT2 gene and regulates its expression, so NMNAT2 is a novel discovered target of p53 and play an important role in p53-mediated signal pathways (Pan et al., 2014).

NMNAT3, the mitochondrial localized NAD+ synthesis enzyme, is a homotetramer (Jayaram et al., 2011; Zhang et al., 2003). The length of this protein is 252 amino acids (28.2 KD).

The three NMATs regulate relatively separated NAD+ pools to meet specific requirement for NAD+ in cells. NMNAT1 and NMNAT2 control the nucleus-cytoplasm NAD+ pool, however, MNAT3 regulates NAD+ level in mitochondria (Berger et al., 2005). The second and third phases (TCA cycle and oxidative phosphorylation) of glucose oxidative pathway happen in mitochondria, and NAD+ participates in both of the phases, so loss of NMNAT3 causes the depletion of mitochondrial NAD+ level and blocks the glucose oxidative pathway, leading the stall of the first stage of glucose oxidative pathway, glycolysis (Hikosaka et al., 2014). But Yamamoto M and his colleagues found that NMNAT3 is dispensable for mediated mitochondrial NAD+ level (Yamamoto et al., 2016). They showed that mitochondrial NAD+ levels and metabolites in glycolysis in nmnat3-KO mice and WT mice are similar. Like NMNAT1 and NMNAT2, NMNAT3 shows the function in axonal protection (Sasaki et al., 2006).

3.2 NMNATs and Disease

NMNATs are relevant to some diseases. In the past, studies from Drosophila and slow Wallerian degeneration (Wlds) mouse model revealed that NMNAT1 have the protection of neurons from degeneration. But there was no report linking the NMNAT1 with human disease directly. In 2012, it was found that the mutation of NMNAT1 can result in the eye disorder Leber congenital amaurosis (LCA) in humans. This disease causes the degeneration of retina. Patients suffering from LAC have various syndromes, such as farsightedness, very sensitive to light, no response for pupils to light, involuntary movements of the eyes, etc. By sequencing the patients' nmnat1 gene, it is now clear that the mutation affects the protein folding and decreased NMNAT1 enzymatic activities (Chiang et al., 2012; Koenekoop et al., 2012). However, the molecular mechanism of the pathology is still unknown.

NMNAT2 is also a critical factor for axons survival. NMNAT2 performs its ability depending on its palmitoylation, so NMNAT2-dependent axon survival can be regulated by NMNAT2 palmitoylation (Milde and Coleman, 2014). Inverse, axon injury

results in rapid depletion of NMNAT2 and this will exacerbate Wallerian degeneration (Loreto et al., 2015). Besides protection of neuron cells, NMNAT2 also protect cardiomyocytes from hypertrophy, this function is associated with NMNAT2 NAD+ synthesis activity (Cai et al., 2012). NMNAT2 also have an important function in regulating lung cancer cell growth. SIRT3 can bind to and deacetylate NMNAT2, this interaction promotes lung cancer cell proliferation (Wu et al., 2013).

Kitaoka and his colleagues (Kitaoka et al., 2013) sought for the reason of NMNAT3 displaying axonal-protective functions by overexpression of NMNAT3. They found that overexpression of NMNAT3 decreased sequestosome 1 (SQSTM1) and increased autophagic flux. SQSTM1 is an autophagy receptor that is involved in the formation of autophagosome (Katsuragi et al., 2015). From these data, they predicted that NMNAT3 exerting its axonal-protective function by altering the formation of autophagy machinery. NMNAT3 is the dominated NMANTs in human red blood cells, in which NMNAT2 is absent (Garwood et al., 2010), so NMNAT3 function is highly related with blood cell health. Deficiency of the mitochondria-localized NMNAT3 shifts the glycolysis pathway to pentose phosphate pathway, this change will dramatically decrease ATP level in blood cell and result in hemolytic anemia (Hikosaka et al., 2014).

So these three NMNATs regulate relatively separated NAD+ pools and play different metabolic roles to meet specific requirements of NAD+ in cells. Based on this information, I wanted to use NMNAT function to explore the compartment specific requirements for NAD+ biosynthesis in *C. elegans* gonad development.

Our lab has examined mitochondrial activity of *pnc-1* and wild-type worms by detecting oxygen consumption. Because mitochondrial NAD+ pool affects the process of TCA cycle and oxidative phosphorylation, the mitochondrial activity reflects the stability of mitochondrial NAD+ pool. Oxidative phosphorylation is the only process that uses oxygen as substrate, and this process takes place in the mitochondria, so the different oxygen consumption between *pnc-1* and wild-type worms would reflect the difference of mitochondrial NAD+ pool in these two groups of worms. However, we found no difference between *pnc-1* mutants and wild-type animals in oxygen consumption (Wang et al., 2015), suggesting mitochondrial NAD+ pool in *pnc-1* mutants is not impaired.

Thus, we hypothesize that the mitochondrial NMNAT will not have an impact on gonad development, but the cytoplasmic NMNAT will affect gonad development.

However, it is unclear which of the two *C. elegans* NMNAT genes, *nmat-1* and *nmat-2*, corresponds to which human gene and which is the mitochondrial and the cytoplasmic enzyme. *nmat-1* and *nmat-2* are both listed as NMAT3 homologs in Wormbase. However, our own phylogenetic analysis failed to substantiate this relationship and did not clarify orthologous relationships (Figure 3-1). We hypothesize that one of the genes is for nucleo-cytoplasmic NAD+ biosynthesis and the other is for mitochondrial NAD+ biosynthesis. To begin investigating this hypothesis, I have created translational fusions of *nmat-1* and *nmat-2* to GFP under the control of the *myo-3* promoter and examined their subcellular localization in muscle cells.

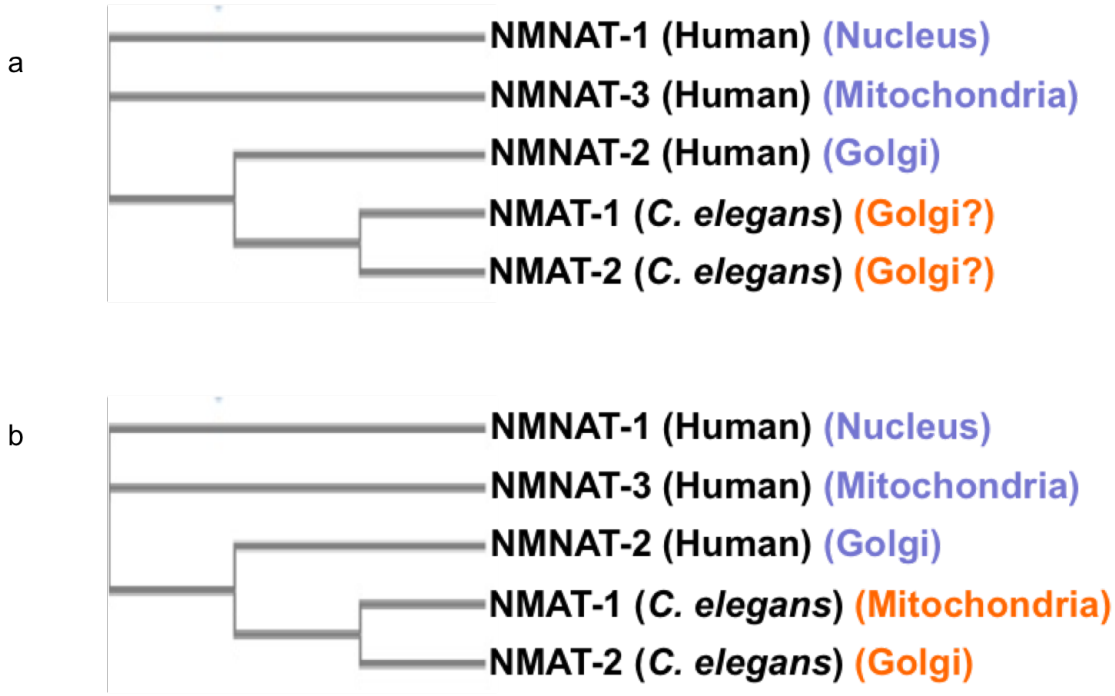


Figure 3-1 Phylogeny of human NMNAT genes and *C. elegans* NMAT genes.

a, the predicted phylogenic relationship of human NMNAT genes and *C. elegans* NMAT genes by EMBL software tool. b, modified phylogenic relationship of NMNAT genes and NMAT genes based on experimental results in Figure 3-2.

Results

My chapter2 experiments showed that a disrupted glycolysis pathway is a cause of gonad development phenotype in *pnc-1* mutants. Glycolysis takes place in the cytoplasm, so this data indicated that cytoplasmic pool of NAD+ is critical for gonad development. Our lab has examined *nmat-2* functionally and found that *nmat-2(tm2905)* animals have a gonad delay development phenotype (Wang et al., 2015). These results suggested that NMNAT2 is required in the cytoplasm. To investigate the hypothesis, I fused NMAT2 with CFP which is driven by the muscle specific *myo-3* promoter. Then I injected the plasmid into worms, selected transgenic animals and observed the subcellular localization of NMAT2::CFP using fluorescence microscopy (Figure 3-2, right). NMAT2::CFP fusion proteins forms sphere-shaped puncta. These puncta are randomly dispersed in the muscle cells and vary in size. The expression pattern of NMAT2 is similar to Golgi protein expression pattern (Broekhuis et al., 2013). According to NMAT2 functional experiment data, we predict that NMAT2 is a Golgi-docked cytoplasmic protein and is in charge of the cytoplasmic NAD+ pool.

I also examined the expression of NMAT1 by applying the same strategy. The expressed NMAT1::CFP fusion proteins looked like small rods that are parallel lying along the muscle mitochondria tubules (Figure 3-2, left). The NMAT1 expression pattern is a typical mitochondrial protein pattern (Han et al., 2012).This result suggested that NMAT-2 is localized in the mitochondria.

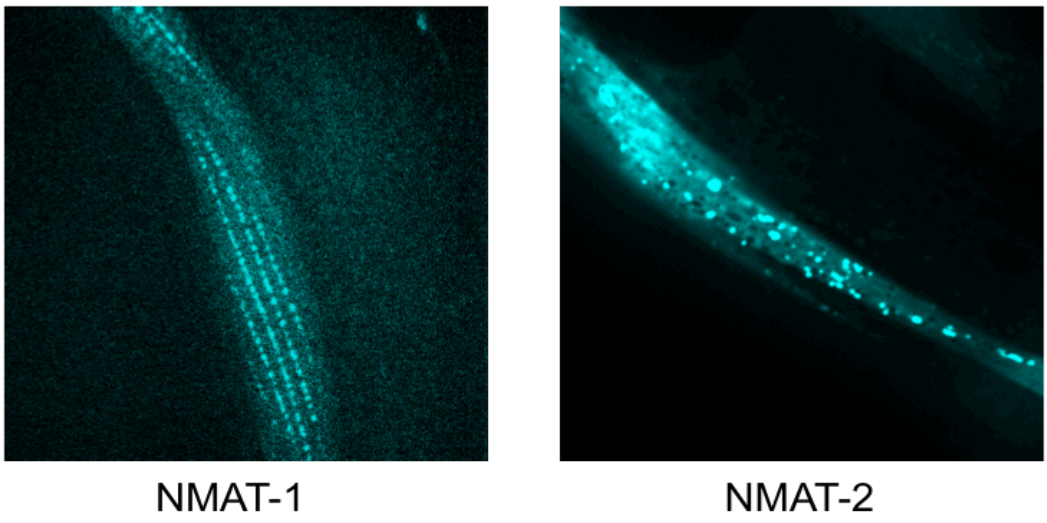


Figure 3-2 Subcellular localization of NMATs in muscle cells.

Left, Subcellular localization of NMAT-1. NMAT1::CFP fusion proteins looked like small rods that are parallel lying along the muscle mitochondria tubules. Right, Subcellular localization of NMAT-2. NMAT2::CFP fusion proteins forms sphere shape puncta. These puncta are randomly dispersed in the muscle cells and the sizes are various.

Discussion

To investigate NMATs localization, NMAT1 and NMAT2 are fused with CFP respectively, and expressed in *C. elegans* muscle cells via muscle specific promoter *myo-3*. NMAT1 is a mitochondrial protein, because the rod-shape protein lays along the muscle mitochondria tubules (Figure 3-2, left). The sphere-shape NMAT2::CFP fusion proteins are dispersed in cytoplasm (Figure 3-2, right), and we predict that NMAT2 is cytoplasmic protein docking on Golgi membrane (Figure 3-1, b).

This result is consistent with other experiments in the lab pointing toward a relationship between glycolysis and gonad development in Chapter 2. Our lab has also examined *nmat-2* functionally via null allele of *nmat-2*. *nmat-2(tm2905)* animals showed a gonad development phenotype. Thus, the *nmat-2* functional experiment is consistent with my observation that NMAT2 is not a nuclear or mitochondrial protein. To further test whether NMAT2 regulates cytoplasmic NAD+ pool, we can feed *nmat-2(tm2905)* animals with 3PG or PEP. Both of the metabolites are predicted to rescue gonad delay in this background. Whether NMAT2 is a real Golgi-localized protein needs to be further

explored by co-location experiments with another known Golgi protein. Nonetheless, our functional experiment and CFP experiment results suggests that the NMAT2 is not a nuclear protein.

