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The subcellular localization of cyclin B2 is required for bipolar spindle formation during *Xenopus* oocyte maturation

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ABSTRACT

Cyclins B1 and B2 are subtypes of cyclin B, a regulatory subunit of a maturation/M-phase promoting factor, and they are also highly conserved in many vertebrate species. Cyclin B1 is essential for mitosis, whereas cyclin B2 is regarded as dispensable. However, the overexpression of the cyclin B2 N-terminus containing the cytoplasmic retention signal, but not cyclin B1, inhibits bipolar spindle formation in *Xenopus* oocytes and embryos. Here we show that endogenous cyclin B2 was localized in and around the germinal vesicle. The perinuclear localization of cyclin B2 was perturbed by the overexpression of its N-terminus containing the cytoplasmic retention signal, which resulted in a spindle defect. This spindle defect was rescued by the overexpression of bipolar kinesin Eg5, which is located at the perinuclear region in the proximity of endogenous cyclin B2. These results demonstrate that the proper localization of cyclin B2 is essential for bipolar spindle formation in *Xenopus* oocytes.

1. Introduction

A maturation/M-phase promoting factor, which consists of cyclin B and Cdc2 kinase, regulates multiple aspects of M-phase, including a nuclear envelope breakdown, the chromosome condensation and spindle formation [1,2]. Although cell division requires a continuity of each mitotic event, little is known about how a maturation/M-phase promoting factor regulates the coordination of these mitotic ones.

In many species, B-type cyclin has several subtypes [3–6]; especially cyclins B1 and B2 have only been described in vertebrates [4]. Cyclin B1-null mice die *in utero*, and cyclin B1/Cdc2 is able to regulate almost all mitotic events in CHO cells [7,8]. In contrast, cyclin B2-null mice develop normally and are fertile, whereas cyclin B2/Cdc2 only disassemble Golgi apparatus in CHO cells [7,8]. These data indicate that cyclin B1 is an essential gene, but that cyclin B2 is regarded as dispensable.

However, cyclin B1 is inessential for *Xenopus* oocyte maturation [5]. Cyclins B1 and B2 are associated with Cdc2 kinase and are active only in mitosis [9]. In human cells, cyclins B1 and B2 differ in their subcellular localization, which is due to a cytoplasmic retention signal (CRS) [10,11]. The overexpression of the cyclin B2 N-terminus containing the CRS domain, but not cyclin B1, inhibits bipolar spindle formation in *Xenopus* oocytes and embryos [12,13]. The antisense RNA-mediated inhibition of cyclin B2 translation, but not cyclin B1, induces a bipolar spindle defect in *Rana japonica* oocytes [14]. These reports suggest that cyclin B2 is essential for bipolar spindle formation in frog oocytes.

In *Xenopus* oocytes, a huge nucleus (i.e., germinal vesicle) is located in the animal hemisphere, while cytoplasm is compartmentalized by the size of the yolk platelets, which causes animal-vegetal polarity [15]. Frog oocytes might thus be more strongly affected by the subcellular localization of various molecules than cultured mammalian cells.

We investigated roles of cyclin B2 localization in bipolar spindle formation in *Xenopus* oocytes in this report. Cyclin B2 was localized in the germinal vesicle and the perinuclear region. This perinuclear localization of cyclin B2 was partially perturbed by the overexpression of the cyclin B2 N-terminus, which resulted in the inhibition of spindle bipolarity regulated by bipolar kinesin, Eg5. These results indicated that the localization of cyclin B2 was essential for the regulation of spindle bipolarity in *Xenopus* oocytes.

Abbreviations: CRS, cytoplasmic retention signal; B2DC, the cyclin B2 N-terminus containing the CRS domain.

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2. Materials and methods

2.1. Preparation, culture, microinjection, and treatment of oocytes

Oocytes were prepared, cultured and microinjected as described [16]. Staging of oocytes was done according to Dumont [17]. To induce maturation, stage VI oocytes were treated with progesterone (5 μg/ml); to inhibit mitotic kinesin Eg5, oocytes were treated with monastrol (150 μM).

