Int. J. Mol. Sci. **2007**, *8*, 399-406

International Journal of
Molecular Sciences
ISSN 1422-0067

www.mdpi.org/ijms

Full Research Paper

Functional Analysis of the *Drosophila Dnop5* Using Targeted RNA Interference

Yan Zhang, Jie Ding, Yongqi Wan, Wei Xie and Liudi Yuan*

Department of Genetics and Developmental Biology, Southeast University Medical School, Ministry of Education, Key Laboratory of Developmental Genes and Human Disease, Nanjing 210009, P. R. China

* Author to whom correspondence should be addressed. 87 Dingjiaqiao Road, Nanjing 210009; P. R. China; Tel: (0086) 25-83272314-812; E-mail: yld@seu.edu.cn

Received: 7 March 2007; in Revised Form: 3 May 2007 / Accepted: 9 May 2007 / Published: *31 May 2007*

Abstract: *Dnop5* is a member of the conserved *nop5/sik1* gene family, which encode components of small nucleolar ribonucleoprotein(snoRNP) complexes. To study the function of DNop5, we generated the polyclonal antibody and determined its expression pattern. It is highly expressed in different periods of the *Drosophila* development. We used heritable RNA interference (RNAi) in combination with the *yeast* GAL4/UAS binary system to knock down the DNop5 protein. It resulted in lethality and dramatic somatic anomalies in RNAi mutant fly, in which the DNop5 protein is reduced efficiently. Northern blotting showed that the processing of 18S rRNA was disrupted in DNop5 knock down fly, but 28S rRNA is normal. These results suggest that DNop5 is essential for the *Drosophila* growth and function in the execution of early pre-rRNA processing steps that lead to formation of 18S rRNA.

Keywords: RNAi; *Dnop5*; 18S rRNA

1. Introduction

In eukaryotes, rRNA genes are transcribed as precursor RNAs (pre-rRNAs) in a specialized nuclear compartment, the nucleolus, and undergo extensive processing that includes covalent modification of highly conserved regions and a series of endo-nucleolytic and exonucleolytic cleavages. Processing

and modification of the pre-rRNA seems to occur simultaneously with the assembly of ribosomal proteins (r-proteins) and large transient RNP (ribonucleoprotein) particles are formed in the nucleolus [1]. Different sets of proteins were found in each complex. *Nop5/sik1* gene encode components of small nucleolar ribonucleoprotein (snoRNP) complexes, snoRNPs mainly catalyze the modification of rRNA. The two major classes of snoRNPs are box H/ACA and box C/D. The box C/D snoRNAs are mainly involved in the 2'-O-methylation of pre-rRNA ribose, and the box H/ACA snoRNAs in the conversion of uridine to pseudouridine residues [2]. Phylogenetic analysis shows that they are highly conserved from plants to human. It is identified that human Nop5/Nop58 is component of the box C/D class of snoRNPs. In *yeast*, Nop5p functions with Nop1p in the execution of early pre-rRNA processing steps that lead to formation of 18S rRNA [3]. It is not clear whether this is the case in *Drosophila*.

Dnop5, a novel gene in *Drosophila*, is the member of the conserved *nop5/sik1* gene family. As other Nop5/Sik1 family members, DNop5 contains multiple KKX motifs at the carboxy terminus, and is the closest relative of Nop5 of *Caenorhabditis elegans*. *Dnop5* cDNA codes for a transcript of approximately 1800 nucleotides. Conceptual translation of its open reading frame predicts a polypeptide of 510 amino acids. As revealed by in situ hybridization, DNop5 protein accumulates in the nucleolus of all cells in the embryo and wing disc [4]. To characterized the expression profile of DNop5, we prepared the polyclonal antibodies against DNop5 and found that it is expressed dynamically and stably during *Drosophila* development. These indicated that DNop5 is maybe very important for growth of *Drosophila*. However, the specific functions of the protein are not known. In this study, we examined the consequences of disrupting the *Dnop5* gene expression in developing and in differentiated cells by using RNAi approach and northern blotting. We present some evidences that DNop5 is associated with fly development and required for 18S rRNA synthesis.