Although NMAT1 expression pattern is as the same as the mitochondrial protein, it also needs to be double-checked with co-location experiments or *nmat-1* functional experiments.

To examine whether NMAT1 is a mitochondrial protein, I used MitoTracker® Red CMXRos as the dye to stain mitochondria in worms. First, I followed the method mentioned by Meissner (Meissner et al., 2011). The dye effectively stained the worm's intestine, but ineffectively stained the muscle cells. I changed the concentration of the dye and soaking time to treat the worms, but still could not get good co-localization image focusing on muscle cells (Figure 3-3).

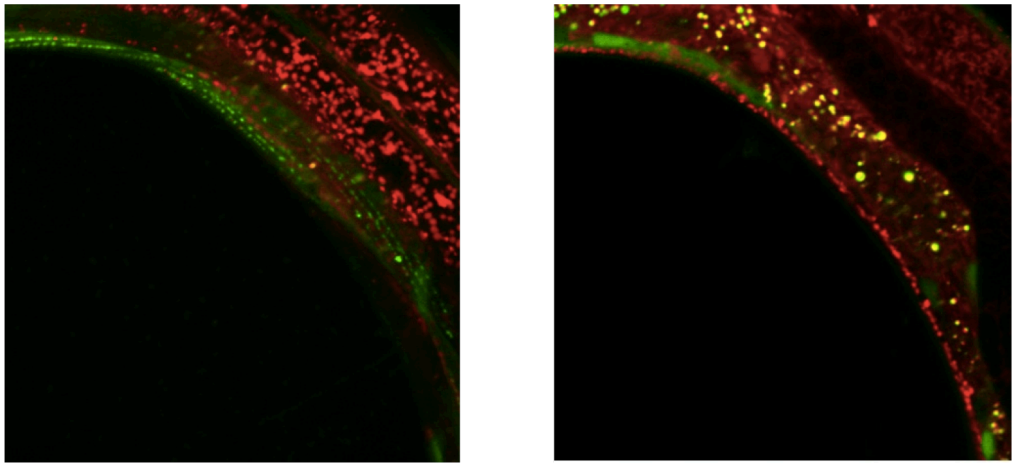


Figure 3-3 Co-localization image of NMAT1-CFP and MitoTracker® Red CMXRos stained mitochondria

These pictures are focusing on the same filed, but from different z stacks.

As an alterative experiment to investigate the subcellular localization of NMAT1, I tested whether NMAT1 is co-localized with a mitochondrial protein. I injected *Pmyo-3*:: NMAT-1::mCherry plasmid and pPD133.54 plasmid into worms. pPD133.54 expresses a protein that can serve as a mitochondrial protein marker; it will drive expression of CFP in muscle mitochondria (the promoter is *myo-3* and the mitochondrial import signal is

from chicken aspartate aminotransferase). I compared the expression patterns of mito-CFP(pPD133.54) and NMAT1::mCherry in animals co-expressing these fluorescent proteins. While the patterns of expression are reminiscent of each other, displaying the tracks or lines normally associated with mitochondria that have lined up along the muscle fiber, they do not perfectly co-localize (Figure 3-4). It seems that NMAT-1 may be in structures adjacent to the mitochondria and perhaps in mitochondria as well. Expression of these transgenes also caused abnormal egg laying behavior. This analysis does not rule out expression of NNMA1 in mitochondria but suggest extra-mitochondrial localization as well.

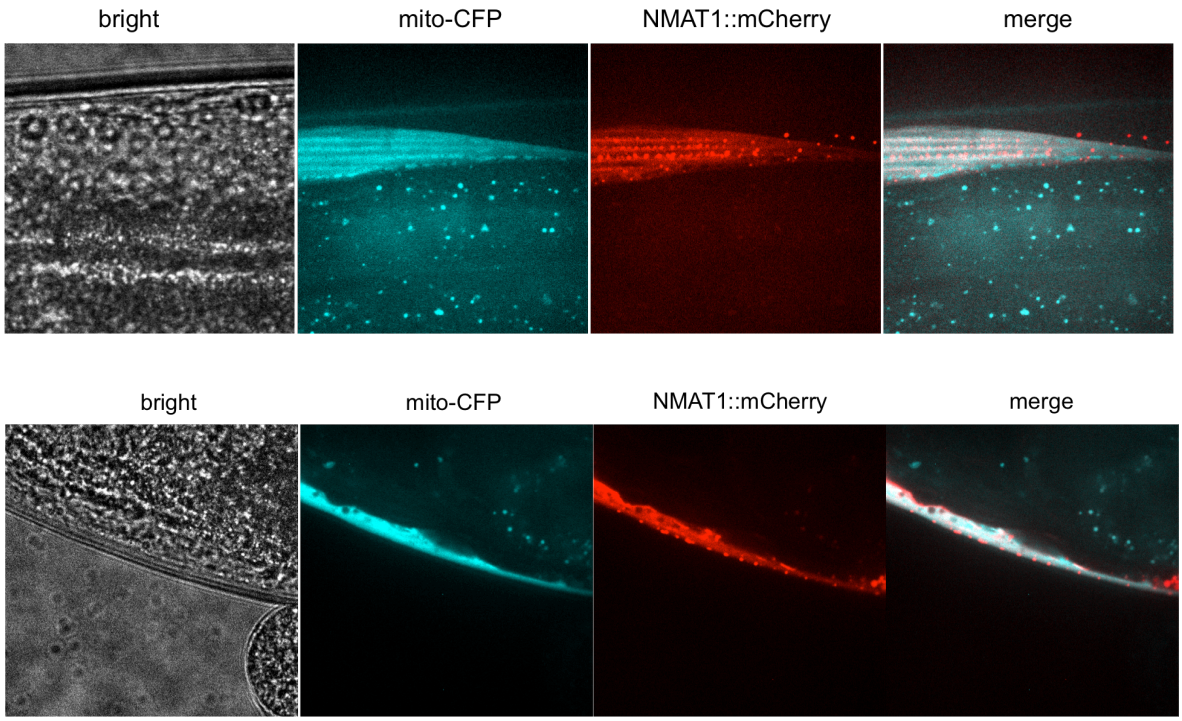


Figure 3-4 Co-localization image of NMAT1-mCherry and mito-CFP

These pictures are focusing on the different fields of worms.

However, we don't have an allele of *nmat-1* to investigate our hypothesis. We aim to use the CRISPR/Cas9 system to create an allele of *nmat-1*. I will discuss these plans in Chapter 4. The data from these experiments will help us to understand the relationship

between the nuclear/cytoplasmic and mitochondrial NAD+ pools in terms of gonad development and will be a useful tool to probe other functions of NAD+ biosynthesis as well.

Material and Methods

Maintenance of *C. elegans*

C. elegans are maintained at 20˚C feeding with *E. coli* strain OP50 as food (Brenner, 1974). N2 is the wild-type strain. The maintenance method is mentioned in chapter2.

Plasmids construction

The backbone for the plasmids construction is from Addgene with the accession number: L4816.

To construct NMAT1-CFP and NMAT2-CFP expression plasmid, I designed primers to amplify the *C. elegans* NMAT1 (W06B3.1) and NMAT2 (F26H9.4) cDNA sequences based on the information from Wormbase.

Primer sequences are for NMAT1 cDNA: forward primer: cgggatccATGGGGACCGAAAAAGTTGT, and reverse primers: ggggtaccttTTCGTAGAGTCTATGATCTT. The amplified fragments with adaptors BamH1 and Kpn1.

Primer sequences are for NMAT2 cDNA: forward primer: cgggatccATGAAACGAGTCGCTCTTCT, and reverse primers: ggggtaccAGAATTTTCTGATACAGATTAT. The amplified fragments with adaptors BamH1 and Kpn1.

The NMAT1 cDNA and NMAT2 cDNA PCR products are inserted into L4816 digested with BamHI and KpnI (NEB). Plasmid L4816 includes myo-3 promoter, which drove the fusion CFP protein expressions in muscle cells.

To construct NMAT1-mCherry expression plasmid, I used NMAT1-CFP expression plasmid as the backbone and digested it with EcoR1 and Kpn1. The plasmid that can express mCherry is a gift from Dr. Chaowen Xiao. I used the forward primers: GGGGTACC AATGGTGAGCAAGGGCGAGGA, and reverse primers: CGGAATTCCTACTTGTACAGCTCGTCCA to amplify the cDNA of the mCherry. The amplified fragments with adaptors EcoR1 and Kpn1.

The mCherry cDNA products are inserted into NMAT1-CFP expression plasmid digested with EcoR1 and Kpn1.

Microinjection

Plasmids are purified by using TIANGEN kit, and then diluted in MilliQ H_2O to approximate 30 ng/ μL. I injected NMAT1-CFP and NMAT2-CFP expression plasmids into the gonad arms of young adult worms (Mello et al., 1991; Mello and Fire, 1995). After the DNA injection, worms are recovered in M9 salt solution (6.8 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, add H_2O to 1 liter) on new OP50 plates at 20˚C. Pick green F1 progeny under GFP microscopes (Zeiss) and put them on OP50 plates individually to screen for stable transgenic strains.

Chapter 4

Targeting *nmat-1* using CRISPR

4.1 Introduction of targeted genome engineering

Numerous human diseases are caused by the mutations in genomic DNA. In order to deal with these diseases, scientists dreamed of correcting of mutations to cure such diseases. In the past decades, they made a big progress in targeted genome engineering. Three wildly applied techniques of targeted genome engineering have been discovered so far, and they are based on Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALEN) and clustered regularly interspaced short palindromic repeat /CRISPR-associated proteins (CRISPR/Cas). All of the methods depend on nucleases to generate site-specific DNA double-strand breaks (DSBs). So, these genome engineering approaches share the same mechanism that produce specific site cleavage on genomic DNA and cause targeted site mutation. However, they take different ways to search for target genomic DNA.

Among these three systems, ZFNs system is the first system that was used for modifying genomic DNA. ZFNs system is based on the chimeric Zinc finger nuclease, which contains two domains: zinc finger DNA-binding domain and FokI restriction enzyme-derived nuclease domain (Kim et al., 1996; Ul Ain et al., 2015). Each zinc finger DNA-binding domain consists of a Cys2-His2 tandem unit, which recognizes three base pairs of DNA and then binds to the major grooves of these DNA (Miller et al., 1985). The combination of six designed zinc fingers DNA-binding domain targets to 18 base-pair of DNA, which is a piece of unique sequence on genomic DNA (Segal et al., 2003). This is the reason behind ZFNs system serving as target genome-editing tool. FokI nuclease domains isolate from *Flavobacterium okeanokoites* (Ma and Liu, 2015). Two FokI nuclease domains are required to form activated dimer to cleave DNA (Bitinaite et al., 1998). Each monomer binds to opposite strand of DNA and span 5–7 base pair (Kim and Kim, 2014). Eukaryotic genomes contain an abundance of sites that can be cleaved by FokI restriction enzyme (Ul Ain et al., 2015), so this system has been applied on various eukaryotes, like plants (Lloyd et al., 2005), mice (Carbery et al., 2010), rats (Mashimo et

al., 2010), and human cells (Soldner et al., 2011; Urnov et al., 2005). Recently, in order to regulate gene expression level, FokI restriction enzyme-derived nuclease domain has been replaced with other functional domains (Vasileva et al., 2015). Compared to the other two genome-editing system, the relative lower targeting density restricts its application in many labs.

Like ZFNs, TALENs also consist of two domains: FokI nuclease domains and DNA binding domains. The DNA binding domain is derived from plant pathogen *Xanthomonas bacterium,* which serves as transcription activator-like effectors to impair host defenses and promote bacterial infection (Cermak et al., 2011). Each of the domains is composed of 33–35 amino acid arranged in tandem. The amino acid residues at 12th and 13th positions are known as repeat variable diresidues (RVD) to establish site specificity (Boch et al., 2009; Moscou and Bogdanove, 2009). For target recognition and editing efficiency, transcription activator-like effector (TALE) binding sites should initiate with a thymine and the spacer between two TALEN arms is from 15 to 21 base-pair (Huang et al., 2011). After TALE binds to target sites, FokI nuclease generate site-specific DNA double-strand break. TALENs genome-editing system has a wider usage spectrum than ZFNs system. Besides higher eukaryotes, even viruses (Bloom et al., 2013) and yeast (Carbery et al., 2010; Li et al., 2011) genomes can be modified by TALENs.