2.2. Construction of recombinant plasmids

All cyclin B N-termini were constructed by PCR, as described previously [12]. Myc-tagged cyclin B N-terminus was constructed by PCR, using a common 5′ primer with *Bam*HI site (5′–CGCGGA TCCATGGCTACTCGTCGCGC–3′) and an appropriate individual 3′ primer containing a stop codon (in a desired place) and a *Bgl*II site. The PCR products subcloned into N-terminal Myc-tagged pT7-G (UK-) vectors. Either N-terminal-truncated *Xenopus* cyclin B1 or cyclin B2 was constructed by PCR (B1ΔN and B2ΔN, respectively). The 5′ primer for B1ΔN was 5′–CGGGATCCTGCAGTGAATATGTCAAG–3′ and the one for B2ΔN was 5′–CGGGATCCTGCAGTGACTATGTGATG–3′, while the 3′ primer was a pT7-G reverse primer (5′–ATGTAGC-CATTCGTATCTGCT–3′). The PCR products subcloned into–pET3a vectors for bacterial expression. A cDNA encoding either *Xenopus Eg5* [18] or *Xklp2* (Clone ID: 3398700; obtained from I.M.A.G.E. Consortium), subcloned into the pGEX and pCMV –SPORT 6 plasmid vectors, respectively. Myc-tagged Eg5 was subcloned into N-terminal Myc-tagged pT7-G (UKII+) transcription vectors.

2.3. In vitro transcription

All of the constructs *in vitro* were transcribed into 5′-capped mRNA using the MEGAscript T7 or Sp6 kit (Ambion), as described [19].

2.4. Antibodies

Anti-B2ΔN antibodies against bacterially produced N-terminal truncation of *Xenopus* cyclin B2 were raised in rabbits by standard methods. The flow-through fraction of anti-B2ΔN antibodies from the B1ΔN column was affinity-purified using Affigel 10 immunoaffinity chromatography (Bio-Rad) of the B2ΔN column as follows [20].

2.5. Western blot analysis

Protein equivalent to one oocyte was subjected to the Western blot analysis with anti-B2ΔN antibodies (1:1000). The secondary antibodies, donkey anti-rabbit IgG antibodies (1:1000; Bio-Rad) was detected by the use of the ECL plus system (GE Healthcare) [21].

2.6. Cytological examination

Either oocytes or matured eggs were fixed in Bouin's solution, dehydrated and embedded in Histosec (Merck). All sections, which were 8 μm thick, were prepared for observing spindles described previously [22]. For immunohistochemistry, sections were rehydrated and autoclaved in a TE buffer (10 mM Tris, 1mM EDTA) for enhancing signals. These sections were incubated with affinity-purified rabbit anti-B2ΔN antibodies (1:50) or anti-Myc antibodies (A-14, Santa Cruz) (1:400). Washed with TBS, they were incubated with EnVision+ Peroxidase (Dako) and stained by 3,3′-diaminobenzidine tetrahydrochloride (DAB) (1 mg/ml) as described previously [23].

3. Results

3.1. The distribution of the cyclin B2 N-terminus differed, depending on the existence of the cytoplasmic retention signal

The overexpression of the cyclin B2 N-terminus containing the cytoplasmic retention signal (CRS) induces a spindle defect during *Xenopus* oocyte maturation [12]. To confirm whether the CRS domain affects the distribution of the cyclin B2 N-terminus in *Xenopus* oocytes, we prepared mRNA encoding myc epitope–tagged cyclin B2 N-terminus with or without CRS (B2DC or B2D, respectively) [10,12]. Immature *Xenopus* oocytes expressing B2D or B2DC were fixed, sectioned, and stained with an anti-c-Myc (A-14) antibody. B2D was distributed on the margin of oocytes, on the basal rim and on the upside of the germinal vesicle, but rarely distributed in the vegetal hemisphere (Fig. 1A). Staining with an anti-c-Myc antibody hardly detect endogenous c-Myc in uninjected control oocytes (Fig. 1C), so that anti-c-Myc staining would reflect the distribution of either B2D or B2DC. In contrast, B2DC was distributed both in the germinal vesicle and in the whole cytoplasm even though it was detected more often in the circumference of the animal hemisphere than in the other region (Fig. 1B). The differences in distribution between B2D and B2DC were observed in the germinal vesicle, the perinuclear region and the vegetal hemisphere (Figs. 1A and B). Especially, in the perinuclear region (including the subnuclear yolk-free zone), B2D was also distributed in the fibrous structure with a zonal pattern, whereas B2DC was distributed in the meshwork with a speckled one (Figs. 1D and E). Comparable amounts of B2D and B2DC were observed by the Western blot analysis (Fig. 1F). These results indicate that the CRS domain affects the subcellular distribution of the cyclin B2 N-terminus in *Xenopus* oocytes.

