# Calcium triggers exit from meiosis II by targeting the APC/C inhibitor XErp1 for degradation

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**Vertebrate eggs awaiting fertilization are arrested at metaphase of meiosis II by a biochemical activity termed cytostatic factor (CSF)[1,2]. This activity inhibits the anaphase-promoting complex/cyclosome (APC/C), a ubiquitin ligase that triggers anaphase onset and mitotic/meiotic exit by targeting securin and M-phase cyclins for destruction[3,4,5]. On fertilization a transient rise in free intracellular calcium[6] causes release from CSF arrest and thus APC/C activation. Although it has previously been shown that calcium induces the release of APC/C from CSF inhibition through calmodulin-dependent protein kinase II (CaMKII)[7,8], the relevant substrates of this kinase have not been identified. Recently, we characterized XErp1 (Emi2), an inhibitor of the APC/C and key component of CSF activity in *Xenopus* egg extract[9]. Here we show that calcium-activated CaMKII triggers exit from meiosis II by sensitizing the APC/C inhibitor XErp1 for polo-like kinase 1 (Plx1)-dependent degradation. Phosphorylation of XErp1 by CaMKII leads to the recruitment of Plx1 that in turn triggers the destruction of XErp1 by phosphorylating a site known to serve as a phosphorylation-dependent degradation signal. These results provide a molecular explanation for how the fertilization-induced calcium increase triggers exit from meiosis II.**

Although it is well established that CaMKII is the essential target of the calcium signal[7,8] on fertilization, the relevant substrates of this kinase have not been identified, and the mechanism(s) leading to APC/C activation have long remained obscure. Recently, we have identified XErp1, a novel component of CSF activity that is both necessary and sufficient to keep the APC/C inactive in CSF-arrested *Xenopus* egg extracts[9] (CSF extracts). In response to calcium, XErp1 is rapidly degraded via a Plx1-dependent mechanism, leading to CSF release and APC/C activation. We have further shown that XErp1 destruction depends on phosphorylation of two critical serine residues within a motif ($DSGX_3S$) known to serve as a 'phosphodegron' for the ubiquitin ligase complex Skp1–Cullin–F-box$^{\beta\text{-TRCP}}$ ($SCF^{\beta\text{-TRCP}}$; ref. 10). Although the identification of XErp1 as a critical substrate of Plx1 provided an attractive explanation for the essential function of Plx1 in APC/C activation[11], the role of the calcium signal remained unclear. In particular, it remained to be explained why XErp1 is not targeted for degradation in CSF extracts, despite the presence of active Plx1 and $SCF^{\beta\text{-TRCP}}$. Here we have explored the hypothesis that a calcium-dependent mechanism could sensitize XErp1 for phosphorylation by Plx1.

We first asked whether XErp1 could be a substrate of CaMKII.