2. Results and Discussion

Most of the steps of ribosome biogenesis in eukaryotic cells take place in the nucleolus. Ribosome biogenesis requires the coordination of many different events, including rRNA transcription, pre-rRNA modification and processing, ribosomal protein production and rRNA-ribosomal protein assembly. In *Drosophila*, a single long 45S pre-rRNA is transcribed by RNA polymerase I and processed to 18S, 5.8S and 28S rRNAs through a series of co- and post-transcriptional steps. The processing of pre-rRNAs require non-ribosomal nucleolar proteins, many of which are associated with small nucleolar RNAs (snoRNAs) in the form of small nucleolar ribonucleoprotein (snoRNP) complexes [1,4]. Functional studies on *yeast nop5/sik* genes have shown that mutations interfere with the processing of the 18S rRNA and cause reduced cell growth and proliferation .It will be interesting to see whether DNop5, the member of the conserved Nop5/Sik1 family, has the same function in *Drosophila*.

2.1. DNop5 is ubiquitously expressed throughout Drosophila development

For functional analysis of DNop5, polyclonal antibody against DNop5 was generated according to standard methods. The polyclonal antibody could specifically detect a 62 KD (calculated molecular weight of DNop5) protein, this data indicates that the antibody is specific to DNop5. Western blot

results show that DNop5 was continuously expressed throughout *Drosophila* development from embryos to adults (Figure 1).

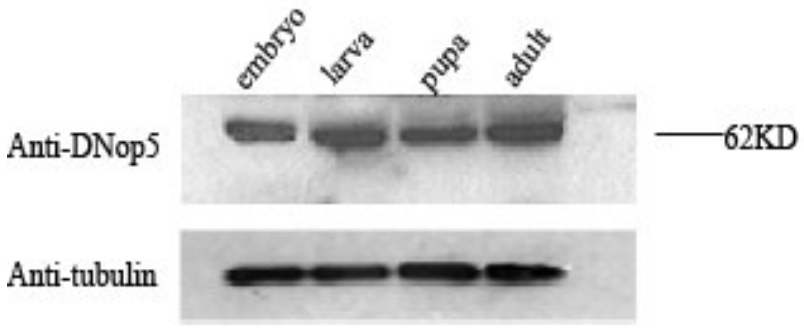

Figure 1. DNop5 is ubiquitously expressed throughout *Drosophila* development by western blotting

2.2 Inducible RNAi targets Dnop5

RNAi is a cellular process of gene silencing in which small duplexes of RNA specifically target a homologous sequence for cleavage, and has proven to be a powerful tool for disrupting gene expression. This technique was initially developed in plants and nematodes and more recently in *Drosophila*, *Zebrafish* and *Mice* [5]. RNAi can be used to interfere with gene expression both transiently by introducing double-stranded RNA (dsRNA) directly into cells and stably by inserting an appropriate construct that produces the targeting dsRNA into the genome. The advantage of using inheritable or inducible RNAi is that the phenomenon becomes a permanent feature of an organism, and the effect can often be triggered under a variety of different conditions and/or at different times of development. In *Drosophila*, combining RNAi with the inducible *yeast* GAL4/UAS binary system [6], proteins can be reduced in a stage and tissue specific fashion, which is not feasible with ubiquitous gene interference or loss of function mutations. Using this approach, we can efficiently reduce the expression of *Dnop5* throughout development and better study the function of this protein.

We generated transformant lines carrying the transgene UAS- Dnop5.IR. This construct led to the synthesis of double-stranded RNA under the control of the GAL4-UAS (Figure 2A). Induction of the *Dnop5* dsRNA synthesis was able to reduce DNop5 protein, as shown in Figure 2B, the expression (using the da-GAL4 driver) of the UAS- Dnop5.IR construct decreased DNop5 protein in larvaes, actin-GAL4/cyo and ptc--GAL4 mediated expression also reduced DNop5 protein levels (data not shown).

2.3 Phenotypic analysis of Dnop5 RNAi mutant flies

UAS- Dnop5.IR flies was crossed to the da-GAL4 and actin-GAL4/cyo driver flies (expressing cells in the body). The progeny flies of UAS- Dnop5.IR/ da –GAL4 and UAS- Dnop5.IR/ actin- GAL4 were missing. The reduction of DNop5 in *Drosophila* can lead to 100 % lethality before the pupal stage. The crossing of UAS- Dnop5.IR flies to the ptc-GAL4 driver line (expressing in dorsal mesothoracic disc that is relative of the wing, costal cells and wing veins) leads to short and thin bristles or lack of bristles. Typical phenotypes are shown in Figure 3.This phenotype was similar with minute phenotype

which can usually discover in mutations of genes encoding ribosomal proteins, known as *minute* mutations, and ribosomal RNA (e.g., the *mini* and the *bobbed* mutations). The *minute* phenotypes have some characteristics, such as delayed larval development, short and thin bristles, reduced fertility and viability, and recessive lethality, which result from a slower rate of cell growth and proliferation [7-9]. In all probability, these phenotypes reflect a reduced rate of protein synthesis resulting from an impaired ribosome biogenesis. Since *Dnop5* is a member of the conserved *nop5/sik1* gene family, we reasoned that DNop5 was likely to play a role in rRNA biosynthesis.

