The APC/C Inhibitor XErp1/Emi2 Is Essential for *Xenopus* Early Embryonic Divisions

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Mitotic divisions result from the oscillating activity of cyclin-dependent kinase 1 (Cdk1). Cdk1 activity is terminated by the anaphase-promoting complex/cyclosome (APC/C), a ubiquitin ligase that targets cyclin B for destruction. In somatic divisions, the early mitotic inhibitor 1 (Emi1) and the spindle assembly checkpoint (SAC) regulate cell cycle progression by inhibiting the APC/C. Early embryonic divisions lack these APC/C-inhibitory components, which raises the question of how those cycles are controlled. We found that the APC/C-inhibitory activity of XErp1 (also known as Emi2) was essential for early divisions in *Xenopus* embryos. Loss of XErp1 resulted in untimely destruction of APC/C substrates and embryonic lethality. XErp1's APC/C-inhibitory function was negatively regulated by Cdk1 and positively by protein phosphatase 2A (PP2A). Thus, Cdk1 and PP2A operate at the core of early mitotic cell cycles by antagonistically controlling XErp1 activity, which results in oscillating APC/C activity.

After fertilization and a prolonged first cell cycle, *Xenopus* embryos progress through 11 rapid divisions devoid of gap phases. Cycle 13 marks mid-blastula transition (MBT), when cycles become longer and gap phases are resumed (1–3). Although it is clear that cyclin-

dependent kinase 1 (Cdk1) is the universal cell cycle regulator, it remains unknown how pre-MBT divisions lacking inhibitory phosphorylations of Cdk1 (2, 4) as well as the anaphase-promoting complex/cyclosome (APC/C)-inhibitory activities of Emi1 and SAC (3, 5, 6) are controlled. Before fertilization, XErp1 mediates the metaphase II arrest of mature *Xenopus* eggs by directly inhibiting the APC/C (7). Surprisingly, XErp1 is completely degraded at fertilization but reaccumulates in early embryos (8–12), yet egg extract studies suggest that XErp1 has no function in mitosis (9).

To understand the regulation of early mitotic divisions, we first examined XErp1 levels in *Xenopus* embryos by immunoblot analyses. After its destruction at fertilization, XErp1 reaccumulated to levels comparable to those in unfertilized eggs and started to disappear again at MBT, which is marked by the destruction of cyclin E1 and replacement of cyclin A1 by cyclin A2 (Fig. 1A). To test whether XErp1 expression is critical for early mitotic cycles, we injected antisense morpholino-oligos (MO) targeting *XErp1* mRNA (XErp1-MO) or control sense MO into one-cell embryos (13). At 24 hours post-fertilization (hpf), 92% of the control MO-injected embryos displayed small blastopores (Fig. 1, B and C), revealing that these embryos completed the first major morphogenetic transformation (i.e., blastopore closure). In contrast, 90% of XErp1-depleted embryos failed to undergo blastopore closure but underwent apoptosis at gastrulation (Fig. 1, B to D; fig. S1, A and B; and movie S1). To confirm that loss of XErp1 accounted for the observed phenotype, we co-injected embryos with XErp1-MO and wild-type (WT) *XErp1* mRNA (myc-XErp1^{WT}) that was not targeted by the MO (Fig. 1D). Expression of myc-tagged full-length XErp1^{WT} efficiently rescued blastopore closure in XErp1-depleted embryos (Fig. 1, B and C). Thus, XErp1 is essential for *Xenopus* early embryonic cycles.

Next, we analyzed whether these divisions depend on XErp1's APC/C-inhibitory activity. Indeed, the majority of XErp1-depleted embryos expressing XErp1 mutated in its zinc-binding region (ZBR⁻) or destruction box (Abox⁻)—both of

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which are deficient in APC/C inhibition (7, 11) (fig. S5)—failed to undergo blastopore closure, whereas Fbox-mutant XErp1, which is not com-

promised in APC/C inhibition (7), efficiently rescued the loss of XErp1 (Fig. 1, B and C, and fig. S1, C to E). Consequently, relative to con-

trol embryos, XErp1-depleted embryos displayed reduced levels of the APC/C substrate cyclin B2 (Fig. 2A) and increased cell cycle lengths (fig.

Fig. 1. XErp1 is essential for early embryonic divisions. **(A)** Immunoblot analyses of calf intestinal phosphatase (CIP)—treated samples harvested at the indicated time points. **(B and C)** One-cell embryos were injected with XErp1-MO or control MO and H₂O or mRNA encoding WT, Fbox⁻ mutant, or ZBR⁻ mutant XErp1; 24-hpf phenotypes were quantified **(B)** or images were taken at the indicated times **(C)**. **(D)** Embryos used in **(B)** were lysed at 6 hpf and immunoblotted. Asterisk marks nonspecific band. Cyc, cyclin.