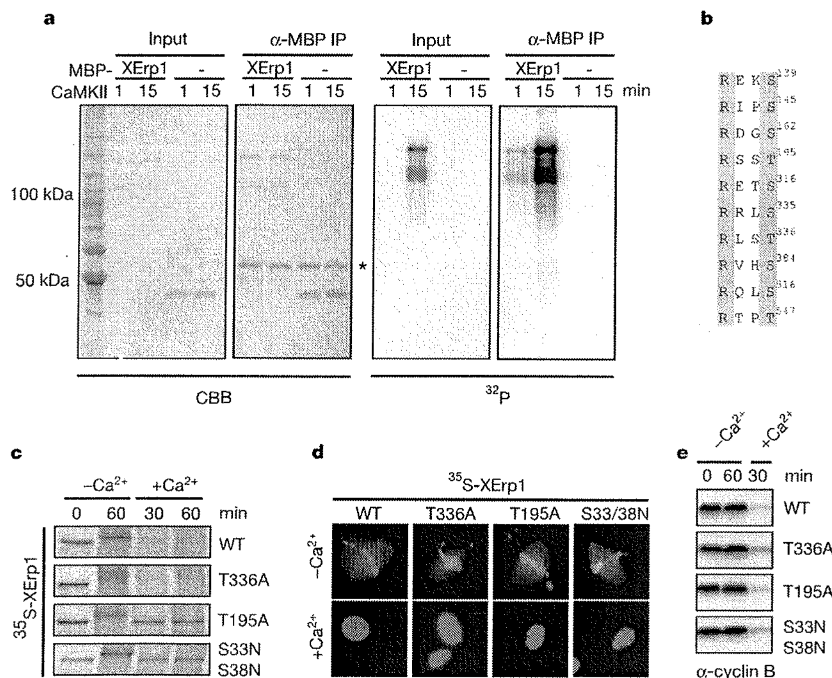


**Figure 1 | XErp1 is a substrate of CaMKII *in vitro*.** **a**, MBP-tagged $XErp1^{WT}$ or MBP alone were subjected to CaMKII phosphorylation reactions for the indicated times and immunopurified using anti-MBP antibodies (asterisk marks heavy chains). CBB indicates Commassie Brilliant Blue. The incorporation of $^{32}P$ was analysed by autoradiography. **b**, Amino acid sequence of all putative CaMKII phosphorylation sites (RXXS/T) present in XErp1. **c–e**, Both $DSGX_3S^{38}$ and $RXST^{195}$ motifs are essential for the degradation of XErp1 on calcium-stimulation. $^{35}S$-labelled IVT XErp1 proteins were incubated in CSF extract in the presence or absence of calcium. At the indicated time points samples were withdrawn for analysis by autoradiography (**c**), microscopic examination of chromatin and spindle structures (**d**) and immunoblotting for cyclin B (**e**).

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Autoradiographic analyses revealed that CaMKII efficiently phosphorylated purified maltose-binding protein (MBP)-tagged wild-type XErp1 but not MBP itself *in vitro* (Fig. 1a). Together with our previous results that XErp1 degradation depends on its prior phosphorylation by Plx1 (ref. 9), this result raised the question of how CaMKII and Plx1 cooperate in response to a calcium signal to target XErp1 for destruction. One attractive model was suggested by recent findings implicating the noncatalytic carboxy terminus of Plks, the so-called polo-box domain (PBD), in both Plk activation and the targeting of these kinases to serine- or threonine-phosphorylated substrates[12]. Based on this concept it was tempting to speculate that CaMKII sensitizes XErp1 for degradation by creating a binding site for Plx1. To explore this hypothesis, we examined the XErp1 sequence for sites that could potentially serve as CaMKII-regulated PBD binding sites (RXST/S; ref. 13). Of the ten putative CaMKII phosphorylation sites (RXXS/T) (Fig. 1b) only two sites ($RXST^{195}$ and $RXST^{336}$) match this optimal consensus. To identify the CaMKII site(s) relevant for calcium-regulated degradation of XErp1 we examined the stability of *in vitro* translated (IVT), $^{35}S$-labelled XErp1 mutated at Thr 195 ($XErp1^{T195A}$) or Thr 336 ($XErp1^{T336A}$) in calcium-supplemented CSF extract. As shown previously[9] IVT wild-type $XErp1^{WT}$ was rapidly degraded on anaphase onset whereas XErp1 mutated at its $DSGX_3S$ motif ($XErp1^{S33N,S38N}$) was not targeted for calcium-induced degradation (Fig. 1c). Notably, IVT $XErp1^{T195A}$, but not $XErp1^{T336A}$, remained stable on calcium addition (Fig. 1c), indicating that Thr 195, but not Thr 336, is essential for the cell-cycle-regulated degradation of XErp1. As shown by microscopic analyses and by immunoblotting for cyclin B, all extracts had entered interphase on calcium addition (Fig. 1d, e), confirming that the trace amounts of IVT products added did not block calcium-induced CSF release. Consistent with our previous observations[9] higher concentrations of $XErp1^{WT}$ and $XErp1^{T195A}$ were capable of blocking calcium-induced CSF release (see Supplementary Fig. S1). Taken together, these data are consistent with the model that CaMKII and Plx1 cooperate to target XErp1 for degradation on a calcium stimulus and that Thr 195 and the $DSGX_3S$ motif are both required for the cell-cycle-regulated destruction of XErp1.