Scientists started to edit genomic DNA via CRISPR-Cas9 system just about four years ago, and due to its precise and efficient genomic editing features, it brings a revolution in biological fields. Actually, CRISPR-Cas9 system was discovered in the last century, it is the immune system in microbes to defend against virus (Anderson et al., 2015; Barrangou et al., 2007). About half of bacteria and about 90% of archaea have the system to defend against virus (Labrie et al., 2010; Ran et al., 2015). This immune system is analogous to vertebrate adaptive immune system that remembers the first infection and generates rapid response for the following infections (Zalatan et al., 2015). The biogenesis of CRISPR immunity includes three stages: adaptation/spacer acquisition, expression/CRISPR RNA (crRNA) biogenesis, interference (Makarova et al., 2011; Zalatan et al., 2015). For spacer acquisition, after virus invasion, the injected DNA from the virus is processed and generates proto-spacers. The proto-spacers are incorporated into the CRISPR array to form new spacers. The next stage is crRNA biogenesis. The

CRISPR locus is transcribed to pre-crRNA, which would be trimmed into short mature crRNAs. Then crRNAs recruit Cas proteins to form the effector complex. This process is known as interference stage. The effector complex recognizes matched foreign viral nucleic acid and degrades it (Deltcheva et al., 2011; Jinek et al., 2012). However, people did not realize its powerful ability in genome engineering until four years ago. In 2012, Jinek et al (Jinek et al., 2012) began to apply CRISPR/Cas system to modify genome in vitro. One year later, Zhang lab (Cong et al., 2013) and Church lab (Mali et al., 2013) have successfully used the system in engineering endogenous genome. In nature, there are six types of Cas proteins (Ran et al., 2015; Zalatan et al., 2015). Among these Cas proteins, Cas9 in type II group has been chosen for CRISPR-Cas system to conduct gene editing. This is because Cas9 participates in all three of the functional steps of CRISPR-based immunity thus the usage of it simplifies the CRISPR–Cas system (Ran et al., 2015).

CRISPR-Cas9 system has three key elements: a nuclease Cas9, a targeting CRISPR RNA (crRNA) and an additional trans-activating crRNA (Cong et al., 2013). If we plan to use this system in worms, we need to inject the 3 plasmids mixture into worms, which decrease the efficiency of the system. To obtain the higher efficiency in creating mutations, the 3' end of crRNA and 5' end of the trans-activating crRNA are fused together and form a single guide RNA (sgRNA). After this step, only two plasmids are needed. The sgRNA specifically targets a sequence in the form of G/A(N)19, which precedes a 3-nt (NGG) protospacer-adjacent motif (PAM) in the targeting sites. After the sgRNA recognizes the target sequence, the Cas9 protein is recruited to this site to generate double-stranded DNA breaks (DSBs). The DSBs will be repaired either by non-homologous end joining (NHEJ), which causes random deletions and/or insertions (InDels), or by homologous recombination, which has been used to generate oligonucleotide-based targeted gene editing in *C. elegans*.

In Chapter 3, I examined the localization of NMAT1, but there is no an allele of *nmat-1* existing to examine the gene functionally. I aimed to use the CRISPR/Cas9 system to create an allele of *nmat-1*. Such an allele would help us to understand the function of NMAT1 and the relationship between the Golgi/cytoplasmic and mitochondrial NAD+ pools in terms of gonad development.

Results

The original guide RNA vector purchased from Addgene contains *unc-119* guide RNA site, so it cannot be used for our experiment directly. To make the vector for a wider use, I modified the original guide RNA vector and changed the *unc-119* guide RNA site, which can be applied to various guide RNA designs (Figure 4-1). Two tandem BsaI sites were introduced to the guide RNA site as the core guide RNA vector. Then we can use this core guide RNA vector for any sgRNA design. But the primers should be: forward primer:TGTT$(N)_{20}$, and reverse primer: AAAC$(N)_{20}$. N_{20} presents the sgRNA. After annealing the forward primer and reverse primer together, we insert the annealed primers into the core guide RNA vector which has been digested by BsaI. The new plasmid would target to any desired target DNA as designed.

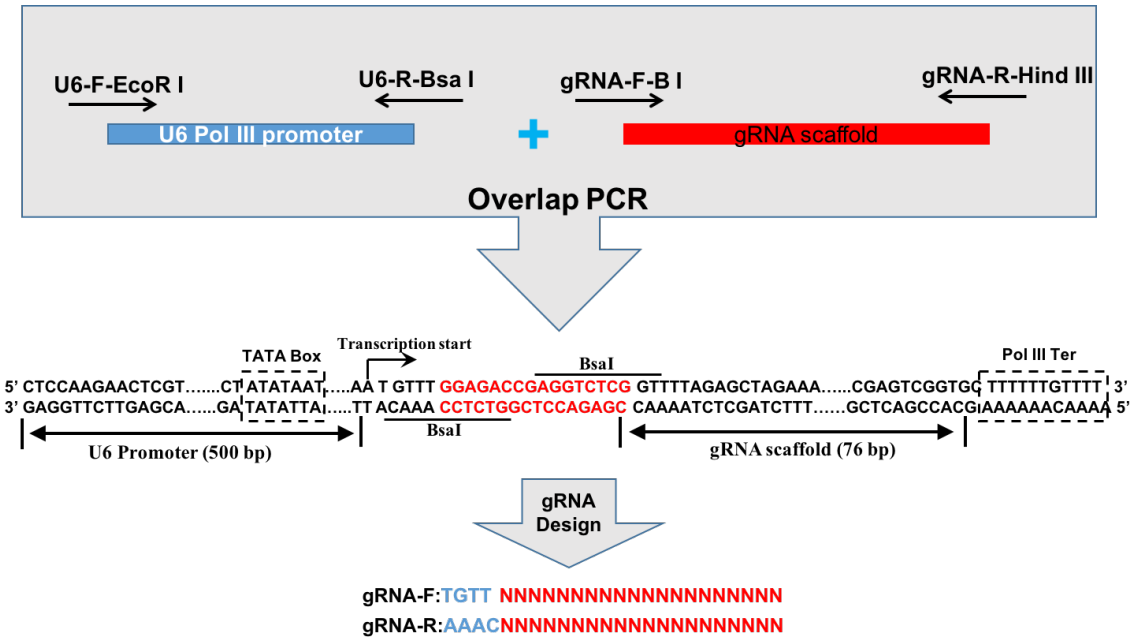


Figure 4-1 Design strategy for guide RNA vector.

Two tandem BsaI sites replace the original guide RNA *unc-119* site as the core guide RNA vector. The new core guide RNA vector can be applied for any sgRNA design. But the primers should be: forward primer:TGTT$(N)_{20,}$ and reverse primer: AAAC$(N)_{20}$. N_{20} presents the sgRNA. After

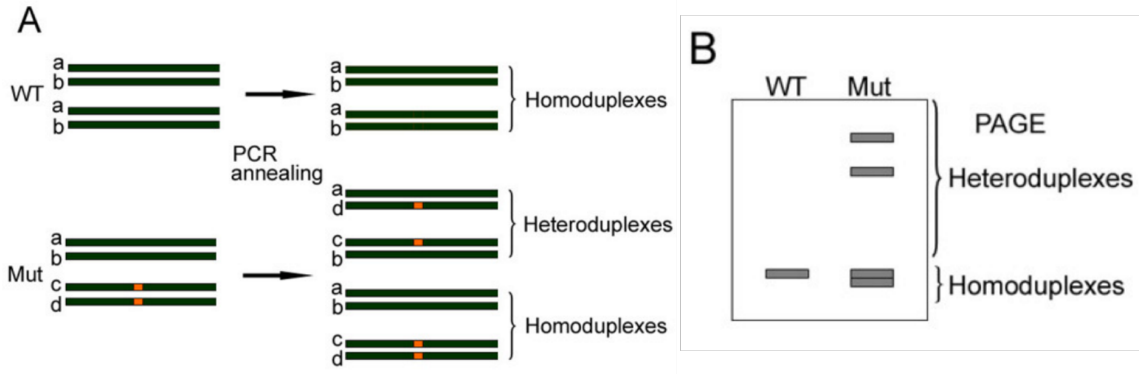
annealing the forward primer and reverse primer together, the annealed primers are inserted into the core guide RNA vector which has been digested by BsaI. The new formed plasmid would target to the target DNA.

To get *nmat-1* mutated worms, I injected the purified Cas9-sgRNA expression vectors mixture into the gonad of 30 F_0 wild-type worms, with a vector for expression of GFP using the ubiquitous promoter *sur-5*. The *sur-5* driven GFP is robustly expressed in nuclei of worms to marker transgenic F_1 progenies. After the microinjection, all of the worms survived and had progeny. Among the F_1 worms, 55 of them expressed green fluorescence. Because we do not know the phenotype of *nmat-1* mutants, we need to screen for mutations. I isolated the 55 green F_1 worms on OP50 plates, kept the F_2 generation and lysed the F_1 worm after in had progeny to identify the mutated strains.

To screen for mutations in these green worms, I applied an efficient genotyping method reported by Zhu Xiaoxiao et al. (Zhu et al., 2014). After I got F_1 worms lysate, I amplified about 1 kb of genomic DNA containing the sgRNA target site by PCR. Then I denatured and annealed the DNA fragments. If the DNA fragments contain InDels mutations, they would form heteroduplex DNA. However, the DNA fragments from wild-type alleles are still homoduplex DNA (Figure 4-2, A). In heteroduplex DNA, the two DNA strands cannot perfectly match to each other due to the InDels mutations, and this causes the formation of an open bubble on the sgRNA target site. Because of the open bubble, heteroduplex DNA have a significantly slower migration speed than homoduplex DNA, and during native 12% polyacrylamide gel electrophoresis (PAGE) heteroduplex DNA lagged behind homoduplex DNA (Figure 4-2, B).

After the 55 annealed DNA fragments and wild-type homoduplex DNA loading into polyacrylamide gel, the InDels mutations showed distinct migration rate on the gel. Notably, #22, #23, #49 and #52 annealed DNA fragments displayed different migration pattern from other DNA fragments (Figure 4-3), suggesting these 4 green worms probably contain InDels mutations at *nmat-1*.

To confirm this, I amplified the genomic DNA containing the sgRNA target site again with PCR and purified the PCR products before sending to sequence. The sequence results showed that #52 have an InDels mutation at 3-nt preceding the PAM position (Figure 4-4).



Zhu et al., 2014

with permission

Figure 4-2 Cartoon of screening of CRISPR/Cas9-mediated mutations by PAGE assay.

(A) Illustration of heteroduplex DNA formation during denaturation and annealing. Dark green bars represent four DNA strands (a–d) in cells harboring monoallelic mutations (orange box). After denaturation and annealing, two types of homoduplex DNA and two types of heteroduplex DNA were formed. (B) Identification of heteroduplex DNA fragments by PAGE assay. heteroduplex DNA migrates slower than homoduplexes due to formation of bubble between matched and unmatched genomic regions. Figure and legend taken from Zhu, X., Xu, Y., Yu, S., Lu, L., Ding, M., Cheng, J., Song, G., Gao, X., Yao, L., Fan, D., Meng, S., Zhang, X., Hu, S. and Tian, Y. (2014) An efficient genotyping method for genome-modified animals and human cells generated with CRISPR/Cas9 system. *Scientific reports* 4, 6420, with permission.

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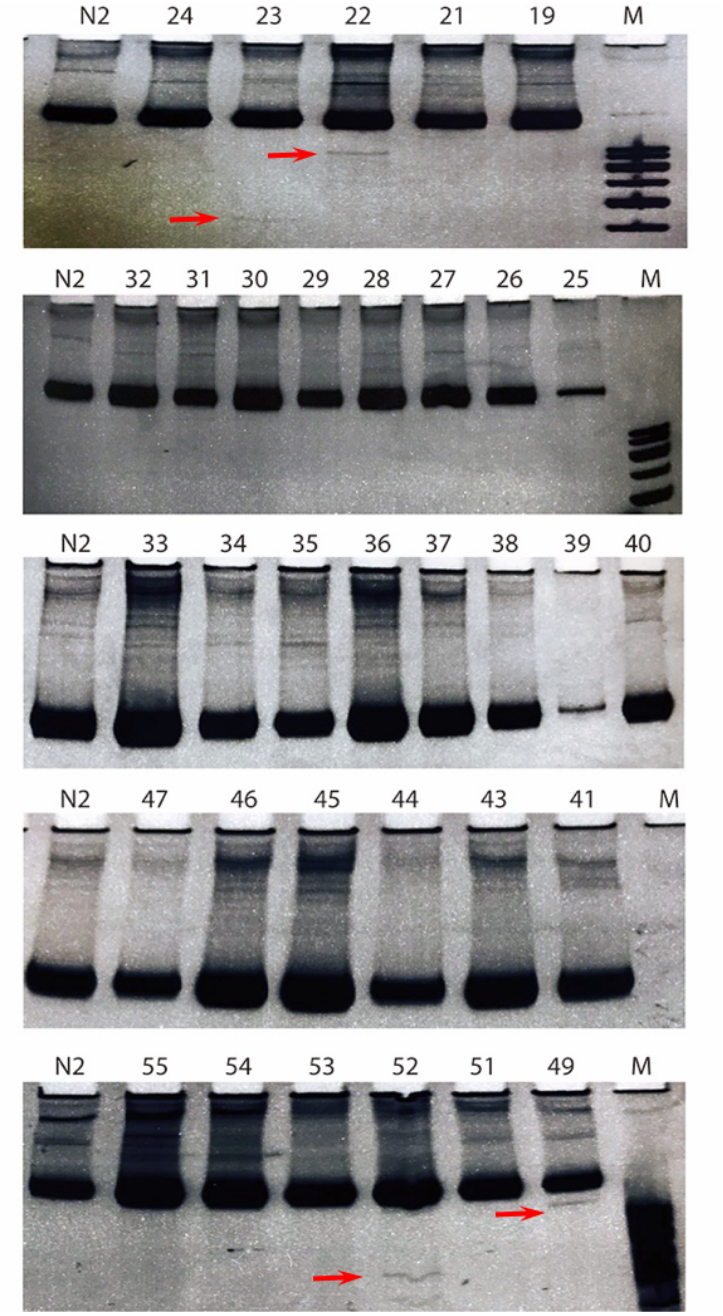

Figure 4-3 Screen *nmat-1* mutations by PAGE assay.