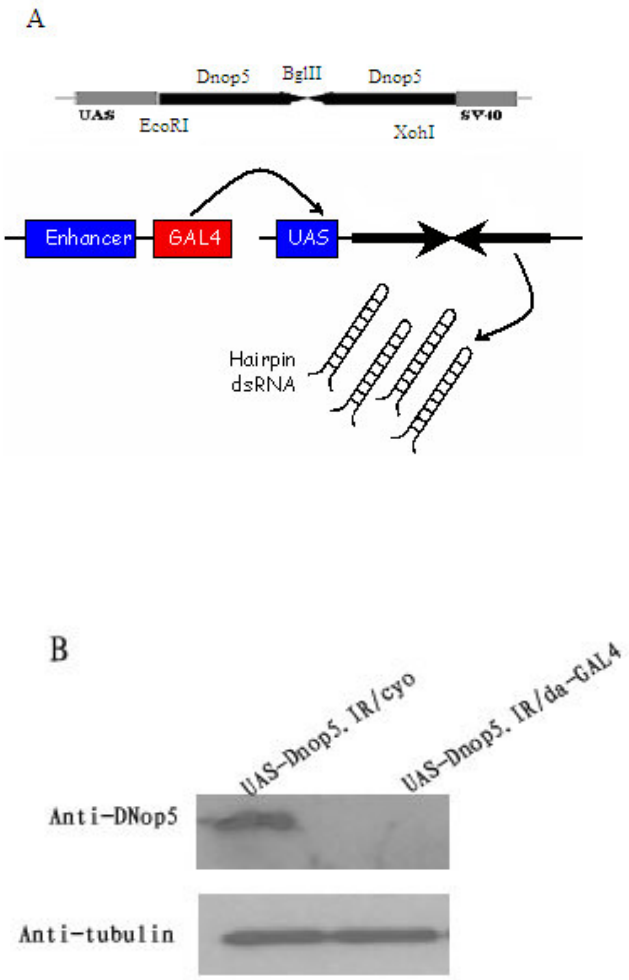

Figure 2. Inducible RNAi targets *Dnop5*. (A) Strategy for inducible dsRNA synthesis.(B) Western blotting for induction of dsRNA by using the da-GAL4 driver.

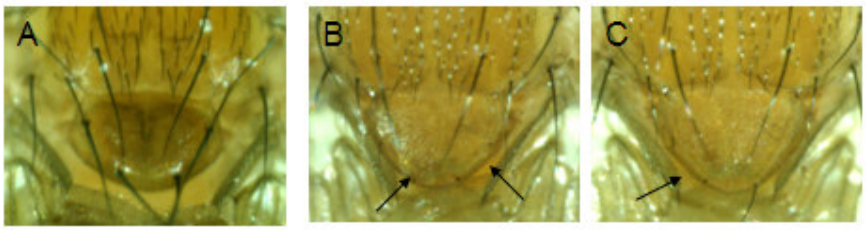

Figure 3. Phenotypes from RNAi flies. (A) W^{1118}; (B,C) UAS- Dnop5.IR /ptc- GAL4

2.4 Depletion of Dnop5 impairs synthesis of 18S rRNA

The 18S rRNA is synthesized by the pathway diagrammed in Figure 4A, the levels of 18S and 28S rRNAs from transgenic flies by using northern blotting analysis were shown in Figure 4B. The 18S rRNA was reduced by approximately 90 % in UAS-Dnop5.IR/da–GAL4 flies, whereas the abundance of the 28S rRNA was unaffected. This indicated that DNop5 depletion leads to a specific reduction of 18S rRNA, which could either be at the level of reduced synthesis or stability, or both.

The defect in production of 18S rRNA suggested an early defect in pre-rRNA processing and the abnormality of ribosome biogenesis, but the question of how DNop5 participates in pre-rRNA processing remains to be uncovered.

