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Staufen1, Kinesin1 and microtubule function in *cyclin B1* mRNA transport to the animal polar cytoplasm of zebrafish oocytes

Kazuki Takahashi [a], Kana Ishii [a], Masakane Yamashita [b, *]

[a] Laboratory of Reproductive and Developmental Biology, Graduate School of Life Science, Hokkaido University, Sapporo, 060-0810 Japan
[b] Laboratory of Reproductive and Developmental Biology, Department of Biological Sciences, Faculty of Science, Hokkaido University, Sapporo 060-0810, Japan

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ABSTRACT

In zebrafish oocytes, *cyclin B1* mRNAs are transported to the animal polar cytoplasm. To elucidate the molecular basis of *cyclin B1* mRNA transport, we analyzed zebrafish Staufen1, a protein known to play a central role in mRNA transport to the vegetal pole of *Xenopus* oocytes. Zebrafish Staufen1 interacts with *cyclin B1* mRNA throughout oocyte growth. Both *cyclin B1* mRNA and Staufen1 are evenly distributed in the cytoplasm of young oocytes but are co-localized to the animal polar cytoplasm in later stages. Real-time imaging showed that the plus ends of oocyte microtubules are free in the cytoplasm in early stages but anchored to the animal polar cytoplasm in later stages. Transport of *cyclin B1* reporter mRNA to the animal polar cytoplasm was inhibited by disruption of microtubules and injection of antibodies against Staufen1 or Kinesin1, a plus-end-directed microtubule motor that interacts with Staufen1, indicating that the transport depends on movement along microtubules toward the plus ends. Reporter mRNAs with an element required for the vegetal localization of *vg1* mRNA in *Xenopus* oocytes were localized to the animal polar cytoplasm in zebrafish oocytes, indicating that the element is functional for animal polar localization in zebrafish oocytes. Our findings suggest that *cyclin B1* mRNA-Staufen1 protein complexes are transported toward the animal pole of zebrafish oocytes by the plus-end-directed motor protein Kinesin1 along microtubules and that a common mRNA transport machinery functions in zebrafish and *Xenopus* oocytes, although its transport direction is opposite due to different organizations of microtubules.

1. Introduction

Specific subcellular localization of mRNAs enables spatio-temporal control of gene expression, which assures normal progression of various biological events. Cyclin B1 protein, a regulatory subunit of maturation/M-phase-promoting factor, is absent in immature zebrafish oocytes [1,2], because its mRNA is translationally repressed and localized as aggregates to the animal polar cytoplasm until the oocytes are stimulated by maturation-inducing hormone [3,4]. We previously reported that *cyclin B1* mRNA localization in zebrafish oocytes and its translational timing after hormonal stimulation are regulated by at least two components, a *cis*-acting element situated in the open-reading frame (ORF) and Pumilio1 (Pum1) bound to the Pumilio-binding element (PBE) in the 3' UTR of *cyclin B1* mRNA, based on the findings that *cyclin B1* reporter mRNA with mutations in the ORF element and PBE failed to stay in the animal polar cytoplasm and underwent precocious translational activation [5–7]. However, the mutant mRNAs were transported from the nucleus to the animal polar cytoplasm, although they were not anchored there [6]. These findings indicate that the mechanisms of *cyclin B1* mRNA transport is different from those of its anchoring to the animal polar cytoplasm. The transport mechanism of *cyclin B1* mRNA in zebrafish oocytes is largely unknown.

We previously identified Insulin-like growth factor 2 mRNA-binding protein 3 (IMP3) as a *cyclin B1* mRNA-binding protein and suggested its involvement in translational regulation [8]. In *Xenopus* oocytes, IMP3 plays a crucial role in the localization of *vg1* mRNA to the vegetal cortex [9,10]. In zebrafish oocytes, however, IMP3 and its binding targets, *vg1* mRNA and *cyclin B1* mRNA, are

* Corresponding author. Laboratory of Reproductive and Developmental Biology, Department of Biological Sciences, Faculty of Science, Hokkaido University, North 10, West 8, Sapporo 060-0810, Japan.
E-mail address: myama@sci.hokudai.ac.jp (M. Yamashita).

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localized to the animal polar cytoplasm but not to the vegetal polar cytoplasm [8]. Therefore, it remains elusive whether a pathway similar to that in *Xenopus* oocytes exists for the animal polar localization of *vg1* and *cyclin B1* mRNAs in zebrafish oocytes.

Staufen1 (Stau1) is thought to play an important role in mRNA transport to the vegetal pole of *Xenopus* oocytes, in collaboration with microtubules and Kinesin1 [11]. In this study, we investigated the involvement of Stau1, Kinesin1 and microtubule in *cyclin B1* mRNA transport to the animal polar cytoplasm in zebrafish oocytes, with reference to the generality and diversity of mRNA transport mechanisms in fish and amphibian oocytes.

2. Materials and methods

2.1. Oocyte culture and extraction

All animal experiments in this study were approved by the Committee on Animal Experimentation, Hokkaido University (permission No. 13–0099). Oocyte culture and extraction were carried out as described previously [8]. For immunoprecipitation analysis, Triton X-100 (1% at the final concentration) was added to the extracts.

2.2. Production of recombinant proteins and antibodies

To produce N-terminally Flag-tagged Stau1 (Flag-Stau1), an ORF of zebrafish Stau1 was amplified with a primer set of Stau1_F and Stau1_R (Supplementary Table 1), which were designed according to a sequence, NM_205561.1, deposited in the NCBI nucleotide database. The PCR product was inserted into the *EcoR*I-digested pCS2+FT-N vector [12] using an In-Fusion HD Cloning Kit (TaKaRa).