Previous studies have shown that phosphopeptide binding to the Plk-PBD is favoured by a serine residue in position $-1$ (SpT/pS; ref. 13). The above model thus predicted that Ser 194 might contribute to sensitizing XErp1 for Plx1-dependent degradation. Indeed, $^{35}S$-labelled IVT $XErp1^{S194A}$ remained stable in calcium-supplemented CSF extract (Fig. 2a, b), similar to $XErp1^{T195A}$ and $XErp1^{S33N,S38N}$. To prove that Plx1-binding to XErp1 depends on both Ser 194 and Thr 195, MBP-tagged wild-type protein and appropriate mutants were subjected to far-western experiments. Indeed, phosphorylation of $XErp1^{WT}$ by CaMKII strongly enhanced the interaction between XErp1 and purified full-length Plx1. As expected, wild-type PBD ($PBD^{WT}$) but not a mutant form of PBD ($PBD^{mut}$) was capable of binding to phosphorylated $XErp1^{WT}$ (Fig. 2c). In contrast, both the S194A and T195A mutants of XErp1 treated with CaMKII failed to interact significantly with full-length Plx1 or $PBD^{WT}$ (Fig. 2c), indicating that $RXST^{195}$ constitutes a CaMKII-regulated Plx1 binding site. Mutation of the $DSGX_3S$ motif did not interfere with the ability of XErp1 to bind to Plx1 or the $PBD^{WT}$ on phosphorylation by CaMKII (Fig. 2c), in line with the expected order of events. Taken together, these data strongly suggest that CaMKII phosphorylation of XErp1 on Thr 195 creates a binding site for Plx1, consistent with the idea that CaMKII sensitizes XErp1 for Plx1-dependent degradation on anaphase onset.

An additional corollary of the proposed model is that PBD docking to XErp1 stimulates the kinase activity of Plx1 towards its substrate XErp1. To test this prediction, we used MBP-XErp1 with or without prior phosphorylation (with unlabelled ATP) by CaMKII to carry out Plx1 kinase assays in the presence of $^{32}P$-labelled ATP. Compared to untreated $XErp1^{WT}$, $XErp1^{WT}$ pre-phosphorylated by CaMKII represented a much better *in vitro* substrate for Plx1 (Fig. 2d) consistent with enhanced Plx1 recruitment after CaMKII phosphorylation. To rule out a contribution of CaMKII to the incorporation of $^{32}P$ into XErp1, parallel phosphorylation experiments were performed in the absence of Plx1. As shown in Fig. 2d, no labelling of XErp1 with $^{32}P$ was observed under these conditions. Notably, pretreatment of $MBP\text{-}XErp1^{S194A}$ or $\text{-}XErp1^{T195A}$ did not result in increased phosphorylation by Plx1 (Fig. 2d), confirming the inability of these mutants to provide a docking site for Plx1. Taken together, these data demonstrate that calcium-activated CaMKII converts XErp1 into an efficient Plx1 substrate.

We next wanted to confirm that the activity of CaMKII is essential for the degradation of XErp1 on anaphase onset. However, as the inhibition of CaMKII prevents CSF release, and thus XErp1 degradation, we analysed the stability of IVT XErp1 in extract that was arrested at anaphase by the presence of non-degradable cyclin B (refs 14, 15). These extracts, called 'Δ90 extracts' (refs 14, 15), retain

