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

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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.

Keywords: Kinesin1, microtubule, mRNA transport, Staufen1, zebrafish oocyte

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 [5,6,7]. 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 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 cyclinB1_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 hr 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, although its level was very low. The expression levels of Stau1 increased rapidly in stage II oocytes but decreased gradually as the oocyte increased in size (Fig. 1A). The expression levels of α-Tubulin were low in stage I oocytes and increased rapidly in stage II. In contrast to Stau1, however, the expression levels of α-Tubulin increased during the oocyte growth (Fig. 1A).

3.2. Interaction of Stau1 with cyclin B1 mRNA in oocytes

Anti-Flag immunoprecipitates from Flag-Stau1-overexpressing oocytes but not control precipitates contained *cyclin B1* mRNA (Fig. 1B), indicating that Flag-Stau1 binds to *cyclin B1* mRNA in oocytes. Since Pum1 [5] and IMP3 [8] are *cyclin B1* mRNA-binding proteins in zebrafish oocytes, we examined the interaction of endogenous proteins, between Stau1and Pum1 (Fig. 1C) or IMP3 (Fig. 1D) in oocytes by a co-immunoprecipitation assay. The results clearly showed that the interaction of Stau1 with Pum1 or IMP3. The interaction disappeared by RNase treatment, indicating that Stau1 does not bind directly to Pum1 or IMP3 but binds via *cyclin B1* or other mRNAs. Interaction of endogenous Stau1 with *cyclin B1* mRNA during oocyte growth (stages I to IV) was analyzed by immunoprecipitation followed by RT-PCR. In all of the stages, the interaction was detected (Fig. 1E).

3.3 Subcellular distribution of Stau1 and cyclin B1 mRNA during oocyte growth

The subcellular distribution of *cyclin B1* mRNA changes during oocyte growth in zebrafish [3]. Since Stau1 continues to interact with *cyclin B1* mRNA (Fig. 1E), we investigated the subcellular distribution of Stau1 in oocytes from stages I to IV by immunostaining with anti-Stau1 (M) antibody. Control experiments without anti-Stau1 (M) antibody showed no clear signals. Control hybridization with the DIG-labeled sense *gfp* probe also showed no specific signals.

Anti-Stau1 (M) immunostaining revealed changes in subcellular distribution of Stau1 during oogenesis (Fig. 2). In stage I oocytes, Stau1 was distributed throughout the cytoplasm like *cyclin B1* mRNA, with strong signals at the cortical cytoplasm of oocytes (Fig. 2A1-3). In stage II oocytes, some signals of both *cyclin B1* mRNA and Stau1 were localized to the animal polar cytoplasm (Fig. 2B1-3). In stage III oocytes, the vast majority of *cyclin B1* mRNA was localized to the animal polar cytoplasm, while Stau1 was distributed around the entire oocyte cortex and the periphery of the nucleus (Fig. 2C1-6). In stage IV oocytes, the animal polar distributions of both *cyclin B1* mRNA and Stau1 became tighter than those in stage III oocytes (Fig. 2D1-6).

3.4. Involvement of microtubule, Kinesin1 and Stau1 in cyclin B1 mRNA transport to the animal polar cytoplasm

When a DNA construct that expresses *gfp-cyclin B1* reporter mRNA (Fig. 3A, 3' UTR) was injected

into the nucleus of full-grown zebrafish oocytes, reporter mRNAs transcribed in the nucleus were transported and anchored to the animal polar cytoplasm and formed aggregates, as in the case of endogenous *cyclin B1* mRNAs (Fig. 3A and B) [6,7]. We therefore used this system to examine the involvement of microtubule, Stau1 and Kinesin1 in *cyclin B1* mRNA transport in zebrafish oocytes, since these molecules are known to function in mRNA transport in *Xenopus* oocytes [11].