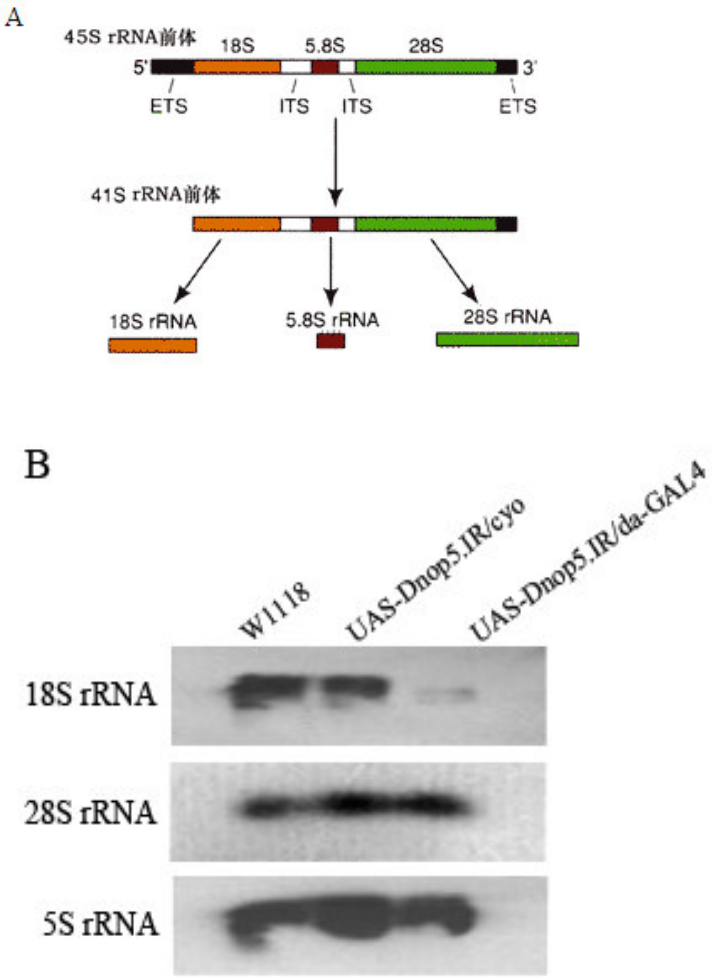

Figure 4. Depletion of DNop5 impairs synthesis of 18S rRNA. (A) The pre-rRNA processing pathway in eukaryotes.(B) Northern blot analysis of rRNA processing during DNop5 depletion.

In summary, we show that DNop5 is very important for growth of *Drosophlia* and its depletion leads to a specific reduction of 18S rRNA in *Drosophila*.

3. Experimental Section

3.1 Fly stocks

Wild-type W^{1118} flies, yw;sb/Tm6b, yw;ady/cyo, da-GAL4, actin-GAL4/cyo, ptc- GAL4 were grown at 25 °C in cornmeal media.

3.2 Plasmid construction

A 460-bp and 560-bp fragments from the coding sequence of the *Dnop5* gene was amplified by RT-PCR and cloned into the pUAST vector as an inverted repeat as described [10-13]. The 560-bp fragment was amplified by using sense primer(5'-gcgc**gaattc**aggtggacaatctgtaccag-3') and antisense primer (5'-gcgc**agatct**cttgatggtcttcacgaagg-3'), which contained *EcoR I* and *Blg II* site respectively. A 460-bp fragment was amplified by using sence primer(5'-gcgc**agatct**aactccttgtccaggtcatc-3') and antisense primer(5'-gcgc**ctcgag**aggtggacaatctgtaccag-3'), which contained *Blg II* site and *Xho I* site respectively. the pUAST vector was digested by *EcoR I* and *Xho I*. The recombinant vector was referred to as pUAST- Dnop5.IR.

3.3 Transgenic Fly Lines

DNA for transformation was prepared using Qiagen Maxi Prep cartridges. pUAST- Dnop5.IR described above was coinjected into W^{1118} embryos with the helper vector pUChsπΔ2–3. Adult G0 transformants were identified by outcrossing to W^{1118} and balanced over cyo or TM6b balancer chromosomes. Transgenic lines were mated with different GAL4 driver strains.

3.4 Antibody generation

To raise antibodies against DNop5, the full length cDNA amplified by RT-PCR was subcloned into the $pET28a^{+}$ vectors fused with $(His)_6$. After expressing in *E.coli* BL_{21}, the DNop5- $(His)_6$ fusion protein was affinity purified by using His-sepharose 4B beads (Amersham Pharmacia) and injected into rabbit to generate polyclonal antibody according to the standard protocol [14]. The antibody was purified through affinity chromatography column with GST-DNop5 expressed and purified from *E.coli* BL_{21}.

3.5 Western blot analysis

Larvaes were lysed in PBT buffer (PBS 0.1 %,Triton X-100). Anti-DNop5 antibody was used at a 1:1,000 dilution for Western blot analysis. HRP-conjugated anti-rabbit antibody (Pierce, 1:1000) was used as a secondary antibody. Anti-tubulin antibody (sigma) was used as a control at a 1:5,000 dilution.