3.2. The subcellular localization of endogenous cyclin B2 was perturbed by the overexpression of cyclin B2 N-terminus

Stage VI oocytes contain a pool of cyclin B2 formed by an inactive pre-maturation/M-phase promoting factor [24]. To detect endogenous cyclin B2 in *Xenopus* oocytes regardless of the overexpression of B2DC, we prepared a specific polyclonal antibody. This anti-B2ΔN antibody, raised against bacterially produced N-terminal-truncated *Xenopus* cyclin B2 protein, did not detect B2DC. As described in a previous report [25], a protein of 51 kDa in mature eggs and a doublet of two close bands of equal abundance in oocytes were detected using the anti-B2ΔN antibody (Fig. 2A). The Western blot analysis demonstrated that the anti-B2ΔN antibody detected a protein in the cyclin B2 mRNA-translated reticulocyte lysates, but not in the cyclin B1 mRNA-translated lysates (Fig. 2B). Therefore, the anti-B2ΔN antibody was able to recognize endogenous cyclin B2, but it was not able to identify cyclin B1.

Next, the distribution of endogenous cyclin B2 in paraffin sections of *Xenopus* oocytes was examined using the anti-B2ΔN antibody. Cyclin B2 was localized in the germinal vesicle and on the animal hemisphere, but was not localized on the vegetal hemisphere (Fig. 2C). In the perinuclear region, cyclin B2 was localized at the meshwork with a simple pattern expanding from the germinal vesicle radially (Fig. 2E). We did not observe this staining with an anti-B2ΔN antibody premixed with a purified antigen (data not shown).

Since the anti-B2ΔN antibody did not detect B2DC, we examined whether B2DC affected the localization of cyclin B2 in oocytes. In the cytoplasm of oocytes injected with B2DC mRNA, endogenous cyclin B2 slightly diffused as compared with uninjected oocytes (Figs. 2C and D). Especially at the perinuclear meshwork in immature oocytes overexpressing B2DC, the distribution of cyclin B2 changed from a simple radial pattern to a speckled one (Figs. 2E

