ショウジョウバエ始原生殖細胞における母性 Nanos タンパク質の新規機能の同定

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Identification of a novel function of maternal Nanos protein in *Drosophila* primordial germ cells

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Introduction

In *Drosophila,* a specialized cytoplasm, or germ plasm localized in the posterior pole region of early embryos is partitioned into the primordial germ cells (PGCs). Germ plasm contains the maternal factors required for germline development. One of the maternal factors, *nanos* (*nos*) mRNA encodes an evolutionary conserved CCHC-type zinc finger protein that plays an essential role in germline development in various animal species. Maternally supplied Nos is partitioned into PGCs and remains detectable in these cells throughout embryogenesis. In the absence of maternal Nos, PGCs enter into apoptotic pathway. When their apoptosis is inhibited, some of the PGCs are able to adopt a somatic cell fate rather than a germline fate. Furthermore, Nos is also required in PGCs for their differentiation into functional germ cells.

Nos, along with a conserved RNA-binding protein, Pumilio (Pum), acts as a translational repressor for specific RNA targets by binding to their 3' untranslated region (3'UTR). In PGCs, Nos represses the translation of *head involution defective* (*hid*) mRNA to suppress their apoptosis during embryogenesis. Furthermore, Nos is required to repress translation of *cyclinB* (*cycB*) mRNA to establish mitotic quiescence during their migration to the embryonic gonads. It has been reported that Nos and Pum bind to Nos-binding sites (UAUU) and Pum-binding sites (UGUA), respectively, in 3'UTR of *cycB* mRNA. They recruit CCR4/NOT deadenylase complex to *cycB* 3' UTR to facilitate deadenylation of its poly(A) tail, which in turn, represses its translation in PGCs. In contrast to this prevailing role of Nos, I report that the 3' UTR from *CG32425*

mRNA mediated Nos-dependent RNA stabilization in PGCs. Although the role of the *CG32425* gene in germline development remains unknown, the expression of maternal *CG32425* mRNA and its protein product was upregulated by maternal Nos in PGCs. This report provides the first evidence that supports a role for Nos in stabilizing RNA in PGCs.

Materials and Methods

Fly stocks

The *nos* allele I used was nos^{BN}. Maternal *nos* RNA is not detected in embryos derived from nos^{BN} homozygous females. As a control, I used embryos from nos^{BN} heterozygous females. These embryos and PGCs formed in the embryos are referred to as normal embryos and normal PGCs, respectively. To generate UASp-EGFP lines, y^1 *M{vas-int.Dm}ZH-2A w[*];; M{3xP3-RFP.attP'}ZH-86Fa* (Bloomington Drosophila stock center; 24486) was used. *nanos*-Gal4-VP16 (*nos*-Gal4) was used to induce the expression of *UASp-EGFP-K10 3'UTR*, *-cycB 3'UTR*, *-CG32425 3'UTR, -R1, -R2, -R3*, and the double-strand RNA against *CG32425* (*P{TRiP.HMC03193}attP40*) (Bloomington Drosophila stock center; 51457) in PGCs. Flies were cultured on standard *Drosophila* medium at 25°C.

Construction of UASp-EGFP lines and EGFP reporter assay

To construct *UASp-EGFP-cycB 3'UTR and –CG32425 3'UTR*, EGFP-coding region was amplified from pEGFP-N1 (Clontech). *cycB* and *CG32425* 3' UTRs were amplified from an embryonic cDNA library. The EGFP-coding region was fused to *CG32425* or *cycB* 3' UTR, and were subcloned into pBluescript II SK(+) vector. The EGFP-*CG32425* 3' UTR and *-cycB* 3' UTR were subsequently inserted into the transformation vector pUAS-K10 attb (a gift from B. Suter). To construct *UASp-EGFP-K10 3'UTR*, EGFP-coding region was amplified from pEGFP-N1. EGFP-coding region was inserted into the vector pUAS-K10 attb. To construct *UASp-EGFP-R1*, *R2*, and *R3*, "region 1", "region 2", and "region 3" were amplified from *UASp-EGFP-CG32425 3'UTR*. These amplified DNAs were inserted into the vector pUAS-K10 attb. For germline transformation, approximately 0.1 nl of solution containing *UASp-EGFP-K10 3'UTR*, *-cycB 3'UTR*, *-CG32425 3'UTR*, *-R1*, *-R2*, and *-R3* in the vector pUAS-K10 attb (200–300 ng/ml in DW) was injected into the embryos

derived from *y*^{1} *M{vas-int. Dm}ZH-2A w[*];; M{3xP3-RFP.attP'}ZH-86Fa* females. The resulting males were mated with *w;; Pr Dr/TM3 Sb* females. Progenies with the desired transgenes were selected by red eyes, and were used to establish stock lines. For the EGFP reporter assay, I used embryos derived from either w;; *nos*^{BN} *nos-Gal4/nos-Gal4* or *w;;nos*^{BN} *nos-Gal4* females mated with males homozygous for each *UASp-EGFP* transgene.