3.6 RNA isolation and northern blot analysis

Total RNA was isolated from larvae of the transgene UAS- Dnop5.IR/ da-GAL4 with Trizol according to the standard protocol. RNAs were separated on 1.5 % formaldehyde agarose gel and transferred to Hybond nylon membrane (Biodyne B). The 400bp fragments of 18S rRNA and 28S rRNA cDNA were amplified by PCR. The 400bp fragments was labeled with biotin-UTP(Roche) by using the T7 transcriptase in vitro according to the manufacturer's instructions and used as probe, the 5S rRNA was used as control. Hybridization was performed at 68 °C in hybridization solution (50 % formamide, 5×SSC,1 % SDS, 5 × Denhardt, 100 mg/ml salmon sperm DNA).Wash the membranes in a biotin binding buffer (0.01 M Na_2PO_4，0.15 M NaCl，pH 6.8) while shaking. Remove the solution, add 4 ml biotin binding buffer and 8 μl 5mg/μl HRP-Strep, incubate at room temperature for 3 hour. Wash 5 times for five minutes per with biotin binding buffer and exposure to X-ray film.

Acknowledgements

This work was supported by National Natural Science Foundation of China Grant 30270309.

References and Notes

1. Gonzales, F.A.; Zanchin, N.I.; Luz, J.S.; Oliveira, C.C. Characterization of Saccharomyces cerevisiae Nop17p, a novel Nop58p-interacting protein that is involved in pre-rRNA processing. *J. Mol. Biol*. **2005**, *346*, 437-455.
2. Lyman, S.K.; Gerace, L.; Baserga, S.J. Human Nop5/Nop58 is a component common to the box C/D small nucleolar ribonucleoproteins. *RNA* **1999**, *5*, 1597-1604.
3. Wu, P.; Brockenbrough, J.S.; Metcalfe, A.C.; Chen, S.; Aris, J.P. Nop5p is a small nucleolar ribonucleoprotein component required for pre-18S rRNA processing in yeast. *J. Biol. Chem*. **1998**, *273*, 16453-16463.
4. Vorbruggen, G.; Onell, S.; Jackle, H. Restricted expression and subnuclear localization of the Drosophila gene Dnop5, a member of the Nop/Sik family of the conserved rRNA processing factors. *Mech. Dev.* **2000**, *90*, 305-308.
5. Hannon, G.J. RNA interference. *Nature* **2002**, *418*, 244-251.
6. Brand, A.H.; Perrimon, N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **1993**,*118*, 401-415.
7. Lambertsson, A. The minute genes in Drosophila and their molecular functions. *Adv. Genet.* **1998**, *38*, 69-134.
8. Cramton, S.E.; Laski, F.A. String of pearls encodes Drosophila ribosomal protein S2, has Minute-like characteristics, and is required during oogenesis. *Genetics* **1994**, *137*, 1039-1048.
9. Schmidt, A.; Hollmann, M.; Schafer, U. A newly identified Minute locus, M(2)32D,encodes the ribosomal protein L9 in Drosophila melanogaster. *Mol. Gen. Genet.* **1996**, *251*, 381-387
10. Enerly, E.; Larsson, J.; Lambertsson, A. Reverse genetics in Drosophila: from sequence to phenotype using UAS-RNAi transgenic flies. *Genesis* **2002**, *34*, 152-155.
11. Enerly, E.; Larsson, J.; Lambertsson, A. Silencing the Drosophila ribosomal protein L14 gene using targeted RNA interference causes distinct somatic anomalies. *Gene* **2003**, *320*, 41-48.

12. Van Roessel, P.; Hayward, N.M.; Barros, C.S.; Brand, A.H. Two-color GFP imaging demonstrates cell-autonomy of GAL4-driven RNA interference in Drosophila. *Genetics* **2002**, *160*, 637–648.
13. Kennerdell, J.R.; Carthew, R.W. Heritable gene silencing in Drosophila using double-stranded RNA. *Nat. Biotechnol.* **2000**, *18*, 896-898.
14. Nishinaka, N.; Hongo, S.; Zhou, C.J.; Shioda, S.;Takahashi, R.; Yamauchi, Y.; Ohashi, T.; Ohki, T.; Nakada, N.; Takeda, F.; Takeda, M. Identification of the novel developmentally regulated gene, Bdm2, which is highly expressed in fetal rat brain. *Devel. Brain Res.* **2000**, *120*, 57–64.