A cDNA fragment of zebrafish Stau1 was amplified with a primer set of Stau1_exF and Stau1_exR (Supplementary Table 1) and inserted into the pENTR/D-TOPO Gateway vector (Invitrogen). The resulting plasmids were recombined with the destination vector pET161-DEST using a Gateway cloning system (Invitrogen) to produce Stau1 tagged with a polyhistidine (His) at the C terminus (Stau1-His). Stau1-His was expressed in *E. coli* BL21 (DE3) and purified according to the method described previously [13]. Polyclonal antibodies against Stau1-His were produced in mice (anti-Stau1 (M)) and guinea pigs (anti-Stau1 (GP)) and affinity-purified with antigenic Stau1-His protein as described previously [8]. Similarly, a mouse polyclonal antibody against End-binding protein 1 (EB1) was raised against zebrafish EB1 (NM_213640.1). In brief, a cDNA fragment of zebrafish EB1 was amplified with a primer set of EB1_exF and EB1_exR (Supplementary Table 1). *E. coli*-produced EB1-His proteins were injected into mice.

2.3. Immunoblotting and immunoprecipitation

Immunoblotting was performed as described previously [14], using anti-Stau1 (M), anti-Stau1 (GP), anti-EB1, anti-IMP3 [8], anti-α-Tubulin (T9026; Sigma-Aldrich), anti-Pum1 [5] or anti-Flag M2 (F1804; Sigma-Aldrich) antibodies. All immunoprecipitation assays in this study were performed using crude extracts equivalent to 10–30 full-grown immature (stage IV) oocytes (100–300 μg proteins). Interaction of Stau1 with Pum1 or IMP3 was examined by co-immunoprecipitation assays, as described previously [8].

2.4. RT-PCR and quantitative real-time RT–PCR (qRT-PCR)

Oocyte extracts (100 μg protein) were subjected to RT-PCR [8], using the primer sequences shown in Supplementary Table 1. For qRT-PCR analysis, cDNA was synthesized with a PrimeScript RT Master Mix (Perfect Real Time) (TaKaRa) according to the manufacturer's instructions. Real-time PCR amplification was performed with a SYBR Premix Ex *Taq*II (Tli RNaseH Plus) (TaKaRa) on Thermal Cycler Dice Real Time System II (TaKaRa).

2.5. Section in situ hybridization, immunostaining and whole-mount in situ hybridization

Histological sections were simultaneously analyzed by *in situ* hybridization and immunostaining [5]. The sections were observed on an LSM 5 LIVE microscope equipped with Plan-Apochromat 10x/0.45 M27 and Plan-Apochromat 20x/0.8 M27 objective lenses (Carl Zeiss). Images were acquired with LSM 5 DUO 4.2 software (Carl Zeiss) and processed with Photoshop and Illustrator (Adobe). Whole-mount *in situ* hybridization was performed according to the procedure reported previously [15].

2.6. Production of reporter mRNAs

A construct containing vg1/vegetal localization element (VLE), a *cis*-acting element responsible for the transport of *vg1* mRNA to the vegetal pole of *Xenopus* oocytes [11], was produced by replacing the *cyclin B1* 3′ UTR of *tgo3*′ [6] with the *Xenopus* VLE sequence [11,16]. Using total cDNA from *Xenopus* oocytes as a template, *Xenopus* VLE was amplified with a primer set of xVg1VLE_F and xVg1VLE_R and cloned into the *tgo3*′ vector amplified with a primer set of cyclin-B1_ORF_R and pT2KXIGΔin_BglII_F (Supplementary Table 1) using an In-Fusion HD Cloning Kit (TaKaRa). The SV40 reporter construct was produced as described previously [7].

For an *in vivo* reporter mRNA localization assay, full-grown immature (stage IV) oocytes were co-injected with distilled water containing 250 pg of reporter vectors that encode *gfp-cyclin B1* mRNA and either 10 ng/μl of nocodazole, 100 ng/μl of anti-GST [14], anti-Kinesin1 (SUK4; DSHB; reported to inhibit the Kinesin1 function in *Xenopus* oocytes [11,17]) or anti-Stau1 (M) antibodies. After injection, oocytes were incubated in culture medium for 4 h at room temperature. The oocytes were fixed with 4% paraformaldehyde and subjected to whole-mount *in situ* hybridization. To quantify reporter mRNAs produced from the injected vectors, total RNA extracted from 10 injected oocytes was subjected to qRT-PCR using primer sets for *gfp* and *β-actin* (Supplementary Table 1). The content of *gfp-cyclin B1* reporter mRNA was normalized to that of *β-actin* mRNA.

2.7. Real-time imaging of the plus end of microtubules in oocytes

A transgenic zebrafish that expresses EB1-GFP [Tg(-0.5zp3b:Mmu.Mapre1-GFP)/nub1Tg] [18] was provided from National BioResource Project (NBRP) Zebrafish, Japan. The movement of EB1-GFP signals in stage I and IV oocytes was traced under a confocal laser microscope LSM 880 with Airyscan (Carl Zeiss).

3. Results

3.1. Behavior of zebrafish Stau1 during oocyte growth

Newly produced anti-Stau1antibodies recognized a single band of ca. 70 kDa, the size of which is comparable to that of Stau1 consisting of 702 amino acids, in oocyte extracts and immunoprecipitates (Supplementary Fig. 1A and B). Since Flag-Stau1 overexpressed in zebrafish oocytes also showed a similar size (cf. Fig. 1B), we conclude that the 70-kDa band is Stau1 in zebrafish oocytes.

The expression levels of Stau1 during oocyte growth were examined by immunoblotting using oocytes classified into 4 stages. Stages I to III are growing and stage IV is full-grown immature oocytes [19]. Zebrafish Stau1 was detected in stage I oocytes,

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