Nocodazole abolished the transport of reporter mRNAs to the animal polar cytoplasm (Supplementary Fig. 2A), suggesting that the transport depends on microtubules, in contrast to anchoring to the animal polar cytoplasm which depends on microfilaments but not on microtubules [3,5,7]. In comparison with water or anti-GST antibody as controls, anti-Kinesin1 or anti-Stau1 antibodies inhibited the transport of reporter mRNAs (Supplementary Fig. 2A). In these experiments, expression levels of reporter mRNAs showed no statistically significant differences (Supplementary Fig. 2B). These results suggest the involvement of microtubule, Kinesin1 and Stau1 in mRNA transport in zebrafish oocytes, as in the case of *Xenopus*.

3.5. VLE-directed mRNA transport in zebrafish oocytes

In *Xenopus* oocytes, Stau1 transports *vg1* mRNA to the vegetal pole via a *cis*-acting element of the mRNA called VLE [11]. We analyzed the behavior of *gfp-cyclin B1* reporter mRNA harboring VLE instead of *cyclin B1* 3' UTR (Fig. 3A-C). Consistent with results previously reported [5,7], *gfp-cyclin B1* reporter mRNAs with the 3' UTR (a positive control) showed aggregated signals localized to the animal polar cytoplasm, whereas those harboring SV40 polyadenylation signal (a negative control) were not localized to any regions. The reporter mRNAs harboring VLE were apparently localized to the animal polar cytoplasm. Although the localization rate was lower than that of the reporter mRNAs harboring *cyclin B1* 3' UTR, this finding suggests that the VLE sequence is functional in zebrafish oocytes as a *cis*-acting element for animal polar localization, different from its original function as an element for vegetal polar localization in *Xenopus* oocytes.

We checked interaction between Stau1 and reporter mRNAs by anti-Stau1 immunoprecipitation followed by RT-PCR. Both VLE and 3' UTR reporter mRNAs, but not SV40 reporter mRNA as a control, were detected in anti-Stau1 immunoprecipitates (Fig. 3D), indicating that Stau1 binds to VLE reporter mRNA. Thus, it is plausible that VLE is recognized by Stau1 that is a component of the mRNA transport machinery to the animal pole of zebrafish oocytes.

3.6. Microtubule orientation in zebrafish oocytes

Since our results suggested that Kinesin1, a motor protein that drives cargos toward the plus ends of microtubules, is involved in *cyclin B1* mRNA transport (Supplementary Fig. 2A), we investigated the orientation of microtubules in zebrafish oocytes by immunostaining of EB1, a microtubule plus-end

tracking protein [20]. A newly produced antibody against zebrafish EB1 (Supplementary Fig. 1C) revealed that EB1 was accumulated in the animal polar cytoplasm of full-grown immature (stage IV) oocytes, whereas it showed no clear accumulation in other regions including the vegetal polar cytoplasm (Fig. 4).

We also observed the behavior of plus end of microtubules by real-time imaging of transgenic zebrafish oocytes that express EB1-GFP [18]. EB1-GFP signals were evenly distributed in stage I oocytes (Supplementary Fig. 3A). In stage IV oocytes, however, some of them were accumulated to the animal polar cytoplasm (Supplementary Fig. 3B). We also found that EB1-GFP signals move randomly in the whole cytoplasm of stage I oocytes (Supplementary Fig. 3C, Supplementary Movie 1), while they are almost immovable in the animal polar cytoplasm in stage IV oocytes (Supplementary Fig. 3D, Supplementary Movie 2). These findings indicate that the orientation of microtubules is not fixed in young oocytes because their plus ends can move freely in the oocytes. In later stages, the plus ends become anchored to the animal polar cytoplasm, which allows microtubules to organize their plus ends toward the animal pole.