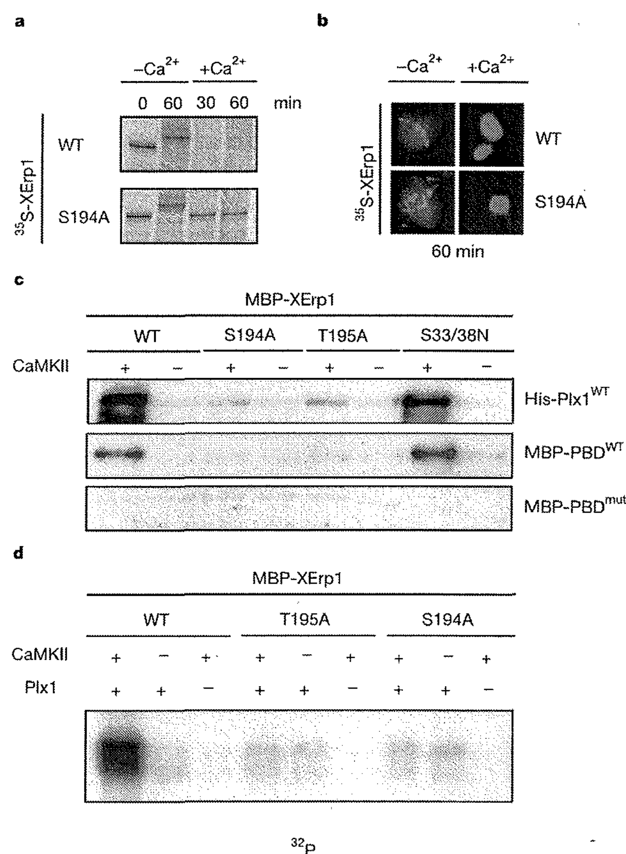


**Figure 2 | CaMKII converts XErp1 into an efficient Plx1 substrate.** **a, b**, Ser 194 is critical for the calcium-induced degradation of XErp1. IVT $^{35}S\text{-}XErp1^{WT}$ or $\text{-}XErp1^{S194A}$ was incubated in CSF extract in the presence or absence of calcium and samples were analysed by autoradiography (**a**) and microscopic examination of spindle and DNA morphology (**b**). **c**, Phosphorylation of XErp1 by CaMKII strongly enhances binding of Plx1 to XErp1. The binding of His-Plx1, $MBP\text{-}PBD^{WT}$ and $MBP\text{-}PBD^{mut}$ to MBP-XErp1 was analysed by far-western ligand blots. MBP-XErp1 proteins were subjected to CaMKII phosphorylation reactions or mock treatments and resolved by SDS–PAGE. Bound Plx1 and PBD were detected using purified anti-Plx1 antibodies. **d**, MBP-XErp1 was subjected to *in vitro* phosphorylation reactions using recombinant Plx1. The incorporation of $^{32}P$ was analysed by autoradiography. As indicated, MBP-XErp1 was pre-incubated with activated CaMKII (or subjected to a mock kinase reaction) and unlabelled ATP. To control for incorporation of $^{32}P$ by CaMKII, parallel reactions were performed in the absence of Plx1.

CaMKII activity (see Supplementary Fig. S2) which can be modulated without affecting cell cycle progression. As expected, IVT XErp1$^{WT}$ was rapidly degraded in Δ90 extracts (see Supplementary Fig. S2). As most available specific CaMKII inhibitors are not able to inhibit activated CaMKII but only to prevent CaMKII activation we first suppressed CaMKII activity in Δ90 extracts by the addition of EGTA (see Supplementary Fig. S2), allowing us to study the effect of a specific CaMKII inhibitor on the stability of XErp1. Consistently, in the absence of an additional calcium stimulus IVT XErp1$^{WT}$ remained stable in EGTA-treated Δ90 extracts, whereas it was rapidly degraded on calcium addition in a Thr 195-dependent manner (Fig. 3a). Notably, the calcium-induced degradation of XErp1$^{WT}$ could be significantly suppressed by the addition of 300 μM CaMKII$^{281-309}$ (Fig. 3a), a peptide known to specifically inhibit the calcium-induced activation of CaMKII (ref. 16). CaMKII activity assays demonstrated that CaMKII$^{281-309}$ prevented the calcium-induced reactivation of CaMKII (Fig. 3c). The rapid degradation of IVT securin (Fig. 3a) under all conditions confirmed that the different treatments did not interfere with anaphase arrest. Furthermore, addition of a constitutively active form of CaMKII (CaMKII$^{1-290}$) induced the rapid degradation of IVT XErp1$^{WT}$ but not of XErp1$^{T195A}$ (Fig. 3b) confirming that CaMKII is the kinase triggering XErp1 degradation on calcium stimulus. In view of an ongoing debate about a possible contribution of Emi1 to CSF activity[17], we also used the above system to examine the fate of this XErp1-related protein. We found that CaMKII activity is not essential for the degradation of Emi1, as indicated by the fact that IVT Emi1 was efficiently degraded in EGTA-treated Δ90 extracts even without calcium addition (Fig. 3a), or when the calcium-induced activation of CaMKII was suppressed by the addition of CaMKII$^{281-309}$ (Fig. 3a). Taken together, these data suggest that XErp1, but not Emi1, is the critical target of calcium-activated CaMKII. This conclusion is in line with results showing that Emi1 is highly unstable in CSF-arrested extract[18,19], from which CaMKII activity is naturally absent. The observation that Emi1 stability does not seem to be regulated by CaMKII, together with results showing that Emi1 protein is unstable in CSF-arrested egg extract[18,19], argues against a critical contribution of Emi1 to CSF activity in *Xenopus* eggs.

