Kaneko, Shunya

Department of Genetics

School of Life Science Graduate University for Advanced Studies, SOKENDAI

Title: Mettl1-dependent tRNA m7G modification controls tRNA expression and fertility in *Drosophila melanogaster*

Introduction

Genetic information had been considered to transmit in a simple flow, DNA to protein in 1950s. The discovery of mechanism which regulates gene expression without any changes of genome sequence and inherits gene expression status across generations of cells has overturned the concept of classical central dogma. This current concept is called epigenetic gene regulation. In these 10 years, RNA modification has been found that it plays a significant role in epigenetic gene regulation because of the development of chemical and antibody-based detection technique for RNA modifications. To date, over 170 different modifications have been identified. They regulate the dynamics and fate of target RNAs to control vital life processes.

Methylation is the one of famous and abundant modification on RNA. To induce various types of methylation on targets, organisms utilize the essential methyltransferases. The methyltransferase like (METTL) family belongs to the seven-beta-strand methyltransferase group but has been less studied than the deeply studied methyltransferase family, like DNMT family. METTL family proteins can transfer methyl groups to DNA, RNA, proteins and any many small molecules using S-adenosyl methionine to supply the methyl group. To date, 35 human METTL genes have been identified, and 12 METTLs are predicted to methylate RNA. Moreover, some METTLs are associated with human disease including cancer, indicating that METTL proteins play a key role in higher eukaryotes. Fruit fly, *Drosophila melanogaster*, also possesses Mettl family, but their biological importance is not understood. Therefore, I decided to address the developmental role of Mettl family using *D. melanogaster* as a model organism.

Initially I performed screen with Mettl genes using CRISPR-Cas9-mediated knockout flies. The human genome contains 35 annotated METTL genes, 23 of which are conserved in *D. melanogaster*. Screening assay revealed seven Mettls are related to normal growth and proliferation. One identified Mettl gene, Mettl1 has shown strong association with fertility. Remarkably, Mettl1-KO fly showed a defect only in spermatogenesis and oogenesis although Mettl1 seemed to express and function in whole body. Therefore, I further analyzed the developmental role of Mettl1 in *D.*

melanogaster.

In eukaryote, METTL1 introduces N7-methylguanosine (m7G) of tRNA, mRNA, and miRNA. To deposit methyl group on target RNAs, METTL1 requires a partner protein, WD repeat domain 4 (WDR4) for their methyltransferase activity. In the case of *D. melanogaster*, Wh is conserved as a homolog of WDR4. Previous studies have shown that Wh mutant flies showed male sterile phenotype and female sterile phenotype, but whether these defects were derived from the disorder of m7G RNA modification. In order to reveal the requirement of Mettl1-dependent m7G modification, I performed the rescue assay in fertility using Mettl1 transgene with mutations which lose catalytic activity of Mettl1. This mutant didn't rescue the sterility of Mettl1-knockout mutant, indicating catalytic activity of Mettl1 and Mettl1-dependent m7G are important for maintaining complete fertility in *D. melanogaster*. I further focused on the mechanism which controls fertility via m7G, and performed m7G site specific reduction and cleavage assay using small RNAs gained from *D. melanogaster* gonads. The cleavage assay revealed Mettl1 methylates 14 of 44 tRNAs in *D. melanogaster*. To examine how the loss of Mettl1 affects tRNAs, I measured tRNAs abundance. The amount of tRNA iMet-CAT, an m7G-modified tRNA, was decreased in male gonads, indicating that m7G modification is required for tRNA stability, as is observed in mammalian cultured cells and *S. cerevisiae*. These results insist Mettl1 stabilizes m7G target tRNAs to maintain complete fertility.

Results

In order to address the biological significance of Mettl genes and Mettl-dependent methylation, I generated and prepared 14 Mettl-knockout flies using CRISPR-Cas9 system and found that seven KO strains have clear defects in the survival rate, growth and fertility. Knock out of CG4045, a putative METTL1 homolog, produced a semi-sterile phenotype, although CG4045 is considered to be expressed in all tissues and developmental stages. From these Mettls, I decided to address the developmental role of CG4045/Mettl1 in *D. melanogaster* gonad, because the link between m7G RNA modification and vital phenomena, such as reproduction, is undetermined.

CG4045 is predicted to be a METTL1 ortholog in *Drosophila* (CG4045 is termed Mettl1 hereafter). I performed fertility assay and found that *Mettl1-KO male and female*. The numbers generated from Mettl1-KO males and females were reduced, indicating that Mettl1 is important for fertility in *D. melanogaster*. *Mettl1*-KO male showed more severe defect in fertility compared with female. To investigate which steps of spermatogenesis is affected in *Mettl1*-KO males, I performed a microscopic analysis and found that *Mettl1*-KO males lost mature sperms from the seminal vesicle, similar to *Wh* mutant males. Mettl1 seemed to be expressed in all testicular cells. To analyze whether somatic or germ cell expression is essential for Mettl1, I prepared a *Mettl1*-transgene conjugated with a *Nanos*-promoter to specifically express Mettl1 in germ cells. *Nanos*P-Mettl1 in a *Mettl1*-KO background rescued the sterile phenotype of *Mettl1*-KO, indicating that the germ cell expression of Mettl1 is important for maintaining fertility and sperm development.