Fig. 2. XErp1 is required for timely destruction of APC/C substrates. **(A)** One-cell embryos were injected with control MO (Ctrl) or XErp1-MO, lysed at the indicated time points, and immunoblotted. **(B)** Embryo extract depleted of XErp1 or control immunoglobulin G extract was supplemented with ³⁵S-labeled securin and at the indicated time points, samples were analyzed by immunoblot and autoradiography analysis. **(C)** XErp1-depleted extract was supplemented with buffer or IVT myc-XErp1^{WT} and samples were analyzed as in **(B)**. **(D)** Extract from **(C)** was treated with CIP and immunoblotted for XErp1. Asterisk marks nonspecific band. **(E)** At 4 hpf, embryos were lysed, and XErp1 and control immunoprecipitates were immunoblotted for XErp1 and Cdc27. **(F)** Non-CIP-treated embryo lysates were immunoblotted. Gwl, greatwall kinase.

S2, A and B). To further confirm this finding, we established an embryonic extract system that allowed us to monitor APC/C activity by analyzing the stability of in vitro translated (IVT) ³⁵S-labeled securin. Depletion of XErp1 caused APC/C activation indicated by the destruction of ³⁵S-labeled securin, and this effect could be rescued by IVT myc-XErp1^{WT} (Fig. 2, B to D). Consequently, XErp1 immunopurified from embryo extract associated with the APC/C core subunit Cdc27 (Fig. 2E). Thus, the APC/C inhibitor XErp1 is critical for timely destruction of APC/C substrates in early *Xenopus* embryos.

In metaphase II, Cdk1 transiently activates the APC/C to compensate for continuous cyclin B synthesis by phosphorylating XErp1 at Ser²¹³, Thr²³⁹, Thr²⁵², Thr²⁶⁷, Thr⁵⁴⁵, and Thr⁵⁵¹ (ST₅), which decreases its half-life and affinity for the APC/C (14, 15). Negative regulation by Cdk1 is antagonized by protein phosphatase 2A (PP2A), which, upon phosphorylation of XErp1 by p90Rsk, binds to XErp1 and keeps dephosphorylating it (8, 11, 14–16). Because XErp1 remained at constant levels during early divisions (fig. S3A) but underwent cell cycle-dependent changes in its electrophoretic mobility (Fig. 2F and fig. S3B), we speculated that XErp1 might be regulated by

Cdk1 and PP2A. If this applies, expression of XErp1 mutated at the six Cdk1 phosphorylation sites (ST₅ → 6A) should cause a cell cycle arrest due to constitutive APC/C inhibition. Indeed, 6-hpf XErp1-depleted embryos expressing myc-XErp1^{6A} displayed gigantic cells and elevated cyclin B2 levels (Fig. 3, A and B, and fig. S3, C and D). This cell cycle arrest was caused by APC/C inhibition, because expression of myc-XErp1^{6A,ZBR} had no effect (Fig. 3, A and B, and fig. S3C). To further confirm these findings, we analyzed the stability of APC/C substrates in embryo extract where we elevated Cdk1 activity by the addition of nondegradable cyclin B (CycB^{Δ90}). Intriguingly, 25 nM CycB^{Δ90} was sufficient to induce the degradation of cyclin B2 and ³⁵S-labeled securin (Fig. 3C). Elevated Cdk1 activity correlated with the phosphorylation of XErp1 (Fig. 3C) and its dissociation from the APC/C, as shown by Cdc27 immunoprecipitation experiments from extract and embryos supplemented with CycB^{Δ90} (Fig. 3, D and G). Thus, Cdk1 negatively regulates XErp1's APC/C-inhibitory function.

An additional corollary of our model is that PP2A activity is required to antagonize Cdk1. In fact, treatment of embryo extract with 1 μM of the PP2A inhibitor okadaic acid (OA) quickly

induced the hyperphosphorylation of XErp1 and the destruction of cyclin B2 and ³⁵S-labeled securin (Fig. 3E). As shown by Cdc27 immunoprecipitation experiments, PP2A inhibition in embryos or extract resulted in the dissociation of XErp1 from the APC/C (Fig. 3, F and G). Notably, prolonged PP2A inhibition by OA induced the destabilization of XErp1 in embryos and extract (fig. S3E). Although this mechanism seems not to be relevant for rapid pre-MBT divisions where XErp1 levels remain constant (fig. S3A), this observation suggests that Cdk1 in the absence of PP2A activity can affect the turnover of XErp1. In *Xenopus* egg extract, the PP2A holoenzyme is targeted to XErp1 via the B'56 subtype of regulatory subunits (15). To test whether the same applies for embryonic divisions, we performed immunoprecipitation experiments from embryo extract expressing FLAG-tagged B'56e. Endogenous XErp1 efficiently coimmunoprecipitated with FLAG-B'56e but not with FLAG-B'55δ (Fig. 3H). XErp1 residues Ser³³⁵, Thr³³⁶, Ser³⁴², and Ser³⁴⁴ (STS₂) are critical for PP2A recruitment in meiosis (14, 15). To test whether these residues are also critical for the mitotic interaction between XErp1 and PP2A-B'56, we performed pull-down experiments from embryo extract using