The above data suggested that the lack of CaMKII activity accounts for the stability of XErp1 in CSF-arrested *Xenopus* egg extract. Consequently, we reasoned that a mutant XErp1 capable of serving as an efficient Plx1 substrate independently of CaMKII should be targeted for degradation in CSF extract even in the absence of a calcium signal. To test this idea, we converted the CaMKII site (RXST$^{195}$LXD) into a consensus site for cyclin dependent kinase 1 (Cdk1; RXST$^{195}$PXK) and examined the stability of the $^{35}$S-labelled

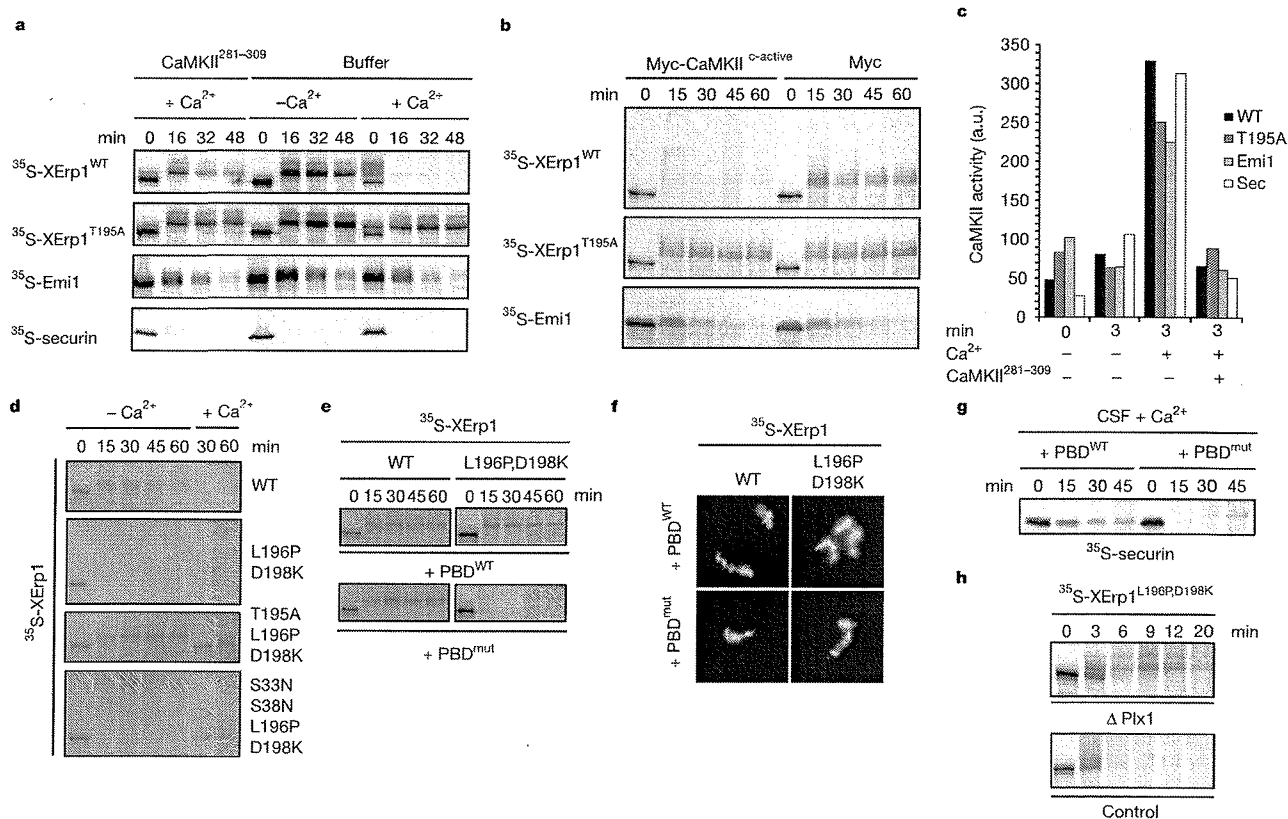


**Figure 3 | CaMKII activity is essential for the degradation of XErp1 in *Xenopus* egg extract.** **a**, IVT $^{35}$S-XErp1$^{WT}$, -XErp1$^{T195A}$, -Emi1 or -securin was incubated in Δ90 extract supplemented with buffer, or calcium in the presence or absence of 300 μM of the CaMKII inhibitory peptide CaMKII$^{281-309}$. Samples were analysed by autoradiography. **b**, IVT $^{35}$S-XErp1$^{WT}$, -XErp1$^{T195A}$ or -Emi1 was incubated in Δ90 extract supplemented with immunopurified IVT constitutively active CaMKII$^{c\text{-active}}$ or immunopurified mock IVT and analysed as in **a**. **c**, Samples from extracts shown in **a** were analysed for CaMKII activity. **d**, Cdk1 can take on CaMKII's function in sensitizing XErp1 for Plx1-dependent degradation. IVT $^{35}$S-XErp1$^{WT}$, -XErp1$^{L196P,D198K}$, -XErp1$^{T195A,L196P,D198K}$ or -XErp1$^{S33N,S38N,L196P,D198K}$ was incubated in CSF extract and samples were analysed by autoradiography. **e–h** Plx1 activity is required for the degradation of XErp1$^{L196P,D198K}$ in CSF extract. **e**, **f**, Samples of CSF extracts supplemented with MBP-PBD$^{WT}$ or MBP-PBD$^{mut}$ were analysed (**e**) as in **d**, and assayed for DNA morphology (**f**). CSF extracts treated as in **e** were supplemented with calcium and assayed for the stability of IVT securin (**g**). Plx1-depleted (ΔPlx1) or mock depleted (control) CSF extract were treated as in **d** (**h**).