4. Discussion

We showed that *gfp-cyclin B1* reporter mRNA harboring VLE, a sequence essential for vegetal polar transport of *vg1* mRNA in *Xenopus* oocytes, was transported to the animal polar cytoplasm in zebrafish oocytes (Fig. 3C). These results suggest that a conserved mechanism functions in the intracellular transport of zebrafish *cyclin B1* mRNA and *Xenopus vg1* mRNA, although the directions of transport are opposite (animal pole in zebrafish and vegetal pole in *Xenopus*). Concerning the difference in direction, it is noteworthy that the microtubule plus end-tracking protein EB1 accumulates in the animal polar cytoplasm of full-grown immature zebrafish oocytes (Fig. 4). In full-grown immature *Xenopus* oocytes, the majority of microtubules are oriented with the minus ends towards the cortex and a subpopulation of microtubules with plus ends directs toward the vegetal pole [17,21]. Although the organization of microtubules in zebrafish oocytes remains to be examined in detail, differences in the direction of mRNA transport might be simply explainable by differences in the microtubule organization between zebrafish and *Xenopus* oocytes.

At least two types of Stau1 can be recognized in zebrafish oocytes: one is co-localized to *cyclin B1* mRNA and the other is not (Fig. 2). Stau1 should interact with various mRNAs in oocytes, besides *cyclin B1* mRNA. Indeed, we found that Stau1 interacts with *zorba*, *mos*, *vg1* and *buc* mRNAs but not with *dazl*, *brul* and *vasa* mRNAs in zebrafish oocytes (data not shown). According to the localization of these mRNAs in oocytes [22,23,24], it is likely that Stau1 preferentially binds to mRNAs that are finally localized to the animal polar cytoplasm of full-grown immature oocytes, although molecular mechanism that allows Stau1 to bind to specific mRNAs remains a mystery. Our findings suggest the involvement of

Stau1 in *cyclin B1* mRNA transport. However, it is also plausible that other molecules including IMP3 contribute to *cyclin B1* mRNA transport, since IMP3 is bound to Stau1 via *cyclin B1* mRNA (Fig. 1D) and localized to the animal polar cytoplasm [8]. In addition, our preliminary experiment showed that an anti-IMP3 antibody inhibits the animal polar localization of *cyclin B1* mRNA. A better understanding of the molecular composition of *cyclin B1* mRNA transport machinery is necessary.

We found that *cyclin B1* mRNA and Stau1 are bound to each other (Fig. 1E) but distributed throughout the cytoplasm of stage I oocytes (Fig. 2A), suggesting that the binding of Stau1 to *cyclin B1* mRNA is insufficient to initiate mRNA transport. What is required to initiate *cyclin B1* mRNA transport? The finding that protein levels of α-Tubulin are low in stage I oocytes and increase with oocyte growth (Fig. 1A), raising the possibility that microtubules are not fully organized to a sufficient level for transporting the Stau1-*cyclin B1* mRNA complex to the animal pole in stage I oocytes. Real-time imaging of the plus-ends of microtubules revealed that they move randomly in stage I oocytes (Supplementary Fig. 3C and Supplementary Movie 1), indicating that the direction of microtubules is not fixed in this stage. Further studies are required to understand what organization of microtubules is required for *cyclin B1* mRNA transport and what molecules are involved in it. Especially, the findings in this study suggest that anchoring of the plus ends of microtubules to the animal polar cytoplasm is a key event for the initiation of *cyclin B1* mRNA transport (Fig. 4). Our next goal is to determine the anchoring mechanism.