Fig. 3. XErp1 is regulated by phosphorylation. (A and B) One-cell embryos were injected with XErp1-MO and H₂O or mRNA encoding WT, Cdk1 phosphomutant ST₅ → 6A (6A), or Cdk1 phosphomutant XErp1 defective in APC/C-inhibition (6A,ZBR). At 6 hpf, images were taken (A) and phenotypes quantified (B). (C) Embryo extract was supplemented with nondegradable cyclin B (CycB^{Δ90}) and analyzed by immunoblot. Time course started with CycB^{Δ90} addition. (D) Cdc27 was immunoprecipitated from extracts treated as in (C) and supplemented with MG-262. Samples were CIP-treated before immunoblotting for Cdc27 and XErp1. (E) Embryo extract was supplemented with 1 μM OA or dimethyl sulfoxide (DMSO) as solvent control, and samples were CIP-treated as indicated and analyzed as in (C). (F) From extracts treated as in (E) and supplemented with MG-262, Cdc27 was immunoprecipitated. Samples were CIP-treated and immunoblotted for XErp1 and Cdc27. (G) One-cell embryos were injected with 50 nM

CycB^{Δ90} or incubated in 2 μM OA for 30 min. Cdc27 immunoprecipitates were CIP-treated and analyzed by immunoblot. (H) mRNA encoding FLAG-tagged PP2A B subunits or H₂O (control) was incubated in embryo extract and immunoprecipitated using antibodies to FLAG. Precipitates were analyzed for XErp1 and FLAG epitope. (I) Extracts were incubated with B'56 mRNAs and GST-tagged XErp1³⁰¹⁻⁴⁰⁰ WT or PKA phosphomutant (4A) XErp1. After reisolation of GST-tagged proteins, interacting B'56 subunits were analyzed by immunoblot. Inputs [(H) and (I)] are shown in fig. S3, F and G. CBB, Coomassie Brilliant Blue.

glutathione *S*-transferase (GST)-tagged WT and mutant (STS₂ → 4A) XErp1 fragments (residues 301 to 400). As shown in Fig. 3I, FLAG-B'56α, FLAG-B'56β, and FLAG-B'56ε efficiently associated with the WT fragment but not the 4A fragment.

Fertilization triggers destruction of cMos (17)—the upstream kinase of the MAPK-p90Rsk pathway—which raises a question about which kinase is critical for XErp1 activity in mitosis. XErp1 residues Ser³³⁵, Thr³³⁶, Ser³⁴², and Ser³⁴⁴, which are critical for PP2A-B'56 recruitment (Fig. 3I), are flanked by highly conserved residues matching the protein kinase A (PKA) consensus phosphorylation motif (18) (fig. S4A). To test whether XErp1 is a substrate of PKA, we performed in vitro assays using full-length XErp1 fused to maltose-binding protein (MBP). MBP-XErp1^{WT} but not MBP-XErp1^{4A} was efficiently phosphorylated by PKA in vitro (Fig. 4A). To analyze whether XErp1 phosphorylation in embryos is mediated by PKA, we incubated MBP-XErp1^{BD} (residues 319 to 375) in [γ -³²P]adenosine triphosphate (ATP)-supplemented control or PKA-depleted embryo extract (Fig. 4B). The WT but not the 4A mutant was efficiently phosphorylated in control-depleted extract (Fig. 4C). PKA depletion diminished MBP-XErp1^{BD,WT} phosphorylation, which was specific for PKA deple-

tion (Fig. 4C). Thus, PKA phosphorylates XErp1 at sites relevant for PP2A-B'56 binding (see Fig. 3I), which suggests that PKA might promote the recruitment of PP2A to XErp1.