InDels mutations in these worms are analyzed by 12% native polyacrylamide gel. #22, #23, #49 and #52 annealed DNA fragments from correlating worms showed different migration pattern from other DNA fragments. Red arrows indicate banding patterns that differ from wild type. M, DNA ladder.

nmat1 sequence

mutant sequence

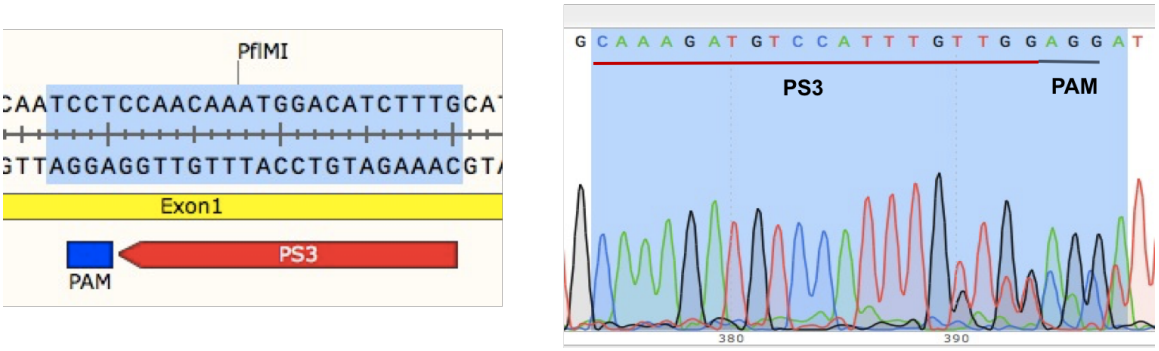

Figure 4-4 Detection of CRISPR/Cas9-mediated genome-modified *C. elegans* targeting *nmat-1* by sequencing.

Left， sequence of target site. Blue bar presents PAM, and red bar presents sgRNA. Right, mutant sequence result. Blue line presents PAM, and red line presents sgRNA.

Discussion

Before I applied the CRISPR/Cas9 system in worms, I edited the purchased guide RNA vector which only target to *unc-119*. Two tandem BsaI sites replaced the *unc-119* sgRNA (Figure 4-1). This kind of modification will allow us to use the vector for any sgRNA.

I got 55 F_1 green worms after I injected the CRISPR/Cas9 system plasmid mixture into 30 F_0 wild type worms. To screen for InDels mutations, I used native PAGE assay to analysis the F_1 green worms. #22, #23, #49 and #52 annealed DNA fragments from correlating worms showed different migration pattern from other DNA fragments (Figure 4-3). The results showed that probably these worms contained InDels mutations. There is another strategy to screen InDels mutations, which the designed sgRNA site need to overlap with the restriction enzyme site, but this kind of strategy has lots of limitation. The native PAGE analysis is faster and cheaper than restriction enzyme screening. So I used native PAGE analysis instead of restriction enzyme screening here.

To further test whether these worms contain InDels mutations, I purified the PCR products and sequenced them. If CRISPR/Cas9 system successfully creates a mutation, there will be a mutation starting from 3-nt preceding the PAM position. Our sequencing results showed the CRISPR/Cas9 system only introduce a mutations in #52 worm and this worm is a heterozygous (Figure 4-4).

I attempted to find homozygous *nmat-1* mutants from #52 worm progeny. 20 F_2 worms were randomly picked and screened for mutant phenotypes. The F_2 worm lysates served as PCR templates and the genomic DNA containing the sgRNA target site was amplified with PCR. Then the PCR products was purified and sent to sequence. However, the sequencing data showed that all of the F_2 worms are still wild-type worms. There are two possibilities to explain the phenomena. The first one is the reversed mutation occurred to fix the mutation, but the chance for this event is rare. The other possibility is *nmat-1* mutation is harmful for worms. When I design guide RNA for *nmat-1*, I chose the guide RNA with a GG motif at the 3' end. The strategy has been successfully applied in worms, which improved editing frequencies from 0.5 to 80% (Farboud and Meyer, 2015). According to the report, among the 55 green F_1 worms, 44 of them would carry mutations.

However, our sequence data showed that only one worm had *nmat-1* mutation. So, I predict that even the heterozygous *nmat-1* mutant is not very viable. The other reason to support this hypothesis is mitochondrial NAD+ pool is particularly important for healthy metabolism in cells and depletion of the pool results in cell death (Alano et al., 2004; Chiarugi and Moskowitz, 2002; Kristian et al., 2011; Pieper et al., 1999). Our data in Chapter 3 showed that NMAT1 locates in mitochondria, so *nmat-1* mutant would have a disrupted mitochondrial NAD+ pool, which ultimately causes the lethal phenotype. To figure out the real reason behind it and obtain the mutant, I need to inject more worms to test my hypothesis.

The nature of the allele will help us to discover the relationship between the nuclear/cytoplasmic and mitochondrial NAD+ pools in terms of gonad development and will be a useful tool to probe other functions of NAD+ biosynthesis as well.

Material and Methods

Maintenance of *C. elegans*

C. elegans are cultured at 20˚C feeding with *E. coli* strain OP50 (Brenner, 1974). N2 is the wild-type strain. The maintenance method is mentioned in chapter2.

Plasmid construction

Cas9-SV40 NLS expression vector (Peft-3::cas9-SV40_NLS::tbb-2 3'UTR, 46168) and PU6::unc-119_sgRNA vector (46169) were purchased from Addgene. PU6::unc-119_sgRNA vector need to modify for our experiment.

To introduce BsaI site for wider use for other sgRNA, U6 promoter was amplified by Forward primer: GCGAATTCCTCCAAGAACTCGTACAAAA.
Reverse primer: ACCGAGACCTCGGTCTCAAACATTTAGATTTGCAATT.
And gRNA scaffold was amplified by Forward primer: TTGAGACCGAGGTCTCG GTTTTAGAGCTAGAAATA.
Reverse primer: CCAAGCTTCACAGCCGACTATGTTTGGC.

Then overlapping PCR U6 promoter and gRNA scaffold by U6 promoter Forward primer and gRNA scaffold Reverse primer. The overlapping PCR products were digested with EcoRI (NEB) and HindIII (NEB) and then inserted into the PU6::unc-119_sgRNA vector that has been digested with EcoRI and HindIII, creating a core guide RNA vector. However, there are other two BsaI sites in the vector. So, I used QuikChange Multi Site-Directed Mutagenesis Kit (Agilent Technologies) and the primers T437C: AAAATGCTCTGAAGTAGGCCTCGAGATC, and A2562G: gcaatgataccgcgggacccacgctcacc to remove the extra two BsaI sites.
nmat1-PS3-F: TTTG CAAAGATGTCCATTTGTTGG
nmat1-PS3-R: GTTT CCAACAAATGGACATCTTTG

DNA microinjection

DNA mixture was microinjected into the germ line of the young adult worms using standard method described previously (Mello and Fire, 1995). The mixture contains a final concentration of Peft-3::cas9-SV40_NLS::tbb-2 3'UTR at 50 ng/ μL, pU6::nmat1 sgRNA at 45 ng/ μL, P_{sur5}::gfp at 20 ng/ μL. P_{sur5}::gfp plasmid is used as maker to test the success of microinjection. Each time, I injected about 30 young adult worms with the DNA mixture. The microinjection method is according to (Mello and Fire, 1995). After the DNA injection, every 5-6 worms are suspended in M9 salt solution to recover on a new OP50 plates at 20˚C. Pick green F1 progeny under GFP microscopes (Zeiss) and put them on OP50 plates individually to screen for stable transgenic strains.

Native PAGE analysis

Worms are lysate by worm lysis buffer (10X PCR buffer 100 μL, Proteinase K 14 μL, add H_2O to 1 ml). Each worm uses 10 μL to yield worm lysate (-80˚C for 15min, 65˚C for 60min, 95˚C for 15min. Or 50˚C for 13hour, 80˚C for 20min). Then PCR the target fragment (95˚C for 30sec, 56˚C for 30sec, 72˚C for 1min, 35X cycles). Denature the PCR products at 95˚C for 10min and cool it down to room temperature to anneal the DNA fragments at 0.1˚C/s speed.

Load the annealed PCR products into 12% native polyacrylamide gel by electrophoresis (acrylamide-bisacrylamide (29:1, w/w), 5X Tris-acetate-EDTA (TAE), ammonium persulfate, and TEMED). After about 2 hours of electrophoresis at 200 V, 30 mA, the 12% native polyacrylamide gel is immersed into ethidium bromide (EB) solution for about 20 minutes before visualization by using Imaging System (Biorad).

CHAPTER 5

Summary

The molecule NAD+ is found in all organisms. It serves as a cofactor for numerous enzymes to fuel redox reactions and as substrates for its consumers, such as the sirtuins and PARPs. Its homeostasis is critical for life span, neurodegenerative diseases and circadian rhythm. However, the mechanism that NAD+ influences development is still unknown.

Our lab has found that blocking salvage NAD+ biosynthesis affects gonad development in *C. elegans*. To explore the mechanisms, I used the *C. elegans pnc-1(pk9506*) mutant as a model, in which the salvage NAD+ biosynthesis has been blocked. I supplied the *pnc-1*(*pk9605*) mutants with glycolytic intermediates 3PG and PEP, which are after the steps using NAD+. These intermediates increased the percentage of normal gonad development in the *pnc-1* population, suggesting disrupted glycolysis lead to the gonad developmental defects.

Eukaryotic cells have relatively separated NAD+ pools, the conclusion comes from the NAD/NADH ratios: cytoplasmic NAD/NADH ratio range is from 60 to 700, but the mitochondrial NAD/NADH ratio is around 7 (Supale et al., 2012). NMNATs regulate these NAD+ pools to meet specific requirements of NAD+ in cells. I would like to use NMNAT function to investigate the compartment specific requirements for NAD+ biosynthesis in *C. elegans* gonad development. I fused *nmat-1* and *nmat-2* to CFP under the muscle specific promoter *myo-3* and examined their subcellular localization in muscle cells. The subcellular localization experiment results showed that NMAT1 is a mitochondrial protein and NMAT2 is a Golgi protein. Our lab has found that *nmat-2* mutants showed severe gonad delay phenotype. So, we predict that cytoplasmic NAD+ is critical for *C. elegans* gonad development. To further explore the function of NMNAT, I applied the CRISPR/Cas9 system to create an allele of *nmat-1*. Now, I am screening for homozygous or heterozygous *nmat-1* mutants.

This work discussed why fix disrupted NAD+ rescue gonad development defects. It will provide us the insights on metabolic products function in animal's development

and also will represent a new therapeutic opportunity for human developmental diseases that are associated metabolism.

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Appendix

Investigation of molecular mechanism of ADSL deficiency-related locomotion in *C. elegans*

Abstract

Purines are a group of important molecules ubiquitously present in all cells that serve many essential biochemical processes. Deficiencies in purine metabolism are associated with a variety of physiological and neurological diseases. Among these deficiencies, ADSL deficiency associates with wide spectrum of symptoms between human patients. There is no effective treatment for ADSL deficiency patients, because we are just now beginning to elucidate the mechanisms of this disease. We found that *adsl-1* mutant worms showed motility problems like human ADSL deficiency patients. This led us to use *C. elegans* as a model to study ADSL disorder, which is caused by locomotion defects and explore the mechanism of the syndromes.

The overarching hypothesis of my study is to investigate how ADSL deficiency causes locomotion defects. The purpose of this study is to understand the mechanism of metabolic disorders of purine metabolism affecting locomotion. My project seeks to solve three questions: 1. Test which tissue function would be impaired by lack of ADSL; 2. Identify the metabolites (accumulating substrate or insufficient product) in *adsl-1* mutant that cause locomotion defects and 3. Investigate hypothesis for molecular mechanism of thrashing deficiency in *adsl-1* worms.

My research will contribute to the understanding of mechanism of ADSL disorder and offer a new strategy for treatment of purine disorder related diseases.