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Figure legends

Fig. 1. Staufen1 protein (Stau1) during zebrafish oocyte growth. (A) Oocyte extracts from stages I to IV were analyzed by immunoblotting with anti-Stau1 (GP) and anti-α-Tubulin antibodies. (B) Stage IV oocytes overexpressed with (+) or without (-) Flag-Stau1 were immunoprecipitated with (+) or without (-) anti-Flag M2 antibody (Flag IP). Flag-Stau1 protein and *cyclin B1* mRNA in the immunoprecipitates were detected by immunoblotting with anti-Flag M2 antibody and RT-PCR, respectively. (C) Extracts from stage IV oocytes were treated with (+) or without (-) RNase A and immunoprecipitated with anti-Pum1, anti-Stau1 or control antibodies (-). Pum1 and Stau1 in the immunoprecipitates were detected by immunoblotting. (D) Experiments similar to those in (C) using anti-IMP3 and anti-Stau1 antibodies. (E) Extracts from stage I to IV oocytes were immunoprecipitated (Stau1 IP) with anti-Stau1 (+) or control antibodies (-) and *cyclin B1* mRNA in the immunoprecipitates was detected by RT-PCR. Extracts from stage IV oocytes before immunoprecipitation were also subjected to RT-PCR to confirm the presence of *cyclin B1* mRNA in the original extracts (initial).

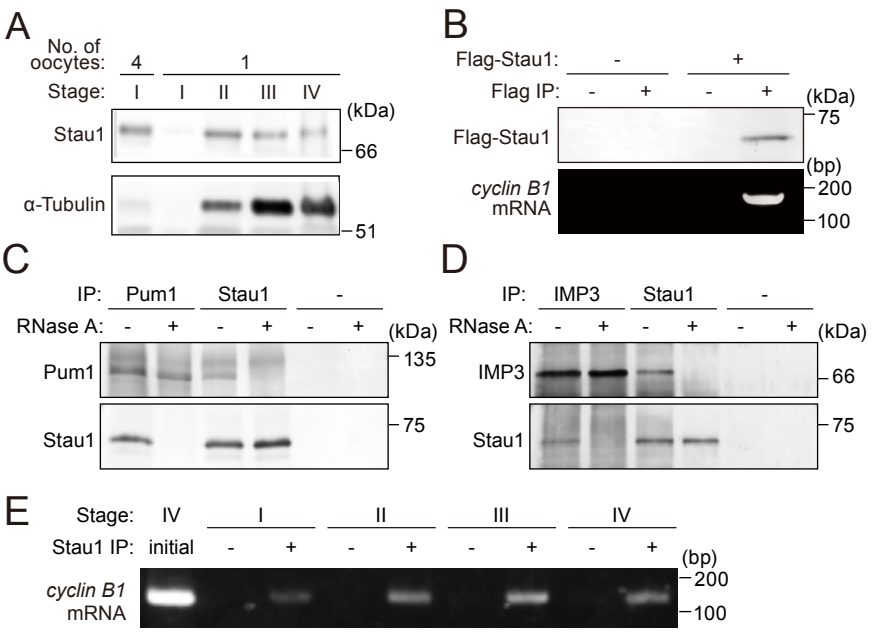
Fig. 2. Distribution of *cyclin B1* mRNA and Stau1 protein during zebrafish oocyte growth. Paraffin sections of zebrafish oocytes were simultaneously analyzed by *in situ* hybridization and immunostaining. Hoechst 33258 staining of DNA (C, D), *in situ* hybridization of *cyclin B1* mRNA (A1; B1; C1, 4; D1, 4), immunostaining of Stau1 (A2; B2; C2, 5; D2, 5) and their merged and magnified images (A3; B3; C3, 6; D3, 6) are shown. CA, cortical alveolus; EC, egg chorion; FC, follicle cell; GV, germinal vesicle; MP, micropyle; YG, yolk granule. Scale bars, 100 μm.