In order to confirm the requirement of catalytic activity of Mettl1 and Mettl1-dependent m7G modification, I prepared *Mettl1*-transgenes with mutation to lose methylation activity, SAM-binding (G79A, G81A) or methylation activity (L157A, D160A), and performed rescue assay in fertility. These transgenes didn't rescue sterility of *Mettl1*-KO male, indicating that Mettl1-dependent m7G RNA modification is important for Drosophila fertility and spermatogenesis. To examine whether *Drosophila* tRNA is modified *in vivo*, I performed m7G site specific reduction and cleavage assay, called TRAC-seq, for detecting internal m7G modification of RNA. TRAC-seq using total RNAs from wild-type and *Mettl1*-KO ovaries revealed that 14 tRNAs among 44 tRNAs are modified by Mettl1. To test if Mettl1 also mediates m7G modification in testes, I performed a similar assay. As expected, the m7G site specific cleavage was caused in wild-type testis but not in *Mettl1*-KO flies, indicating that Mettl1-mediated tRNA modification occurs in both female and male gonads.

In *S. cerevisiae* and mice, m7G modification promotes tRNA stabilization. I therefore checked the abundance of m7G target tRNAs in *Mettl1*-KO flies. Northern blotting showed that the amounts of tRNA iMet-CAT was decreased in *Mettl1*-KO testes, but the amount of tRNA Trp-CCA was not changed. These results indicate that Mettl1-dependent m7G modification is required for some m7G target tRNA expression in testes.

Discussion

Drosophila Mettl1 and m7G modification seemed to be important for elongation step of spermatogenesis and for maintaining fertility. In *Mettl1*-KO testes, round spermatids accumulated in the region near the seminal vesicle. This indicates that loss of m7G did not affect entry or progression of meiosis but that spermatogenesis was arrested. It is difficult to determine the cell types in which Mettl1 plays a critical role during mitotic division of spermatogonia; however, this could be addressed using a cell type-specific GAL4/UAS expression system.

It remains unclear how controlling tRNA expression is involved in the maintenance of spermatogenesis. Recent studies have shown that stabilization of tRNA Arg-TCT by ectopic expression of mouse Mettl1 enhances translation of cell-cycle genes, like such as CDK6 and others. It is therefore possible that *Drosophila* Mettl1 positively regulates translation of key germ cell genes that have cognate mRNA codons recognized by stabilized tRNAs. Northern blot analysis showed decreased levels of tRNA iMet-CAT in *Mettl1*-KO testes, suggesting that regulation of translation initiation via Mettl1-dependent m7G is also important for testis. It is still an open question whether translation initiation is impaired and affects the fertility in *Mettl1*-KO males. To gain an insight into this issue, I am currently examining Ribo-seq analysis and OPP (O-propargyl-puromycin) staining to evaluate the translation levels in *Mettl1*-KO male testis.

Materials and methods

Northern blotting, plasmid construction, generation of Mettl1 rescue strain, immunostaining, and microscopic analysis of gonad in this thesis are basically performed as described previously. A part of the materials and methods is summarized as follows.

Fly stocks

$y^1 w^{1118}$, FM7/Y and FM7C/Y were treated as a WT lines. *Mettl1-KO* and *Mettl*-KO flies were: $y^1 w^{1118}$ *Mettl1*^{D1}*/FM7, Kr>GFP,* $y^1 w^{1118}$*; Mettl*/CyO* (in the case of MettlXX located on the 2nd chromosome), and $y^1 w^{1118}$*; + ; Mettl*/TM6C* (in the case of MettlYY located on the 3rd chromosome). These mutant flies were generated using the transgenic CRISPR-Cas9 method. To generate mutants, I used the following strains: $y^1 v^1$ *nos-phiC31; attP40*, y^2 *cho*^{2} v^1*; Sp hs-hid/CyO* , $y^1 w^{1118}$; +; *attP2{nos-cas9}*. Depletion of Mettl region in Mettl mutants was checked by PCR and sequencing.

Fertility assay

To test for male fertility, single sample or control (y^1w^{1118}, WT) males were mated with three control (y^1w^{1118}, WT) females at 25°C for three days. After mating, the parental flies were removed and incubation continued for 11 days. Progeny were counted and the average number per vial calculated. Ten independent vials for each strain were prepared for this assay. The female fertility assay followed the method of the male fertility assay except that mating was allowed for 10 days.

m7G site specific reduction and cleavage

Total RNA from ovaries and testes were isolated using Isogen (Nippon Gene, 311-02501) following the manufacturer's instructions. Ten micrograms of total RNA were incubated with 0.1 M NaBH4 and 1 mM free m7GTP for 30 min on ice in the dark. Reduced RNAs were precipitated with 3 M sodium acetate pH 5.2 (Thermo Fisher), glycogen (Nacalai Tesque) and ethanol at -20°C for at least 1 hr. RNA samples reduced by NaBH4 were then reacted with aniline-acetate solution (H2O:glacial acetate acid:aniline, 7:3:1) at room temperature in the dark for 2 h to cause m7G site-specific cleavage. The cleaved RNAs were detected by northern blotting.

TRAC-seq

TRAC-seq in this thesis is basically performed as described previously. Small RNAs (< 200 nt) were isolated from total ovary RNAs using a mirVana miRNA Isolation Kit (Thermo Fisher, AM1561) following the manufacturer's instructions.

Sequencing data were trimmed of 3′ adapter sequences and 5′-end barcode sequences by CLC-Genomics Workbench ver.22.1 and mapped to Drosophila tRNA reference sequences (dm6-mature-tRNAs) from GtRNAdb. The read coverage and mapping read number were measured by CLC-Genomics Workbench. From read coverage number, I calculated the cleavage score of each

sample. The cleavage scoreWT or *Mettl1*-KO of site i in tRNA was defined as the ratio between the number of read starts and number of total reads, as previously described. The cleavage score was further defined by the difference between cleavage scoreWT and cleavage score *Mettl1*-KO The sites with cleavage score > 15 were regarded as m7G-dependent cleavage sites. After identifying potential m7G-modified tRNAs by calculating the cleavage score, I confirmed the sequence of ± 6 bases around the m7G site and checked whether the candidate tRNAs had the “RAGGU” motif.