INTRODUCTION

Purine Biosynthesis Pathways

Purine metabolism plays an important role in cells and has diverse biological functions. Purine nucleotides can be synthesized through two pathways (Figure A-1). One is the de novo synthesis pathway, which uses CO_2, glycine, and glutamine as precursors to build the phosphorylated ring (Nyhan, 2005). The other pathway is the salvage pathway that reuses a phosphorylated ring in a catabolic manner. The compound inosinic acid (IMP) is the hub for these two pathways, and can be converted into either guanine or adenine nucleotides. IMP is synthesized via a ten-step process during de novo synthesis. The first step is that 5-phospho-α-ribosyl-1-pyrophosphate (PRPP) is catalyzed to 5-PRA by PRPP amidotransferease; this is a rate-limiting step of purine metabolism. The enzyme phosphoribosylglycinamide formyltransferase (GART) participates in steps 2, 3, and 5. As GART, phosphoribosyl aminomidazole succinocarboxamide synthetase (PAICS) shows muti-functions, it catalyzes steps 6 and 7, and IMP cyclohydrolase (ATIC) catalyzes steps 9 and 10. The enzymes involved in the rest of the steps are phosphoribosyl formylglycinamidine synthase (FGAMS) in step 4 and adenylosuccinate lyase (ADSL) in step 8 (Alexiou and Leese, 1992; Fang et al., 2013; Watts, 1983).

Disorders of purine metabolism cause a wide spectrum of clinical syndromes in humans, some of the severe phenotypes are lethal (Jurecka, 2009). Although these clinical presentations vary among patients, these presentations are mainly on developmental and neurological problems (Alexiou and Leese, 1992; Jinnah et al., 2013; Nyhan, 2005; Watts, 1983). However, purine metabolism disorders attract limited attention from the medical and research communities. The reasons mainly lie in two aspects: First, the low prevalence among the population (Jurecka, 2009; Nyhan, 2005). Second, extreme challenges for medical diagnosis, the purine disorder brings variable symptoms, for example, more than 14 different disorders have been reported (Jurecka, 2009; Nyhan, 2005). Although there are some ‘effective’ therapeutic treatments for purine disorders, these treatments cause gout. So it is imperative to explore the pathophysiology of purine disorder, and find new effective therapeutic method without side effects for this disease.

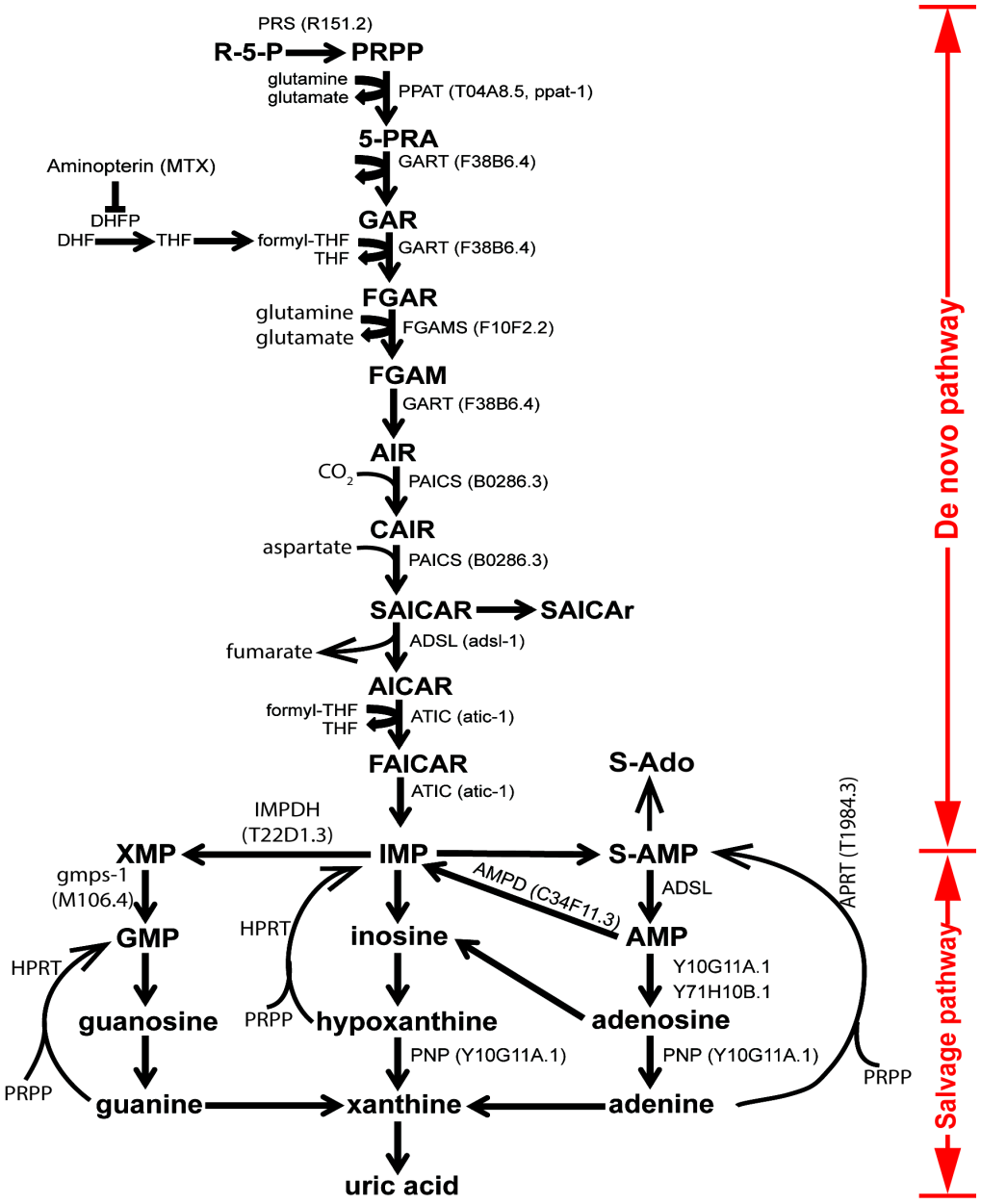

Figure A-1 Purine de novo synthesis pathway and salvage pathway.

Purine nucleotides can be synthesized through two pathways. One is the de novo synthesis pathway, which uses CO_2, glycine, and glutamine as precursors to build the phosphorylated ring. The other pathway is the salvage pathway that reuses a phosphorylated ring in a catabolic manner. The compound IMP is the hub for these two pathways, and can be converted into either guanine or adenine nucleotides. IMP is synthesized via a ten-step process during de novo synthesis. The first step is that PRPP is catalyzed to 5-PRA by PRPP amidotransferease; this is a rate-limiting step of purine metabolism. The enzyme GART participates in steps 2, 3, and 5. As GART, PAICS shows muti-functions, it catalyzes steps 6 and 7, and ATIC catalyzes steps 9 and 10. The enzymes involved in the rest of the steps are FGAMS in step 4 and ADSL in step 8.

Abbreviations:R-5-P, Ribose 5-phosphate; PRPP, 5-phosphoribosyl-1-pyrophosphate; 5-PRA, 5-phospho-d-ribosylamine; GAR, glycinamide ribonucleotide; FGAR, N-formylglycinamide ribonucleotide; FGAM, N-formylglycinamidine ribonucleotide; AIR, aminoimidazole ribonucleotide; CAIR, carboxyaminoimidazole ribonucleotide; SAICAR, N-succinocarboxamide-5-aminoimidazole ribonucleotide; AICAR, aminoimidazole-4-carboxamide ribonucleotide; FAICAR, 5-formamido-4-imidazolecarboxamide ribonucleotide; IMP, inosine monophosphate; XMP, Xanthosine monophosphate; GMP, Guanosine monophosphate; S-Ado, succinyladenosine; S-AMP, adenylosuccinate; AMP, adenosine monophosphate; THF, tetrahydrofolate; PRS, Ribose-phosphate diphosphokinase; PPAT, glutamine phosphoribosylpyrophosphate amidotransferase, GART, phosphoribosylglycinamide formyltransferase; FGAMS, phosphoribosylformylglycinamide synthase; PAICS, aminoimidazole ribonucleotide carboxylase; ADSL, adenylosuccinate lyase; ATIC, 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase; HPRT, Hypoxanthine-guanine phosphoribosyltransferase; PNP, purine nucleotide phosphorylase, IMPDH, IMP dehydrogenase; APRT, adenine phosphoribosyl transferase; MTX, Methotrexate.

ADSL Deficiency

ADSL catalyzes two steps in the purine synthesis pathways: in the de novo pathway, it converts succinylaminoimidazolecarboxamide ribose-5'-phosphate (SAICAR) to AICAR and produces fumarate. Also, in the salvage pathway, it cleaves adenylosuccinate into AMP and fumarate. The human ADSL protein is a ~50 kDa homotetramer (Figure A-2). This protein has three domains and its catalytic domain is on C-terminus (Kmoch et al., 2000). Recent research indicates that the severity of the ADSL deficiency is related to ADSL enzymatic activity and stability (Kmoch et al., 2000; Ray et al., 2013).

ADSL deficiency is associated with large heterogeneity between human patients (Jurecka et al., 2015). Based on the levels of severity, the disease can be cataloged into three forms: severe type I form, milder type II form and fatal neonatal form (Jinnah et al., 2013; Jurecka et al., 2008b). Patients suffering from type I form of ADSL deficiency show severe psychomotor retardation, microcephaly, developmental arrest, and patients suffering from type II form of ADSL deficiency show ataxia, hypotonia and gait disturbance (Jurecka et al., 2012a; Jurecka et al., 2012b; Jurecka et al., 2015). Patients suffering from fatal neonatal form are lack of spontaneous movement, seizures and respiratory failure, and they will die within first weeks of lives (Jurecka et al., 2012a; Jurecka et al., 2012b; Jurecka et al., 2015). Patients suffering from ADSL deficiency

reported with motility problems, like hypokinesia, ataxia, arthrogryposis, gait disturbance (Jinnah et al., 2013).

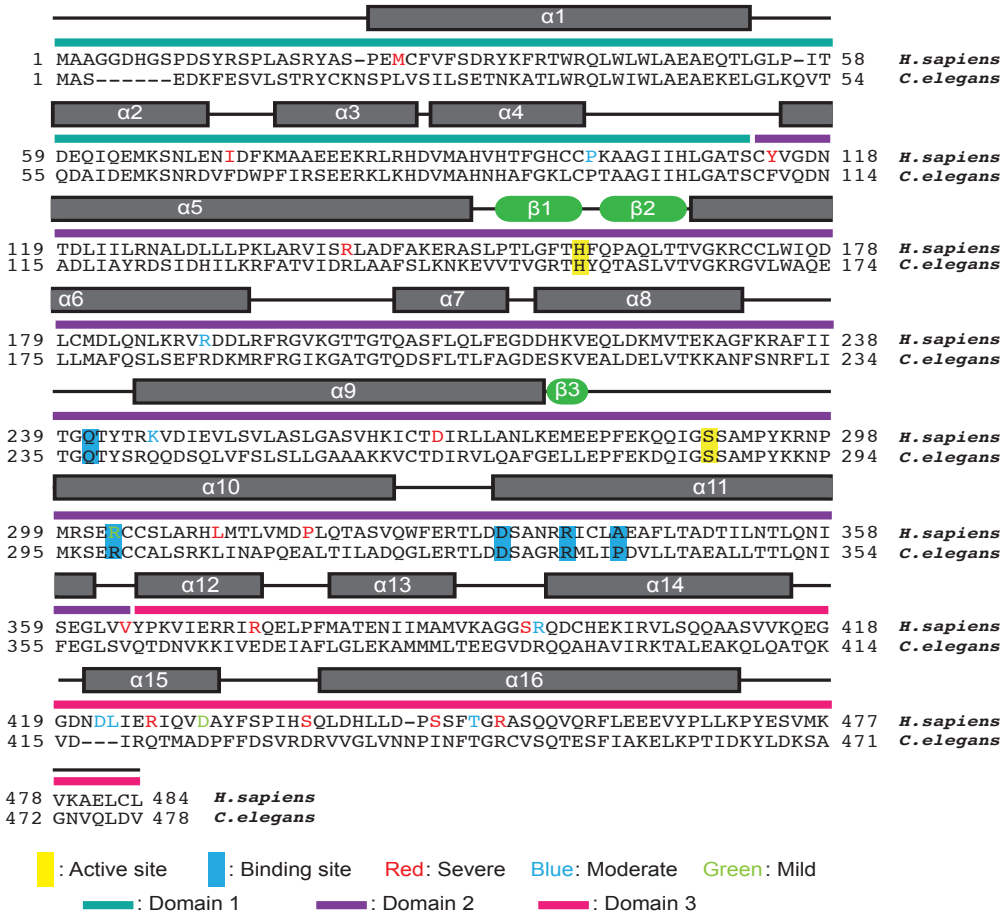

Figure A-2 Alignment of human ADSL protein and *C. elegans* ADSL-1 protein.

Black rectangles indicate the secondary structure of *C. elegans* ADSL-1 protein.

For the ADSL disorder study, I would like to use *C. elegans* as a model. The reason is that *C. elegans* is a good model for neurons research. It only contains 302 neurons and the main interactions between them have been well studied (Calahorro and Ruiz-Rubio, 2011; White et al., 1986). As a result, *C. elegans* can simplify the complex neurological diseases of human and offer a fundamental conserved model for the disease. In addition, *C. elegans* is a perfect system for muscle disease. The muscle cells in *C.*

elegans cannot regenerate (Carre-Pierrat et al., 2006; Gieseler et al., 2000), so quantification of the functional muscle cells is easily detectable.