To test this idea, we incubated in vitro PKA-phosphorylated MBP-XErp1^{BD} in embryo extract and analyzed PP2A levels associated with reisolated MBP-XErp1^{BD}. Under stringent conditions where PP2A did not associate with unphosphorylated MBP-XErp1^{BD,WT}, prephosphorylation of MBP-XErp1^{BD,WT} but not of XErp1^{BD,4A} drastically increased the amount of copurified PP2A (Fig. 4D). Consequently, expression of full-length myc-XErp1^{4A} failed to rescue loss of endogenous XErp1 in embryos (Fig. 4E and fig. S4, B and C). Expression of full-length myc-XErp1 mutated at either motif Ser³³⁵/Thr³³⁶ (S335/T336A) or Ser³⁴²/Ser³⁴⁴ (S342/S344A) was also inefficient in complementing loss of endogenous XErp1, and the corresponding MBP-XErp1^{BD} fragments were less efficiently phosphorylated by PKA in vitro than were MBP-XErp1^{BD,WT} fragments (fig. S4, D to G). Thus, although Ser³⁴²/Ser³⁴⁴ does not match the PKA consensus motif, our data—in line with previous reports (11)—suggest that phosphorylation of both motifs is important for the APC/C-inhibitory function of XErp1. Finally, we tested the prediction that PKA inhibition should result in a phenotype reminiscent of that of XErp1-

depleted embryos. To this end, we analyzed embryos treated with the PKA inhibitors H89 or PKI (19, 20). At 24 hpf, the majority of PKA-inhibited embryos failed to undergo blastopore closure and died (Fig. 4F and fig. S4H). Immunoblot analyses confirmed efficient PKA inhibition in these embryos (Fig. 4G). Prolonged PKA inhibition—like that of PP2A (fig. S3E)—resulted in reduced levels of XErp1 (Fig. 4G), further confirming our idea that PKA, via PP2A-B'56, antagonizes Cdk1's negative effect on XErp1 in mitosis.

Our studies identify XErp1 as a mitotic APC/C inhibitor essential for the fast embryonic pre-MBT divisions. According to our model (Fig. 4H), XErp1-mediated APC/C inhibition allows cyclin B accumulation and thus entry into mitosis. At early mitosis, Cdk1's inhibitory effect on XErp1 is efficiently antagonized by PP2A-B'56. Once Cdk1 reaches maximal activity, it prevails over PP2A and thereby triggers APC/C activation. During mitotic exit, XErp1 must remain inactive despite decreasing Cdk1 activity, which suggests the existence of a molecular switch that inactivates PP2A-B'56 and/or PKA at anaphase. In embryo extract, PKA activity remained constant during early divisions (fig. S4I), indicating that PP2A-B'56 might be the target of such a mechanism. At mitotic entry, Cdk1 promotes inactivation of its

Fig. 4. PKA phosphorylates XErp1 at sites critical for PP2A B'56-recruitment. (A) In vitro PKA phosphorylation assay using [γ -³²P]ATP and MBP-tagged full-length WT or PKA phosphomutant (4A) XErp1. γ -³²P incorporation was analyzed by autoradiography. (B) Embryo lysate depleted of PKA (Δ PKA) or treated with control antibodies (Δ Ctrl) were immunoblotted for PKA and tubulin. (C) Embryo lysates from (B) were supplemented with [γ -³²P]ATP and phosphorylation of MBP-XErp1^{BD} was analyzed by autoradiography. Recombinant PKA was added to Δ PKA lysate. (D) MBP-XErp1^{BD} was phosphorylated by PKA in vitro and incubated for 20 min in embryo extract. After repurification of MBP-XErp1^{BD}, the associated PP2A catalytic subunit (PP2A-C) was analyzed by

immunoblot. (E) One-cell embryos were injected with XErp1-MO or control MO and H₂O or mRNA encoding WT or PKA phosphomutant (4A) XErp1; at 24 hpf, the indicated phenotypes were quantified. Images and immunoblots are shown in fig. S4, B and C. (F) One-cell embryos were incubated with H89 or injected with PKI to inhibit PKA; at 24 hpf, the indicated phenotypes were quantified. Images are shown in fig. S4H. (G) Embryos were treated as in (F); samples were taken at 6 hpf, CIP-treated as indicated, and immunoblotted for XErp1 and the phosphorylated PKA consensus motif (RXXP/pT, where X stands for any amino acid and pS/pT for phosphorylated serine or threonine) to monitor PKA activity. (H) Model of APC/C regulation in early mitotic divisions.

antagonist PP2A-B55 δ via the Gwl-Ensa-Arpp-19 pathway (21–23). Perhaps Cdk1 itself could inactivate PP2A-B'56 once it reaches maximal activity in metaphase. By identifying XErp1 as a mitotic APC/C inhibitor, our studies provide a framework for understanding how different regulatory modules composed of phosphorylations of Cdc20, APC/C, XErp1, and possibly PP2A-B'56 are interconnected to create oscillatory Cdk1 activity driving cell cycle progression.

References and Notes

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