In situ hybridization for *CG32425* mRNA

Whole-mount in situ hybridization of embryos was performed. To synthesize an RNA probe for *CG32425* mRNA, I used cDNA generated from the PGCs purified from stage 4–17 embryos by a vector-capping procedure. A *CG32425* cDNA corresponding to the protein-coding region (1443 bp; 1–1443 in *CG32425*-RC, FlyBase: http://flybase.org) was amplified. EGFP-coding region (720 bp) was amplified from pEGFP-N1 (Clontech). The amplified cDNAs were inserted between the T7 and SP6 promoters in the pGEM-T Easy Vector (Promega). Templates for RNA probes were amplified from these plasmids by using T7 and SP6 primers. Digoxigenin (DIG)-labeled RNA probes were synthesized from the fragments using either T7 or SP6 RNA polymerase (Roche). Signals were detected using either an alkaline phosphatase–conjugated anti-DIG Fab fragment (Roche) or a horseradish peroxidase–conjugated anti-DIG antibody (Roche). In the latter case, signal was amplified using the TSA Biotin System and Streptavidin- FITC (PerkinElmer).

Immunostaining

Immunofluorescence staining of embryos was carried out as previously describe. I collected embryos derived from *nos*^{BN}*/TM3* or *nos*^{BN} females mated with *w*^{1118} males. The following primary antibodies were used at the indicated dilutions: rabbit anti-GFP (1:500, A11122; Life Technologies), chick anti-Vasa (1:500), and guinea pig anti-CG32425 antibody (1:200). Anti-CG32425 antibody was raised against a synthetic peptide (STAAQNLRNSNGQE) (GL Biochem). To detect primary antibodies, the following secondary antibodies were used: Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:500, A11034; Molecular Probes), Alexa Fluor 546-conjugated goat anti-chick IgY (1:500, A11040; Molecular Probes), and Alexa Fluor 488-conjugated goat anti-guinea pig IgG antibody (1:500, A11073; Molecular Probes). Stained embryos were

mounted in Vectashield (Vector Laboratories) and observed under a confocal microscope (Leica Microsystems).

Functional analysis of *CG32425*

To reduce maternal *CG32425*, I induced the expression of a double-strand RNA during oogenesis. I obtained female adults either with (control females) or without *Cy* phenotype (maternal-RNAi females) derived from *w;; nos-Gal4* parental females mated with *y w; UAS-CG32425 dsRNA/CyO* (Bloomington Drosophila stock center; 51457) males. Control and maternal-RNAi females were mated with w^{1118} males, and their adult progeny were dissected in order to inspect the morphology of their ovaries and testes. Ovaries with no mature eggs and regressed testes shorter than 1 mm in length were considered dysgenic gonads.

Results

***CG32425* 3'UTR mediated Nos-dependent upregulation of RNA and protein expression in PGCs**

In the course of examining the role of the 3' UTR from the mRNAs immuno-precipitated with Pum protein in embryos, I have obtained a preliminary result suggesting that *CG32425* 3' UTR acts as an RNA region that enhances protein production from mRNA that contains it in normal PGCs, but not in PGCs lacking maternal Nos (*nos* PGCs). Since this result was in contrast to the prevailing role of Nos, which binds to the 3' UTR of target mRNAs to repress their protein production in PGCs, I focused on the role of *CG32425* 3' UTR. To confirm this preliminary results, I generated a fusion gene, which carries *CG32425* 3' UTR at a location downstream of EGFP-coding region (*UAS-EGFP-CG32425 3'UTR*), and the resulting fusion gene was inserted into the cytogenetic locus, 86, located on the right arm of the third chromosome by using the P [acman] recombination technique. This fusion gene was transcribed in PGCs by using Gal4/UAS system, and EGFP protein expression was examined in PGCs of late embryos (stage 12-15). I found that EGFP expression from

UAS-EGFP-CG32425 3'UTR was much lower in *nos* PGCs than in normal ones. Furthermore, mRNA expression from *UAS-EGFP-CG32425 3'UTR* was significantly reduced in *nos* PGCs compared to normal ones.

Identification of regions required for Nos-dependent upregulation of RNA and protein expression in *CG32425* 3'UTR

To identify the regions required for Nos-dependent upregulation of RNA and protein expression in PGCs, *CG32425* 3' UTR was deleted to produce three regions, 1, 2, and 3, and each 3' UTR region was fused downstream of UASp-EGFP (*UASp-EGFP-R1*, *-R2*, and *-R3*, respectively). These transgenes were transformed and activated in PGCs by maternal *nos*-Gal4, and these regions were tested for their effects on RNA and protein expression in the EGFP-reporter assay system described above. RNA and protein expression from *UAS-EGFP-R3* was reduced in *nos* PGCs, compared with that in normal PGCs. In contrast, RNA and protein expression from *UAS-EGFP-R1* and *-R2* were slightly higher in *nos* PGCs than in normal ones.