The most interesting part of our research on ADSL is that we found similar locomotion deficiency phenomenon in *C. elegans*. Compared to the wild-type worm's thrashing rate, *adsl-1* knockout mutants have a slower thrashing rate when suspended in M9 solution (Figure A-3) and they are uncoordinated.

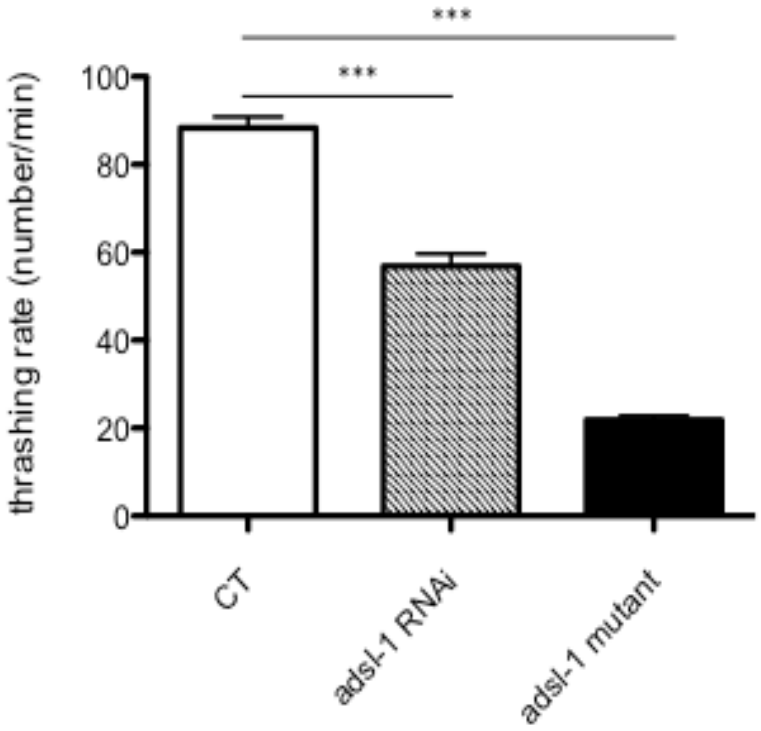

Figure A-3 Thrashing rate in wide-type worm, adsl-1 RNAi worms and null adsl-1(tm3328) mutant.

Thrashing rate significantly decreased in adsl-1 RNAi worms and null *adsl-1(tm3328)* mutant. Error bars are S.E. ***, $p < 0.001$, calculated using Student's t-test.

According to our preliminary data and literature, I propose three specific aims for my project:

Which tissue function, neurons and muscle, would be impaired by lack of ADSL?

Locomotion requires neurons and muscle cells to coordinate with each other; therefore, dysfunction of one type of these cells will cause the abnormal thrashing phenotype. Because thrashing requires muscular and neuronal functions, I want to test whether the thrashing defect in *adsl-1* knockout mutant is caused by lack of ADSL-1 function in muscle or neurons.

Experimental Strategy:

First, I will specifically knockdown (KD) *adsl-1* in neuron or muscle and examine which kind of worms would show thrashing defects and then I will restore ADSL-1 in neuron or muscle in *adsl-1* mutant worms to check which transgenic strain has rescued thrashing defects.

Results

NR350 worms are used as muscle specific RNAi worms (Qadota et al., 2007). To knock down ADSL level in muscle cells, I fed NR350 with *adsl-1* RNAi, and found that thrashing rate decreased slightly but the difference is significant in statistical test in RNAi animals (Figure A-4, A). This result suggests that lack of ADSL-1 in muscles leads to locomotion deficiency. TU3311 worms are considered as neuron-specific RNAi strain (Calixto et al., 2010), so supplying the worms with *adsl-1* RNAi specifically knocked down ADSL level in neuron. However, neuron specific ADSL-1 KD worms (TU3311) have similar thrashing rate to wild-type animals (Figure A-4, B), suggesting lack of ADSL-1 in neurons is not relevant to thrashing defects. The data indicated that the locomotion phenotype in *adsl-1* mutants arise from autonomous lack of ADSL-1 in muscles.

To further investigate my hypothesis, I tried to restore ADSL-1 protein in muscle cells or neuronal cells in null *adsl-1* animals and check whether locomotion defect could be rescued in these transgenic worms. I attempted to use muscle specific promoter (*myo-3*) and pan-neuron specific promoter (*unc-14*) (Ogura et al., 1997; Wolkow et al., 2000) to express ADSL-1 in muscle and neurons respectively in null *adsl-1*(*tm3328*) mutants. The

adsl-1 mutants we use here is the strain tm3328, in which the third exon is deleted. ADSL-1::CFP driven by promoter *myo-3* partially rescued thrashing defects (Figure A-5). However, the partial rescue result is probably due to the mosaic expression pattern of ADSL-1::CFP in the muscle cells. Not all of the muscle cells have been restored with ADSL-1 protein (data not shown).

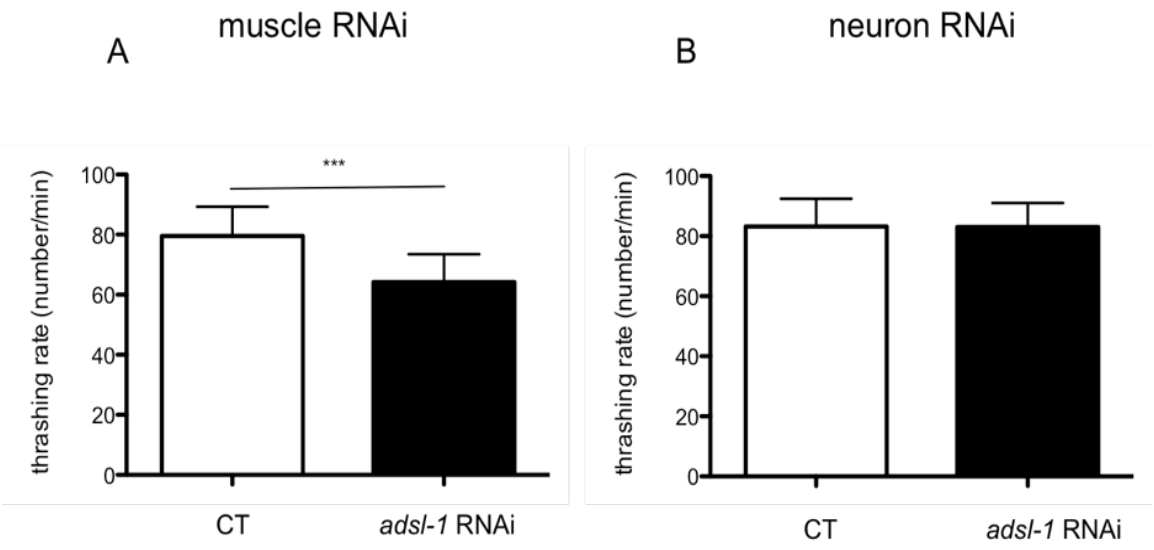

Figure A-4 Knocking down ADSL-1 in muscle affects locomotion.

(A) Thrashing assay showing muscle specific RNAi worms feed with *adsl-1* RNAi demonstrated slower thrashing rate than the worms fed with control RNAi. In histograms, error bars are S.E. ***, $p < 0.001$, calculated using Student's t-test. (B) Thrashing assay showing neuron specific RNAi worms feed with *adsl-1* RNAi demonstrated similar thrashing rate to the worms fed with control RNAi.

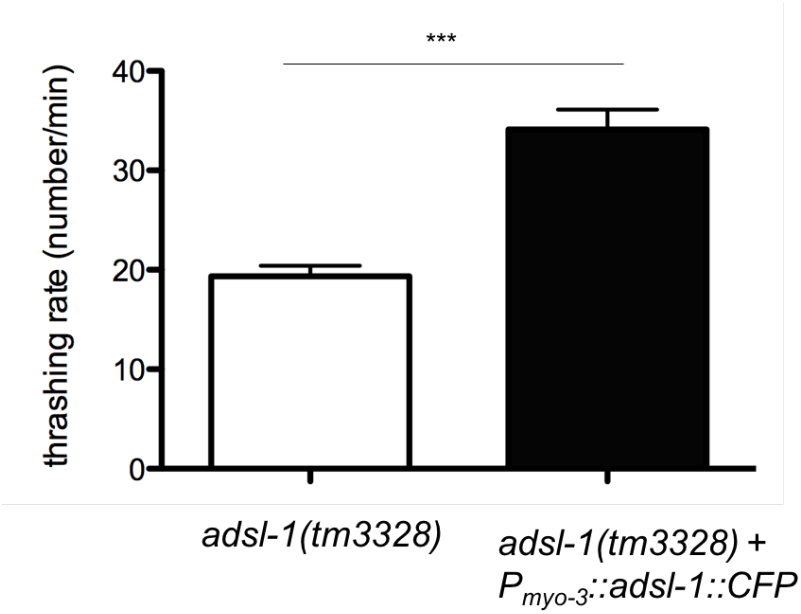

Figure A-5 Restoring ADSL-1 in muscle rescued thrashing defects in *adsl-1(tm3328).*

In histograms, error bars are S.E. ***, $p < 0.001$, calculated using Student's t-test.

Discussion

Locomotion requires two types of cells – neurons and muscle cells to coordinate with each other. So, dysfunction of either one type of the cells will results in the abnormal locomotion. To test which tissue function would be impaired by lack of ADSL, I specifically knockdown *adsl-1* in muscle or neuron by using muscle-specific RNAi worms (NR350) and neuron-specific RNAi worms (TU3311). Thrashing rate is decreased in muscle-specific RNAi animals (Figure A-4, A). This result suggests that lack of ADSL-1 in muscles leads to locomotion deficiency. However, neuron-specific RNAi worms have similar thrashing rate to wild-type animals (Figure A-4, B), suggesting lack of ADSL-1 in neurons is not relevant to thrashing defects. The data indicated that the locomotion phenotype in *adsl-1* mutants arise from autonomous lack of ADSL-1 in muscles.

I tried to restore ADSL-1 protein in muscle cells, however, the ADSL-1::CFP driven by promoter *myo-3* partially rescued thrashing defects (Figure A-5). Then I checked the ADSL-1::CFP expression pattern in muscle cells and found that the expression of ADSL-1::CFP lost in some of the muscle cells. Therefore, probably the mosaic expression of ADSL-1::CFP driven by promoter *myo-3* cannot have normal thrashing rate as wild-type worms. To solve the problem, we can inject the plasmid to worms again and obtain the transgenic worms which have ADSL-1::CFP in all muscle cells or integrate the plasmid into worms' genomic DNA. Also I will restore neuron ADSL-1 in *adsl-1* mutant background to further confirm that thrashing defects of P_{unc14}::adsl-1 *adsl-1* mutant worms could not be rescued and thrashing rate should be similar to *adsl-1* mutant.

Our data indicated that the abnormal thrashing phenotype in *adsl-1* mutants is caused by lack of ADSL-1 in muscle cells, suggesting loss of ADSL-1 damages the proper function of muscle cells in adsl-1 mutants. Adam Fenton in our lab tested the function of muscle cells in *adsl-1* mutants by levamisole. Levamisole can be used to examine the function of muscle cells while bypassing the neurons. It specifically binds to acetylcholine receptors on muscle cells and which leads to a hyper-contracted paralysis of worms (Lewis et al., 1980a; Lewis et al., 1980b), and therefore by monitoring the time-course of paralysis, we can analyze physiological function of muscle cells. He found that *adsl-1* mutants are more resistant to levamisole than wild-type worms. After he put wild-type worms in 2 mM levamisole solution, the average paralysis time of wild-type worms is 330s. Comparing to wild-type worms, *adsl-1* mutants are more resistant to levamisole, they take 680s to be paralyzed (Data not shown). This result also demonstrated that the function of muscle of *adsl-1* mutants is impaired.

Is the thrashing deficiency in *adsl-1* worms caused by accumulating substrates or impaired synthesis of purine nucleotides?

ADSL-1 deficiency would lead to two results: accumulating substrates and impaired synthesis of purine nucleotides, and either of the results could cause the locomotion defect in *adsl-1* worms. I plan to investigate which one is the cause for the thrashing deficiency in *adsl-1* worms.

In human, the pathophysiological mechanism of ADSL deficiency has not been deciphered yet, some people have suggested that neuron/muscle dysfunction caused by ADSL deficiency is due to insufficient purine nucleotides. Because some reports showed that purine nucleotides supplements relieved the syndrome, but the effects are not sustained (Jinnah et al., 2013; Jurecka et al., 2008a). But some data suggested accumulating SAICAR/ SAICAr causes the dysfunction of neuron/muscle. It has been found that dephosphorylated ADSL's substrates (SAICAR and S-AMP), succinylaminoimidazole-carboxamide riboside (SAICAr) and succinyladenosine (S-Ado), are accumulated in patients' body fluids (Jurecka et al., 2015). In type I form patients S-Ado/SAICAr ratio is around 1. In type II form patients, although the level of SAICAr is similar to type I form patients, S-Ado level is higher, so the S-Ado/SAICAr ratio is more than 2. In neonatal fatal patient, the S-Ado/SAICAr ratio is less than 1. The data suggested that SAICAr is toxic, but S-Ado may have protection effect from SAICAr. In 2010, Marie Zikanova et al. (Zikanova et al., 2010) analyzed 14 patients' ADSL mutant proteins, and they found that the affinities and the proportional catalytic activities between the substrates and mutant proteins didn't change. These findings indicated that S-Ado/SAICAr ratio is not cause of severity level, but the result of severity level. All the literature suggested that the accumulation of SAICAR/SAICAr might attribute to the severity level.