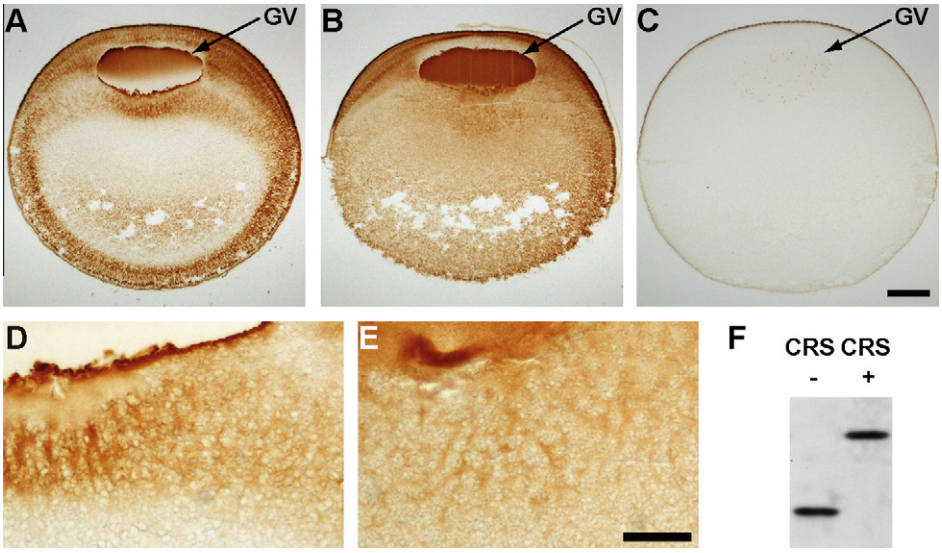

Fig. 1. Distribution of Myc-tagged cyclin B2 N-terminus. Immature oocytes expressing B2D (A) or B2DC (B) are stained with anti-Myc antibodies. Immunostaining of uninjected oocytes as a control is shown (C). A higher magnified image of the perinuclear region of oocytes injected with B2D or B2DC (D and E) mRNA. The Western blot analysis of immature oocytes injected with Myc-tagged B2D or B2DC (F). GV represents germinal vesicle. All Figures are meridional sections, and their animal pole points toward the upside. Scale bars represent 150 μm (A–C) and 50 μm (D and E).

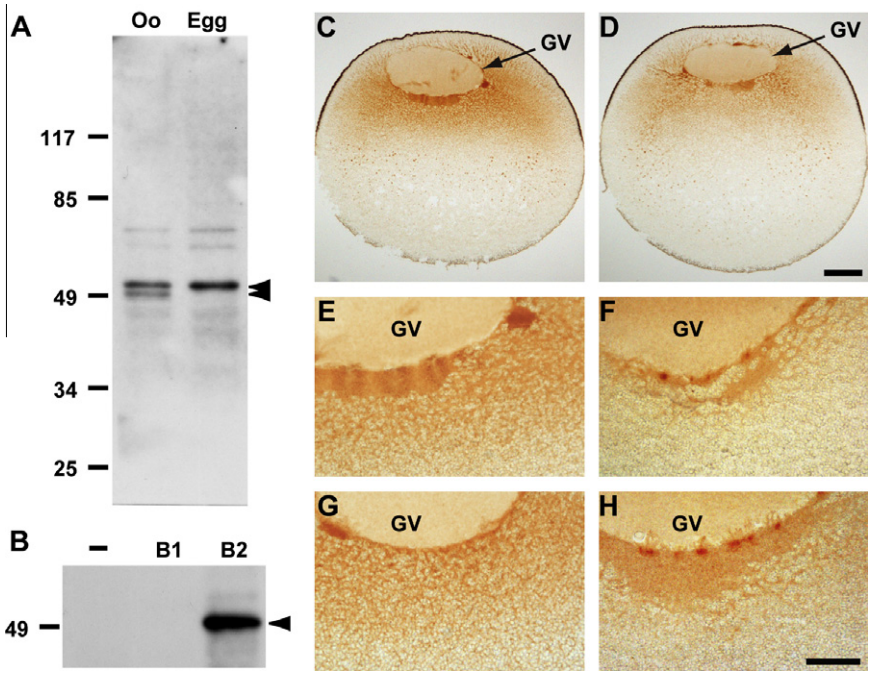

Fig. 2. The subcellular localization of endogenous cyclin B2 and the effect of the overexpression of the cyclin B2 N-terminus. *Xenopus* immature oocyte (Oo) and matured egg (Egg) extracts (A), and *in vitro*-synthesized cyclins B1 and B2 proteins in a reticulocyte lysate (B) were immunoblotted with affinity-purified anti-B2ΔN antibodies. As a control, the same volume of a reticulocyte lysate to which no mRNA was added was used. Arrowheads represent endogenous cyclin B2. Sizes of protein markers (kDa) are indicated on the left side. Immature uninjected oocytes (C) or oocytes injected with B2DC (D) are stained with anti-B2ΔN antibodies. A higher magnified image of the perinuclear region of uninjected oocytes is also shown (E). The same perinuclear regions of immature oocytes injected with B2DC (F), B2D (G) or B1DC (H) are stained with anti-B2ΔN antibodies. GV represents germinal vesicle. All Figures are meridional sections, and their animal pole points toward the upside. Scale bars represent150 μm (A and B) and 50 μm (C–F).

and F). This change in the cyclin B2 distribution was not observed in the overexpression of B2D or a cyclin B1 N-terminus containing of its own CRS domain (B1DC; Figs. 2G and H, respectively). This region corresponds to the one in which B2DC is distributed (Fig. 1E). In contrast, no significant difference in the germinal vesicle was observed (Figs. 2C and D). Therefore, these results reveal that the subcellular localization of endogenous cyclin B2 at the perinuclear meshwork might be perturbed by the overexpression of B2DC.