IVT product in CSF extracts. As Cdk1 is active in CSF extract and able to create docking sites for Plk-PBDs (ref. 13), the prediction was that Cdk1-mediated phosphorylation of this mutant XErp1 would result in the docking of Plx1, the phosphorylation of the amino-terminal phospho-degron and the destruction of the protein. Indeed, whereas IVT XErp1$^{WT}$ remained stable in CSF extract, XErp1$^{L196P,D198K}$ was rapidly degraded even in the absence of calcium (Fig. 3d), indicating that Cdk1 or any other proline-directed kinase active in CSF extract could substitute for CaMKII and sensitize this XErp1 mutant for Plx1-dependent degradation. As predicted by our model, the degradation of these XErp1 forms in CSF extract could be prevented by mutating either critical residues within the CDK1 consensus site (XErp1$^{T195A,L196P,D198K}$) or the $DSGX_3S^{38}$ motif (XErp1$^{S33N,S38N,L196P,D198K}$) (Fig. 3d). To demonstrate that the activity of Plx1 was essential for the degradation of XErp1$^{L196P,D198K}$, we examined the stability of IVT XErp1$^{L196P,D198K}$ in CSF extract supplemented with an excess of MBP-PBD$^{WT}$, which we have shown to exert a dominant-negative effect on Plx1 function[9]. As expected, the addition of MBP-PBD$^{WT}$ resulted in a significant stabilization of IVT XErp1$^{L196P,D198K}$ in CSF extract, as compared to a control-treated extract (Fig. 3e) but had no effect on the stability of IVT XErp1$^{WT}$ (Fig. 3e). Analyses of the chromatin structures revealed that the extracts remained CSF-arrested under all conditions (Fig. 3f). The dominant-negative effect of MBP-PBD on Plx1 function was confirmed in parallel experiments in which we examined the stability of IVT securin on calcium addition. PBD$^{WT}$- supplemented CSF extract, but not PBD$^{mut}$-treated extract, failed to exit meiosis on calcium treatment, as indicated by stable IVT securin (Fig. 3g). Consistently, MBP-PBD$^{WT}$ but not PBD$^{mut}$ prevented the calcium-induced degradation of endogenous XErp1 (see Supplementary Fig. S3). Finally, a similar stabilization of IVT XErp1$^{L196P,D198K}$ was observed in CSF extract immunodepleted of Plx1 but not in mock-depleted extract (Fig. 3h). Taken together, these data demonstrate that the creation of a Plx1-PBD docking site on XErp1, an event normally brought about by calcium-activated CaMKII, defines the timing of Plx1-dependent degradation of XErp1.