According to the literature, I favor hypothesis that thrashing deficiency in *adsl-1* worms is due to toxic effects of accumulating SAICAR, not insufficient purine nucleotides. But I will investigate both hypotheses.

In order to investigate whether decreased purine in adsl-1 worms is the related to locomotion defects, Wenqing in our lab tried to increase purine in *adsl-1* knock-down worms by feeding *adsl-1* RNAi worms with purine salvage pathway intermediates hypoxanthine or adenosine (0.5-1 mM) (Data not shown). Thrashing defects cannot be rescued in these worms. However, the sterility phenotype of ADSL-1 KD worms can be rescued. This result suggested that sterility phenotype, not thrashing defect, is caused by impaired synthesis of purine nucleotide. She also tried to decrease purine level in wild-type by individually suppressing three purine biosynthesis genes (*ppat-1*, F10F2.2

(FGAMS), and B0286.3 (PAICS)) with RNAi. These genes are above ADSL-1 in the de novo pathway, and knocking down these genes should reduce purine level without increasing SAICAR in worms. However, in these RNAi worms no obvious thrashing deficiency phenotypes were found (Data not shown). These results suggested that reduced concentration of purine nucleotides is insufficient to cause locomotion deficiency in worms.

To investigate the relationship between SAICAR and locomotion. Wenqing supplemented *adsl-1* RNAi worms with aminopterin or methotrexate (MTX) to block accumulation of SAICAR. Both of them block the formation of 5'-phosphoribosyl-N-formylglycineamide (FGAR), an upstream intermediate of purine biosynthesis leading to a block prior to production of SAICAR. The drugs rescued thrashing defects in *adsl-1* RNAi worms (Data not shown). These data suggested that elevated SAICAR caused thrashing deficiency in *adsl-1* RNAi worms.

ADSS-1 is the downstream enzyme of ADSL-1, and knock down this gene with RNAi could decrease S-Ado and affects ratio of SAICAr to S-Ado. *adss-1* RNAi worms showed same phenotype as *adsl-1* RNAi worms (Data not shown), which suggested decreasing S-Ado affects ratio of SAICAr to S-Ado and that changed ratio of SAICAr to S-Ado is relevant to locomotion defects.

All our results indicated that the accumulating SAICAR is related to worm locomotion defect, SAICAR/SAICAr is the toxic intermediate for thrashing deficiency in *adsl-1* worms, and S-Ado have the protective effect from SAICAR/SAICAr.

Experimental Strategy:

Genetic or chemical approaches will be used to change the SAICAR levels or purine level in both *adsl-1* mutant and wild type worms and then examine whether these metabolites changes affect muscle/neuron function. Moreover, SAICAR, S-Ado and purine levels will be evaluated by LC-MS/MS to confirm the effectiveness of the approaches in each experiment.

Results

All of Wenqing's results indicated that accumulation of SAICAR is harmful for worm locomotion. I further tested this hypothesis by *atic-1* RNAi. In purine de novo synthesis pathway, the enzyme ATIC is also a good target to test our hypothesis. Because ATIC is right downstream enzyme of ADSL, suppress the expression of this enzyme may increase SAICAR and decease S-Ado that would change the ratio between SAICAR and S-Ado in worms. the *atic* RNAi worms would mimic the phenotype of *adsl-1* RNAi and. After I constructed the *atic* RNAi plasmid to decrease ATIC level in worms. The *atic* RNAi worms mimiced the thrashing phenotype of *adss-1* RNAi worms and *adss-1* RNAi worms (Figure A-6). The result indicated that SAICAR/SAICAr is the toxic intermediate for thrashing deficiency in adsl-1 worms, and S-Ado have the protective effect from SAICAR/SAICAr.

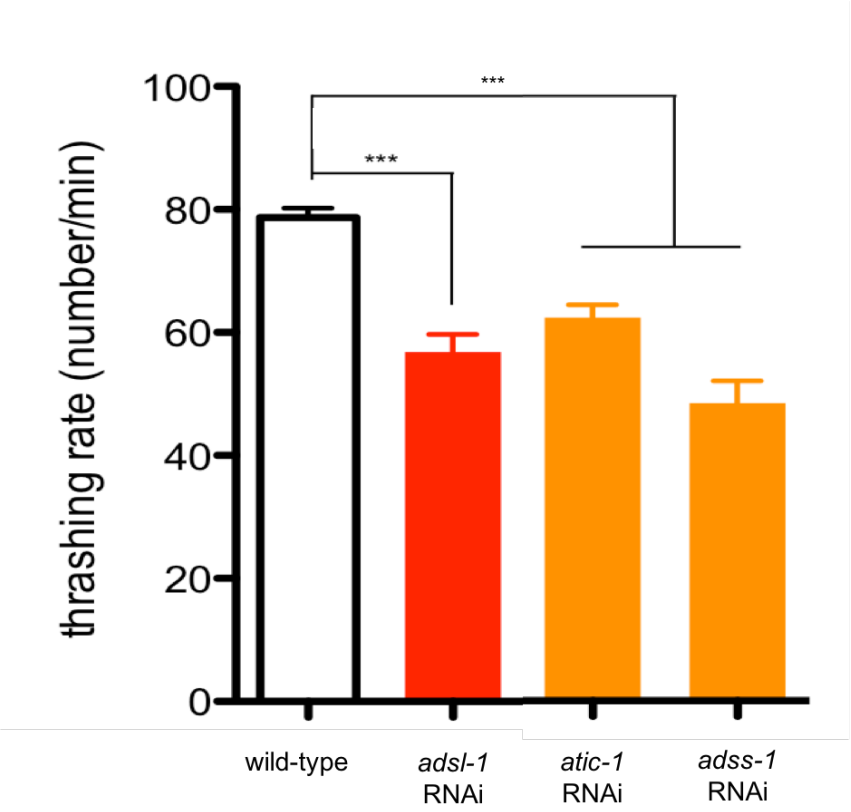

Figure A-6 *atic-1* RNAi effect on thrashing rate.

Knocking down *atic-1* with RNAi decreased the thrashing rate in worms. ADSS-1 & ATIC-1 are the downstream of ADSL-1, and knock down these genes with RNAi would decrease S-Ado and affect ratio of SAICAr to S-Ado. *adss-1* and *atic-1* RNAi worms showed similar thrashing phenotype as *adsl-1* RNAi worms**.** *adss-1* RNAi data is from Wenqing Wang.

Discussion

According to the literature, we hypothesize that The previous data in our lab showed that accumulating SAICAR/SAICAr is the toxic intermediate for thrashing defect in *adsl-1* worms, and S-Ado have the protective effect from SAICAR/SAICAr. Some of our lab previous data supported the hypothesis. Wenqing changed the purine levels in worms (data not shown) and found that reduced concentration of purine nucleotides is insufficient to cause locomotion deficiency in worms. Our lab also decreased the SAICAR/SAICAr level in *adsl-1* RNAi worms with aminopterin or MTX, and these two compounds rescued thrashing defects in *adsl-1* RNAi worms. All of the data indicated that not the insufficient purine nucleotides, but the elevated SAICAR caused thrashing deficiency in *adsl-1* RNAi worms.

To further test our hypothesis, I knocked down ATIC level by RNAi technique. The *atic* RNAi worms showed the thrashing defects as of *adss-1* RNAi worms and *adss-1* RNAi worms (Figure A-6). The result also indicated that SAICAR/SAICAr is the toxic intermediate for thrashing deficiency in *adsl-1* worms.

Although our finding demonstrated boosted SAICAR has a toxic effect to worm locomotion, SAICAR levels or the ratio of SAICAr to S-Ado have not been measured in these worms. We will measure SAICAR level and the ratio of SAICAr to S-Ado in our worms to support our hypothesis.

Furthermore, the combinations of double RNAi to block accumulation of SAICAR will be done to support our hypothesis, like RNAi ADSL-1 with another upstream gene, for example, FGAMS (F10F2.2). The thrashing rate of these double RNAi worms should be similar to wild-type worms. Because these worms cannot

accumulate toxic SAICAR in their body, the thrashing defect shouldn't be observed in these worms.

We attempted to confirm SAICAR is toxic by soaking hatched L1 stage WT worms in up to 200 uM of SAICAR solution for up to 72 hours, but no thrashing defects were observed. It probably the concentration of SAICAR is not high enough to create the thrashing defect or SAICAR cannot get into cells. We will figure out this in the future. We can also use other metabolites instead of SAICAR. The compounds glycinamide ribonucleotide (GAR) and 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR), which are two metabolites in de novo pathway, can be purchased from company. We will feed worms with GAR, the intracellular SAICAR may be elevated and the worms would show thrashing deficiency. However, AICAR, which is the downstream metabolite of SAICAR, cannot affect SAICAR level in these worms, and these worms will show normal SAICAR level and thrashing rate.

Investigate a hypothesis for molecular mechanism of thrashing deficiency in *adsl-1* worms.

Purines are used by all kinds of cells in organisms (Fang et al., 2013; Jinnah et al., 2013), and purine de novo synthesis and salvage synthesis pathways precisely correlate with each other to supply the different nucleotides demands in different tissues (Fang et al., 2013). For example, de novo synthetic pathway is highly active in liver and muscle cells, and salvage synthetic pathway is active in erythrocytes and mature neuron cells (Dudzinska et al., 2006; Gordon et al., 1979).

In 2012, Keller found that in cancer cells SAICAR can activate pyruvate kinase isoform M2 (PKM2) (Keller et al., 2012). Pyruvate kinase is the final enzyme participating in glycolysis, which transfers a phosphate group from phosphoenolphruvate (PEP) to ADP and yields pyruvate and ATP. Four pyruvate kinases have been found in humans: PKM1, PKM2, PKL and PKR. PKM1 has high activity and is present in differentiated tissues to produce ATP efficiently (Gui et al., 2013; Ikeda et al., 1997). PKM2 can switch to high- or low-activity state depending on different physiological states, and presents in proliferating cells and cancer cells (Gui et al., 2013). Recent

research found that PKM2 is not only responsible for proliferating cells and cancer cells, but a dominant pyruvate kinase present in most tissue and cells (Bluemlein et al., 2011).

Two pyruvate kinases have been characterized in *C. elegans*, they are encoded by genes *pyk-1* and *pyk-2*. The results from high-throughput in vivo analysis of gene expression informs that PYK-1 is expressed in body muscle, body wall and vulval muscle, and PYK-2 is expressed in the intestine (Hunt-Newbury et al., 2007).

According to the information: (1) PYK-1 is the homolog of pyruvate kinase and presents in muscle cells, (2) de novo synthetic pathway is highly active in muscle cells, (3) our previous data showed KD *adsl-1* in muscle, which would increase SAICAR in muscle, demonstrated locomotion defects, (4) SAICAR can activate PKM2, we predict that SAICAR is the activator of PYK (likely PYK-1) and affects thrashing via increasing PYK activity. (SAICAR→ PYK1----| thrashing rate)

Experimental Strategy:

Change the activity of PYK-1 or the endogenous concentration of PYK-1 in worms and check whether change of PYK-1 could influence locomotion in worms.

Results

I tested our hypothesis by knocking down *pyk-1* in *adsl-1*(*tm3328*) mutants or wild-type worms. We found that *pyk-1*(RNAi) *adsl-1* mutant worms have higher thrashing rate than *adsl-1* mutants (Figure A-7, A). This thrashing assay suggested that SAICAR affects thrashing phenotype partially through PYK-1. *pyk-1*-KD WT worms showed normal thrashing rate like wild-type worms (Figure A-7, B). This could be muscle cells are already optimized; taking the suppressor PYK-1 away could not make muscle functionally better. However, more experiments are needed to investigate our hypothesis.

Because SAICAR is an activator of PYK-1 and SAICAR affects thrashing phenotype partially through PYK-1, we hypothesize that altering the activity of PYK-1 would change the thrashing rate in worms. To decrease the activity of PYK, I fed WT or

adsl-1(*tm3328*) mutants with 1mM alanine, which is a negative modulator for pyruvate kinase (Richard et al., 1998). It showed that alanine boosted thrashing rate in *adsl-1*(*tm3328*) mutants (Figure A-8, A), but had no effect on wild-type worms (Figure A-8, B). These results are similar to the counterpart in *pyk-1*-kd worms (Figure A-7), suggesting that SAICAR weak the thrashing rate by activating PYK-1.

FBP is an activator of PKM2 (Anastasiou et al., 2012; Jurica et al., 1998; Keller et al., 2012), and PYK-1 is a homolog of PKM in worms, so I supplemented wild type worms with 1 mM FBP to active PYK-1. Wild-type worms treated with FBP demonstrated slower thrashing rate than control group worms (Figure A-9). The data also indicated that PYK-1 mediates thrashing rate.