3.3. Eg5 can rescue a spindle defect induced by the cyclin B2 CRS domain

A spindle defect caused by B2DC indicates monopolar spindles (monaster) in oocytes, whereas the overexpression of B2DC induces a multipolar spindle in embryos [12,13]. The meiotic spindle formation differs from the mitotic one, because microtubules are reorganized around condensed chromosomes in oocytes without

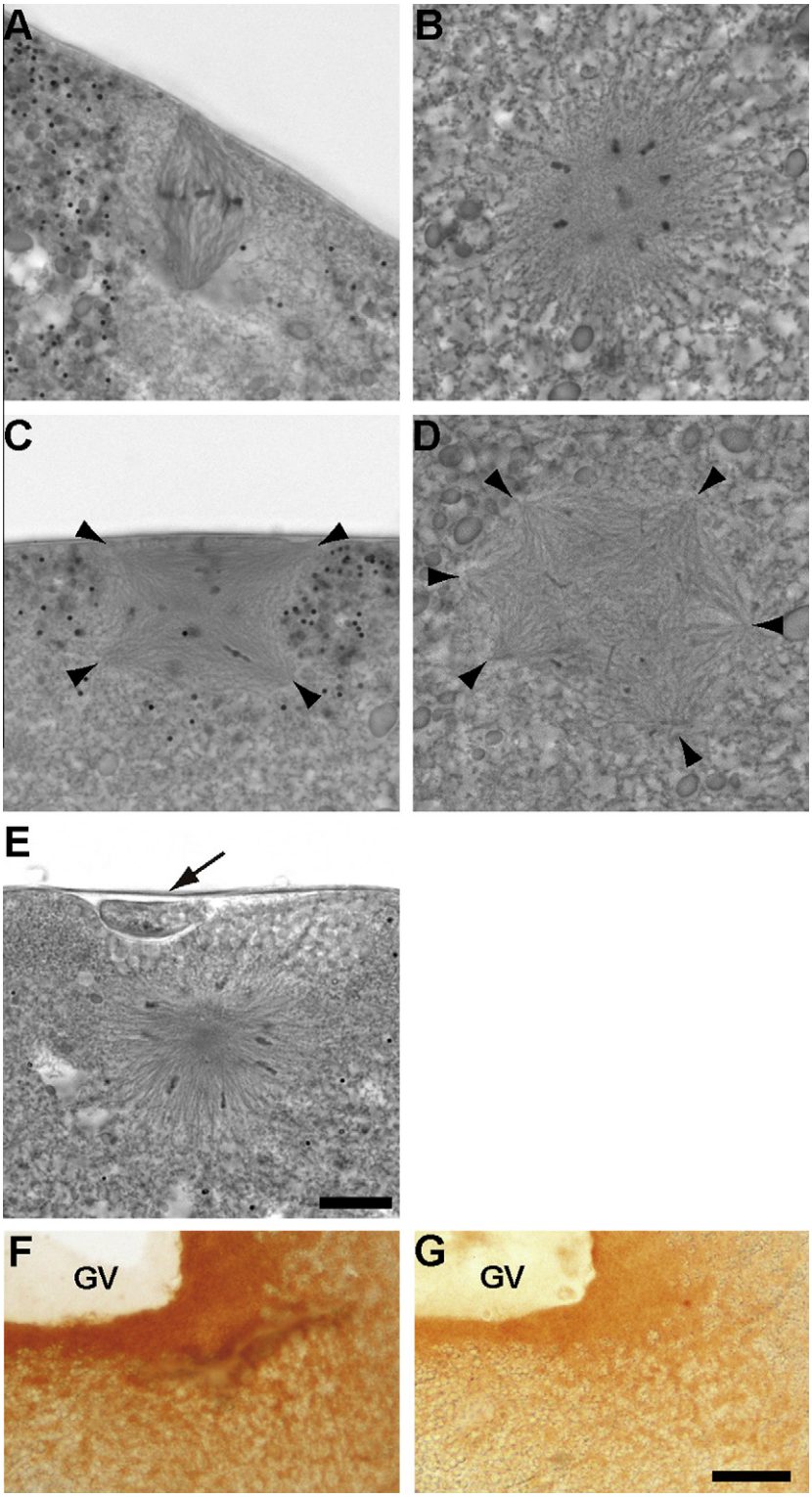

Fig. 3. Spindle morphology of oocytes injected with Eg5 or treated with monastrol, and the localization of Myc-tagged Eg5. Xklp2 mRNA alone was injected into oocytes (A). B2DC mRNA was first injected and then Xklp2 mRNA was injected into oocytes (B). Eg5 mRNA was injected into oocytes (C). B2DC mRNA was injected and then Eg5 mRNA was injected into oocytes (D). The oocytes were treated with monastrol (E). These oocytes were harvested four hours after germinal vesicle breakdown and examined cytologically. An arrowhead represents a spindle pole; an arrow shows a first polar body. Immature oocytes expressing Eg5 are stained either with anti-Myc antibodies (F) or with anti-B2ΔN antibodies (G). GV represents germinal vesicle. These figures are serial meridional sections, and their animal pole points toward the upside. Scale bars represent 10 μm (A–E) and 50 μm (F, G).

centrosomes [26]. These observations suggest that bipolar kinesins such as the BimC kinesin subfamily, Xklp2 and Eg5, might be inhibited by the overexpression of B2DC [13,18,26,27]. To examine the effects of Xklp2 or Eg5 on spindle formation during oocyte maturation, we injected mRNA encoding either *Xklp2* or *Eg5* mRNA into oocytes. After hormonal stimulation, the spindle morphology was examined four hours after germinal vesicle breakdown, when normal mature oocytes are arrested again at the second meiotic

metaphase [19]. Almost all (92.3%) of the oocytes injected with *Xklp2* mRNA formed a normal bipolar spindle and the majority (67.7%) of the oocytes injected with *Eg5* mRNA formed a multipolar spindle (Figs. 3A and C). Next, to investigate whether Xklp2 or Eg5 is able to rescue a spindle defect by the overexpression of B2DC, we injected mRNA encoding either *Xklp2* or *Eg5* into oocytes that had been injected