Expression of endogenous *CG32425* mRNA and its protein product in PGCs

To examine the expression of CG32425 mRNA with the 3' UTR in PGCs, I performed in situ hybridization of whole embryos using a digoxygenin (DIG)-labeled probe. *CG32425* mRNA was distributed throughout cleavage and syncytial blastodermal embryos at stage 4. RNA signal rapidly decreased in the somatic region, but was enriched in PGCs of cellular blastodermal embryos at late stage 5. During the rest of embryogenesis, the signal remained detectable in PGCs. An essentially identical expression pattern was observed by fluorescence in situ hybridization (FISH) with probes for the region identical to the DIG-labeled one. CG32425 protein expression was almost undetectable in PGCs from embryonic stages 4 to 8. The protein expression was later increased in PGCs from stage 9 until the end of embryogenesis. In contrast, in *nos* PGCs, expression of *CG32425* mRNA and its protein product was much lower than that observed in normal ones.

Discussion

Stabilization of *CG32425* mRNA in PGCs by maternal Nos protein via *CG32425* 3' UTR

In this study, I found that the expression of *EGFP CG32425 3'UTR* mRNA increased in PGCs in a Nos-dependent manner, while the expression of *EGFP-K10 3'UTR* and -c*ycB 3'UTR* mRNAs was similar in normal and *nos* PGCs. I expected that these three mRNAs are initially produced at an identical level in normal and *nos* PGCs, because they are transcribed from the same promoter activated by *nos*-Gal4. Thus, the alteration of *EGFP-CG32425 3'UTR* mRNA expression in *nos* PGCs occurs at a post-transcriptional level. Therefore, it is reasonable to conclude that *EGFP-CG32425 3'UTR* mRNA is stabilized in PGCs in a Nos-dependent manner via its 3' UTR.

***CG32425* 3' UTR contains a region that promotes RNA stabilization in PGCs**

In deletion experiment of *CG32425* 3' UTR, I found that the expression level of *EGFP-R3* mRNA, but not *EGFP-R2*, was reduced in *nos* PGCs compared with normal PGCs. This strongly suggests that the regulatory element for RNA stabilization is present in the region, which is included in region 3, but not in region 2. I also found that the levels of RNA and protein expressed from *UASp-EGFP-R1* and *-R2* transgenes in *nos* PGCs was higher than those in normal PGCs. This suggests that the regulatory region, which is included in both region 1 and region 2, contains the element for Nos-dependent RNA degradation. Interestingly, this region contains one Nos- and one Pum-binding site separated by a short intervening sequence. A similar arrangement of these binding sites is observed in *cycB* 3' UTR, which is required for Nos-dependent translational repression. Nos and Pum proteins bind to *cycB* 3' UTR through the Nos- and Pum-binding sites, respectively. These proteins recruit the deadenylase CCR4-NOT complex to shorten poly(A) tails, which consequently represses translation of *cycB* mRNA. I speculate that a similar interaction among Nos, Pum, and the CCR4-NOT complex occurs in this regulatory region to cause the deadenylation of EGFP-R1 and -R2 mRNA, which, in turn, induces their destabilization and translational repression. By contrast, the region, which is included in region 3, but not in region 2 contains four Nos-binding sites and only one Pum-binding site that overlaps with one of the four Nos-binding sites. This leads us to speculate that Nos protein, along with a cofactor other than Pum, binds to the region included in region 3 to stabilize the mRNA. Thus, I assume that Nos protein stabilizes mRNA in a Pum-independent manner.

Nos-dependent stabilization of *CG32425* mRNA in PGCs

CG32425 mRNA is maternally supplied throughout early embryos, but is degraded rapidly in the somatic region at stage 5. The remaining *CG32425* mRNA in PGCs persists until at least the end of embryogenesis. Translation of *CG32425* mRNA initiates after stage 9, which in turn accumulates CG32425 protein in PGCs. During embryogenesis, maternal Nos protein is critical for the expression of *CG32425* mRNA and its protein product in PGCs. My observation that the maternal RNAi for *CG32425* eliminated CG32425 protein expression in PGCs throughout embryogenesis indicates that maternal *CG32425* mRNA is dominant in PGCs, and the zygotic level of *CG32425 mRNA* is low, if any, in these cells. Thus, it is reasonable to conclude that maternal *CG32425* mRNA is stabilized in PGCs by maternal Nos protein. In the somatic region of early embryos, maternal *CG32425* mRNA was rapidly degraded. Maternal RNAs are degraded in early embryos during the maternal to zygotic transition (MZT) by specific decay mechanisms that involve RNA binding proteins [e.g., Smaug (Smg) and Pum], and small noncoding RNAs [e.g., microRNAs and Piwi-interacting RNAs (piRNAs)] to recruit the CCR4-NOT complex to the target RNAs. Although it remains unclear whether these decay mechanisms work in PGCs, Nos protein may associate with a cofactor other than Pum on 3' UTR of *CG32425* mRNA to repress or antagonize the CCR4-NOT-dependent decay mechanisms.

Nos has long been known as a translational repressor in various animal species, including *Drosophila melanogaster*. However, I report for the first time here that maternal Nos acts to stabilize specific mRNAs. Thus, Nos protein has dual functions in translational repression and the stabilization of specific RNAs.