Fig. 3. Effects of 3' UTR sequences on the *gfp-cyclin B1* reporter mRNA localization to the animal polar cytoplasm. (A) Structures of reporter mRNAs. The *cyclin B1* 3' UTR sequence was replaced with either *Xenopus* vg1/vegetal localization element (VLE) or SV40 polyadenylation signal (SV40) sequences. (B) Reporter mRNAs were detected by whole-mount *in situ* hybridization using a *gfp* probe, and the resulting signals were classified into 5 groups (Aggregated, Class I, II, III and Not localized: Class I, signal restricted to less than 1/4 of the oocyte hemisphere; class II, signal ranging from 1/4 to 1/2 of the hemisphere; and class III, signal expanding over 1/2 of the hemisphere [6]). Localization of endogenous *cyclin B1* mRNAs is also shown (Wild type). Arrows indicate localized signals. Scale bars, 100 μm. (C) The numbers of oocytes showing each localized signal were counted. Data were collected from three independent experiments. The numbers in parentheses indicate the total numbers of oocytes analyzed. (D) Extracts from oocytes injected with reporter constructs (3' UTR, VLE, SV40) were subjected to immunoprecipitation with (+) or without (-) anti-Stau1 antibody. The reporter mRNAs in the immunoprecipitates (Stau1 IP) and extracts before immunoprecipitation (initial) were detected by

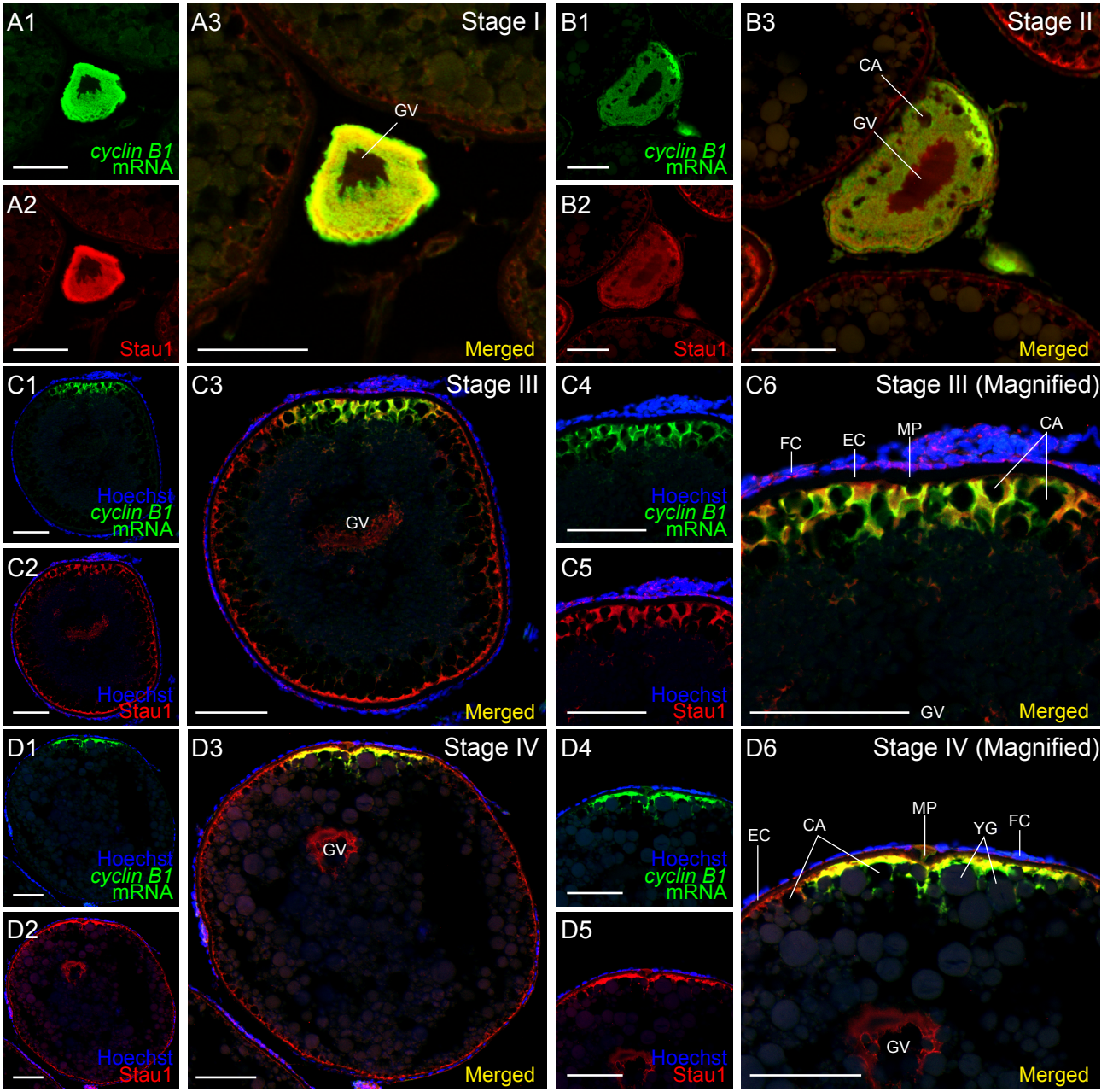
RT-PCR using the *gfp* sequence.