with B2DC mRNA. Half (50.0%) of the oocytes formed a monopolar spindle following injection of both B2DC mRNA and *Xklp2* mRNA (Fig. 3B), just as in the oocytes overexpressing B2DC. In contrast, one third (34.2%) of the oocytes formed a multipolar spindle when injected with both B2DC mRNA and *Eg5* mRNA (Fig. 3D), and a little (2.6%) of the oocytes formed a monopolar spindle. These results suggest that the overexpression of Eg5 was able to rescue a spindle defect induced by B2DC.

The mitotic kinesin Eg5 is specifically inhibited by monastrol which inhibits bipolar spindle formation in *Xenopus* egg extracts [28]. To examine whether monopolar spindle formation following injection of B2DC mRNA is caused only by the failure of an Eg5 function, stage VI oocytes were treated with both progesterone and monastrol. The majority (69.4%) of oocytes treated with monastrol showed a monopolar spindle with a rosette-like structure just as in oocytes overexpressing B2DC (Fig. 3E). This observation indicated that bipolar spindle formation during *Xenopus* oocyte maturation was inhibited only by the failure of an Eg5 function.

Eg5 is phosphorylated by Cdc2 kinase, and thereby begins to bind microtubules at the early prophase [29,30]. After translocation, Eg5 is further phosphorylated by pEg2 and regulates spindle bipolarity [31]. This translocation of Eg5 to spindle microtubules might require the proper localization of cyclin B2/Cdc2 at the prophase, and the failure of phosphorylation of Eg5 by the perturbation of this complex might cause a spindle defect. To examine the state of the localization of Eg5, Myc-tagged Eg5 was made to express in immature *Xenopus* oocytes. To avoid the ectopic expression of Eg5 by its overexpression, we injected a lower dose (13.8 ng) of Myc-tagged Eg5 mRNA than that in rescue experiments (18.4 ng). Myc-tagged Eg5 was localized at the perinuclear meshwork (Fig. 3F) and close to endogenous cyclin B2 (Fig. 3G). The localization of Myc-tagged Eg5 was not affected by the overexpression of B2DC (data not shown). The results mentioned above suggest that the perturbation of the localization of endogenous cyclin B2 might attenuate phosphorylation and subsequent translocation of Eg5.