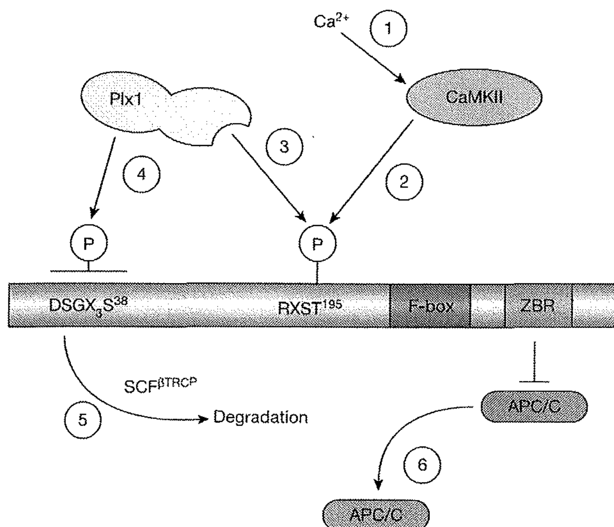


**Figure 4 | Model of how calcium triggers release from CSF arrest.** (1) A transient rise in free calcium activates CaMKII. (2) Activated CaMKII phosphorylates XErp1 at Thr 195, thereby creating a docking site for Plx1. (3) Plx1 binds to XErp1 via its PBD. (4) On binding to its substrate, Plx1 becomes activated and phosphorylates XErp1 at Ser 33/38 of the $DSGX_3S^{38}$ motif. (5) Phosphorylated $DSGX_3S^{38}$ is recognized by $SCF^{\beta\text{-}TRCP}$ leading to the destruction of XErp1. (6) XErp1 degradation leads to APC/C activation.

In conclusion, our study identifies the critical substrate of the calcium-activated CaMKII that triggers exit from meiosis II in response to fertilization. Specifically, our data demonstrate that CaMKII phosphorylates the APC/C inhibitor XErp1, thereby acting as a novel priming kinase for the recruitment of Plx1 (Fig. 4). The bound Plx1, previously shown to be essential for causing release from CSF arrest[11], then triggers the degradation of XErp1 by phosphorylation of a $DSGX_3S$ degron. Our findings thus explain how a calcium signal prompts the exit from meiosis through the spatiotemporal integration of the action of two key kinases, CaMKII and Plx1, both converging onto XErp1, a critical inhibitor of APC/C.

## METHODS

**Plasmids, proteins and antibodies.** XErp1, Emi1 and securin constructs were as described[9]. Site-directed mutagenesis was performed using the QuikChange kit (Stratagene). MBP-tagged proteins were purified following published protocols[9]. Purification of His-tagged Plx1 from SF9 cells was performed as described[11]. *In-vitro* translation experiments using $^{35}$S-labelled methionine were performed according to manufacturer's protocol (Promega). Full-length Plx1 purified from SF-9 cells was used to generate rabbit antibodies to Plx1. MBP-PBD$^{WT}$ and MBP-PBD$^{mut}$ (W408F, H532A and K534A) were used as described previously[9].

***In vitro* kinase assays.** CaMKII was purchased from New England biolabs. *In vitro* CaMKII assays were performed at 30 °C in kinase reaction buffer containing 45 U of activated CaMKII, 0.1–1 mM ATP, 4 μCi [$\gamma$-$^{32}$P]ATP and 200 ng of purified MBP-tagged XErp1 protein. Samples were taken at the indicated time points. CaMKII activity assays were performed based on published protocols[8] using Autocamtide-2 (New England Biolabs) as substrate. Plx1 kinase assays were performed for 5 min at 30 °C using kinase reaction buffer supplemented with 4 μCi [$\gamma$-$^{32}$P]ATP and MBP-tagged XErp1 proteins which had previously been incubated with CaMKII or buffer (control) in the presence of 1 mM ATP.

**Far-western ligand blots.** Far-western ligand blots were performed in TBS supplemented with 0.1% Tween-20 and 5% wt/vol skim milk powder. For each assay 500 ng MBP-XErp1, treated with CaMKII or buffer (control), was subjected to SDS polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to membranes. These were then incubated overnight at 4 °C with 2 μg ml$^{-1}$ of MBP-tagged PBD[9] or His-Plx1. Bound protein was detected using affinity-purified rabbit antibodies to Plx1.

***Xenopus* extracts.** *Xenopus* CSF egg extracts were prepared as described previously[20]. CSF release was induced by adding 1 mM $CaCl_2$ to the extract. DNA and spindle morphology were examined as described previously[21]. CSF-released extract was arrested at anaphase by the addition of non-degradable cyclin B (Δ90 extract) as described[14,15]. The Δ90 extract was treated with 300 μM EGTA and CaMKII was re-activated by the addition of 600 μM calcium. Where indicated, MBP-PBD$^{WT}$ or MBP-PBD$^{mut}$ was added to the extract to a final concentration of 400 μg ml$^{-1}$. For some experiments extract was treated with cycloheximide. The immunodepletion of Plx1 was performed as described[22].


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