Fig. 4. Distribution of EB1 in zebrafish stage IV oocytes revealed by *in situ* hybridization of *cyclin B1* mRNA, immunostaining of EB1 and merged images. The animal (upper box) and vegetal (lower box) polar regions in (A) are magnified in (B) and (C), respectively. Hoechst 33258 staining of DNA is shown in blue. Broken lines indicate the boundary between the oocyte and follicle layer (B, C). Scale bars, 100 μm (A), 50 μm (B, C).

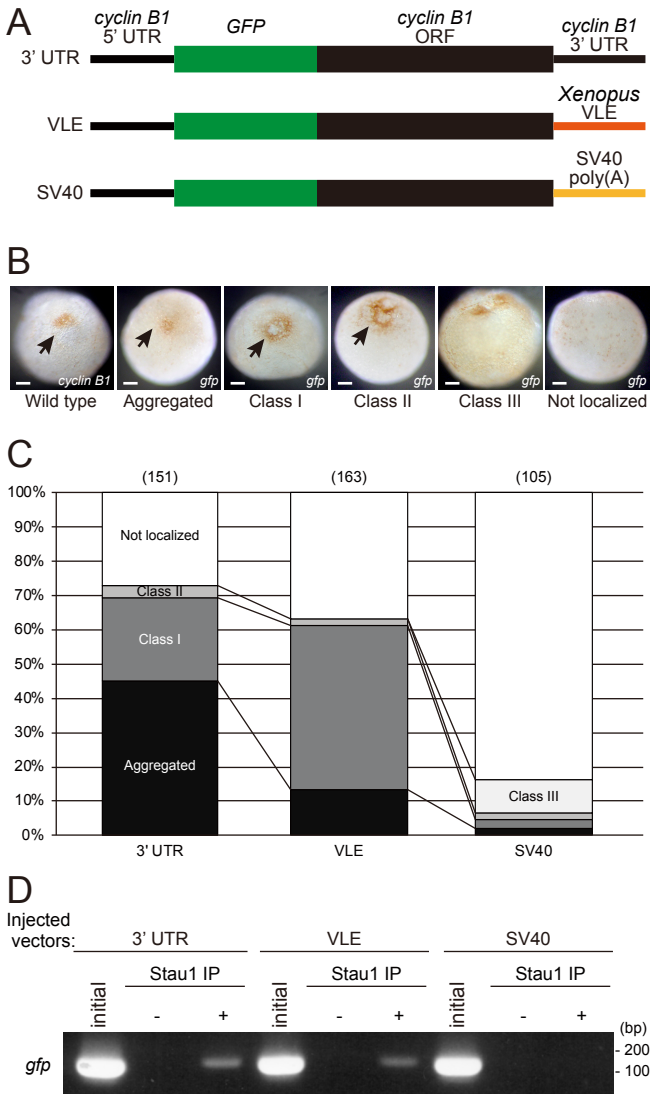
Takahashi et al. Fig. 1



Takahashi et al. Fig. 2



Takahashi et al. Fig. 3



Takahashi et al. Fig. 4