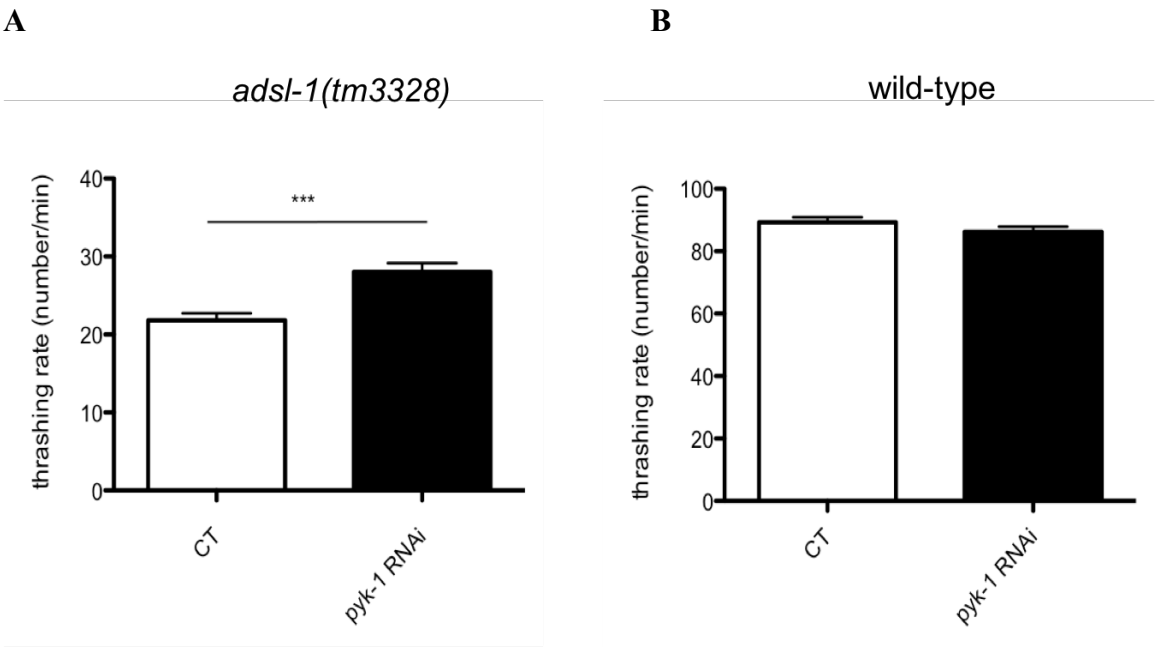

Figure A-7 SAICAR affects thrashing rate via PYK-1.

(A) *pyk-1*(RNAi) *adsl-1* mutant worms have higher thrashing rate than *adsl-1* mutants. (B) *pyk-1*-KD WT worms shows similar thrashing rate as wild-type worms. In histograms, error bars are S.E. ***, $p < 0.001$, calculated using Student's t-test.

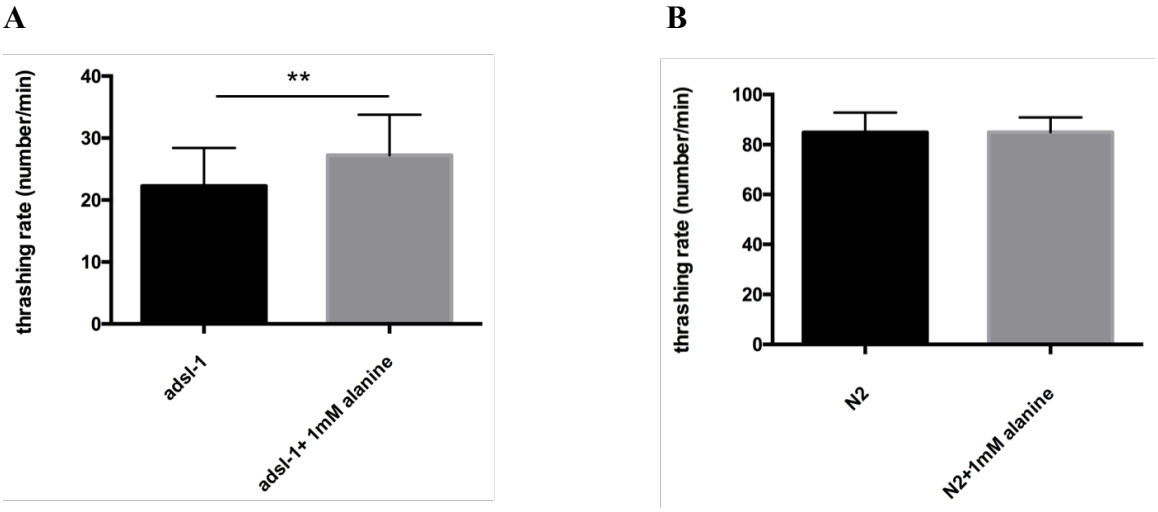

Figure A-8 Alanine effect on thrashing rate.

Alanine is an inhibitor of PYK. (A) Alanine has no effect on wild-type worms. (B) Alanine increased the thrashing rate in *adsl-1(tm3328)* mutants. Worms are treated with 1mM alanine. In histograms, error bars are S.E. **, $p < 0.01$, calculated using Student's t-test.

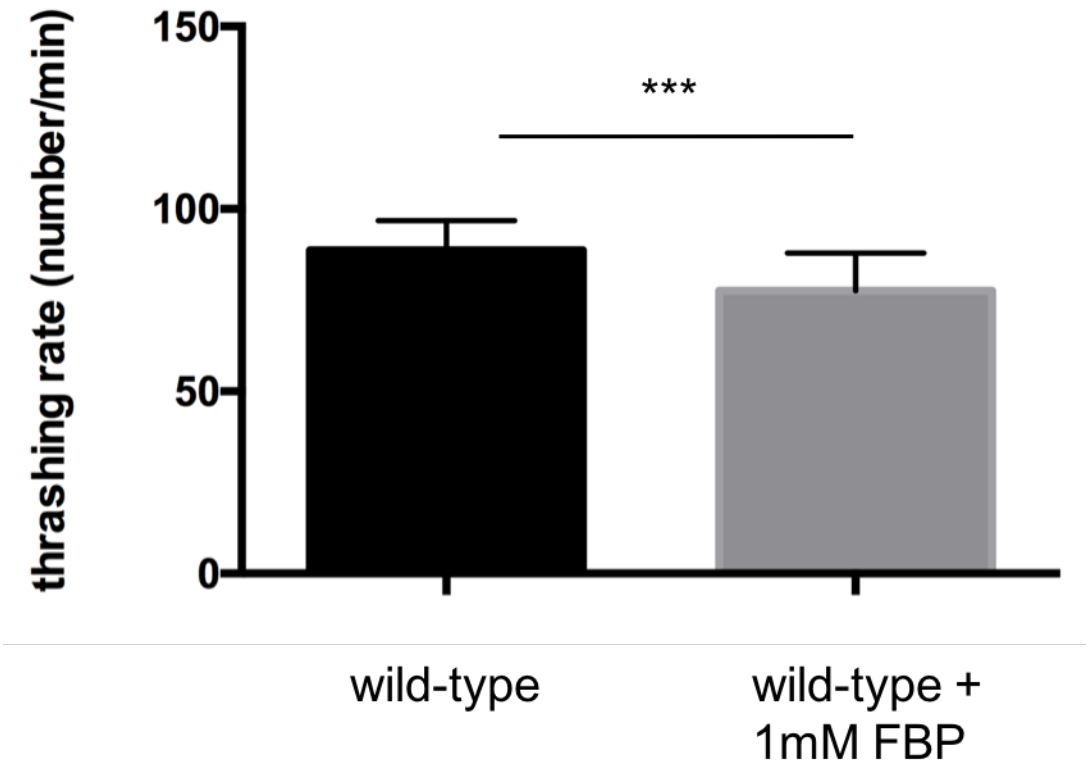

Figure A-9 Fructose-1,6-phossphate effect on thrashing rate.

Fructose-1,6-phossphate hampers thrashing rate in wild-type worms. Fructose-1,6-phossphate (FBP) is an activator of PYK. Worms are treated with 1mM FBP. In histograms, error bars are S.E. ***, $p < 0.001$, calculated using Student's t-test.

Discussion

To test whether SAICAR affects thrashing phenotype partially through PYK-1, I changed the activity of PYK and examined the thrashing rate in these worms. Wild-type worms or *adsl-1*(*tm3328*) mutants were fed with 1mM alanine to lower the activity of PYK. Alanine boosted thrashing rate in *adsl-1*(*tm3328*) mutants (Figure A-8, A), but had no effect on wild-type worms (Figure A-8, B). These results are similar to the counterpart in *pyk-1*-kd worms (Figure A-7). However, FBP, the activator of PKM2, decreased thrashing speed of wild-type worms (Figure A-9), suggesting that SAICAR weak the thrashing rate partially through activating PYK-1.

The another way to increase total PYK-1 activity is to overexpress PYK-1. We plan to overexpress *pyk-1* in muscle using *myo-3* promoter to increase PYK-1 level in wild type and *adsl-1* mutant worms. These transgenic worms would have more PYK-1 than their counterparts, so the total activity of PYK-1 is potentially higher in these transgenic worms. P_{myo-3}::pyk-1 worms will show thrashing defects, and the thrashing rate would be slower than WT worms. Also, adsl-1 mutants with overexpressed PYK-1 would have slower thrashing rate than adsl-1 mutants.

In order to confirm that PYK-2 is not relevant to thrashing defects in *adsl-1* mutants, we need to knock down *pyk-2* in *adsl-1* mutants or wild type worms via RNAi and compare the thrashing rate of theses worms with adsl-1 mutants or WT worms. We've already showed that decreased muscle ADSL-1 level by RNAi causes thrashing defect in worms (Figure 4), and the predicted expression pattern of PYK-2 is in worms' intestine. Accordingly, I predict that SAICAR affects on intenstival PYK-2 would not affect thrashing rate in muscle. So, the thrashing speed of pyk-2-kd worms in WT or *adsl-1* mutant background would be similar to their counterparts.

Our results pinpoint out that besides PYK-1, SAICAR affects locomotion defect also through other pathways. So, to seek the other pathways participating in locomotion with SAICAR, I will apply the global metabolomics approach to measure metabolites changes in different conditions, find out the overlapping metabolites and narrow them down to specific pathways.

CONCLUDING REMARK

Disorder of purine metabolism is a kind of refractory disease; the main aim of my study is to build up a purine metabolic disorder model in *C. elegans* to explore pathophysiological and molecular mechanisms of this disease. My study focuses on metabolite SAICAR in ADSL deficiency worms and using molecular and metabolic approaches finds out molecular mechanism in the regulation of purine disorder related diseases. This would help us find a new strategy for treatment of purine disorder related diseases.

Material and Methods

Maintenance of *C. elegans* and Supplements Treatment

C. elegans are cultured at 20˚C feeding with *E. coli* strain OP50 (Brenner, 1974). N2 is the wild-type strain. The maintenance method is mentioned in chapter2.

Alanine (Santa Cruz) and FBP (Santa Cruz) are dissolved into distilled H_2O and filtered to sterilize. Add appropriate volume of these solution on seeded OP50 plates. Then place the plated at room temperature for 1-2 day. The purpose of this step is to make final concentration of these solutions are 1 mM evenly on the whole plates.

To prepare worms for thrashing assay, I transferred 5 N2 adult worms or balanced *adsl-1* worms on a seeded NGM plates with metabolites supplements and cultured them at 20 ˚C until the next generation of worms reached mid-L4 stage.

Thrashing Assay

In my experiments, mid-L4 stage worms are picked for thrashing assay. Place a single worm in M9 solution for 1 min to let the worm get used to the solution, then count the frequency of thrashing movements for 1 min. One single thrashing movement is considered as a complete change in the direction of bending of the head.

RNAi Assay

Most of RNAi constructs were obtained from *C. elegans* RNAi Library (Source BioScience UK). RNAi feeding assays were carried out as described in (Gengyo-Ando et al., 2006). Adult worms were plated on RNAi plates and incubated at 20℃ until the next generation of worms reached mid-L4 stage. Then mid-L4 stage worms are picked for thrashing assay.

For *atic* RNAi plasmid construction, forward primer: CCCAAGCTTGTGCAGACAGAATGTCATCT, and reverse primer: CGGGGTACCCTCTGAACCAAGAACTCCAC are used to amplify 584 bp of *atic* DNA, then insert the piece of DNA into L4440 vector which has been digested by KpnI and HindIII. The created plasmid is transformed into HT115 *E. coli.* Using the same

strategy for *pyk-1* RNAi plasmid. forward primer: CCCAAGCTTGTTCGTGAAGCTGCCGATTC, and reverse primer: CGGGGTACCCTTTTGTGCAAGGAACACCT.

Statistic Analysis

The *p* values of gonad delay phenotype assessment are calculated by using Fisher's exact test. Error bars are S.E. $0.05 < p < 0.1$; *, $0.01 < p < 0.05$; **, $0.001 < p < 0.01$; ***, $p < 0.001$.