4. Discussion

This study showed that bipolar spindle formation during meiosis I required the proper localization of cyclin B2 in *Xenopus* oocytes. The localization of cyclin B2 at the perinuclear meshwork was partially perturbed by the overexpression of B2DC (Figs. 2C and D), which resulted in a spindle defect. In amphibian oocytes, not only yolk protein but also germ plasm is regularly arranged [15,32]. Therefore, the subcellular localization of molecules might be important for maturing *Xenopus* oocytes.

Cyclin B2 was localized both in the germinal vesicle and at the perinuclear meshwork in *Xenopus* oocyte (Fig. 2E). At this perinuclear meshwork, both B2DC fragment and Eg5 were localized (Figs. 1E and 3F) and the localization of cyclin B2 was perturbed by B2DC (Fig. 2F). Beckhelling et al. [33] reported that cyclin B2 was associated with a specific endoplasmic reticulum (ER) subdomain to form "annulate lamellae" in *Xenopus* oocytes. Eg5 is localized near the ER until the early prophase to translocate microtubules in *Xenopus* oocytes [30]. The first phosphorylation of Eg5 by a cyclin B/Cdc2 for translocation would be dependent on their relative locations in *Xenopus* oocytes. Because not only a mimic phosphorylation mutant of Cdc2 kinase recognition site in Eg5 but also a nonphosphorylation mutant of it was able to rescue the spindle defect induced by B2DC (data not shown), the bipolar activity of ectopically expressed Eg5 could not be regulated by Cdc2 kinase. These results suggest that the timing of endogenous Eg5 translocation would be strictly regulated by the correct localization of cyclin B2/Cdc2. In contrast, the overexpression of Xklp2, a member of the BimC subfamily, had no effect on spindle formation and did not rescue the spindle defect induced by B2DC (Figs. 3A and B). This observation is consistent with the previous finding that immunodepletion of Xklp2 from egg extracts has no effect on bipolar spindle formation using DNA beads [34].

Unlike cyclin B1 [35], we did not observe the staining with an anti-B2ΔN antibody on the spindle apparatus during *Xenopus* oocyte maturation (data not shown). Hence, cyclin B2 might regulate other event(s) during oocyte maturation besides spindle formation. The overexpression of B2DC showed the dose-dependent inhibition of the meiotic resumption as well as spindle formation in *Xenopus* oocytes (data not shown). Cyclin B2 is highly expressed in meiosis I in porcine oocytes, but not in meiosis II [36]. These observations suggest that cyclin B2 might be involved in the resumption or the progress of oocyte meiosis I and regulate the establishment of spindle bipolarity in this process. In *Xenopus* oocyte, meiosis I could be regulated more precisely than mitotic cell division, because a local perturbation of cyclin B2 could disturb this progress. This assumption is consistent with the findings described in previous reports that cyclin B2 does not play a distinct role during mitosis in cultured cells [8]. Myt1, associated with the membrane similarity to cyclin B2, mediates prophase I arrest by inhibiting Cdc2 in *Xenopus* oocyte, but does not regulate mitosis in HeLa cells [21,37]. On the other hand, although cyclin B2-null mice develop normally and are fertile, murine oocytes injected with cyclin B2 mRNA induced the breakdown of the germinal vesicle more efficiently than those injected with cyclin B1 mRNA [38]. These results suggest that mammalian oocytes may have diminished the subcellular environment in which cyclin B2 is involved in meiosis I, because their size is far smaller than that of frogs. If the proper localization of cyclin B2 is not required for oocyte meiosis I, cyclin B1 may function as cyclin B2.

In conclusion, bipolar spindle formation is regulated by the proper localization of cyclin B2 in *Xenopus* oocytes. The local perturbation of cyclin B2 affects the bipolar activity of Eg5. These results support the view that the intracellular signal transduction might be highly influenced by the subcellular localization of a maturation/M-phase promoting factor and so on.

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