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# Non-proteolytic ubiquitylation counteracts the APC/C-inhibitory function of XErp1

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**Mature *Xenopus* oocytes are arrested in meiosis by the activity of XErp1/Emi2, an inhibitor of the ubiquitin-ligase anaphase-promoting complex/cyclosome (APC/C). On fertilization, XErp1 is degraded, resulting in APC/C activation and the consequent degradation of cell-cycle regulators and exit from meiosis. In this study, we show that a modest increase in the activity of the ubiquitin-conjugating enzyme UbcX overrides the meiotic arrest in an APC/C-dependent reaction. Intriguingly, XErp1 remains stable in these conditions. We found that UbcX causes the ubiquitylation of XErp1, followed by its dissociation from the APC/C. Our data support the idea that ubiquitylation regulates the APC/C-inhibitory activity of XErp1.**

Keywords: APC/C; CSF; XErp1/Emi2; UbcX/UbcH10

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## INTRODUCTION

In vertebrates, mature oocytes await fertilization when they are arrested at metaphase of meiosis II. This resting state depends on the cytostatic factor (CSF), which maintains the anaphase-promoting complex/cyclosome (APC/C)—a ubiquitin E3 ligase—in an inactive state (Maller *et al*, 2002). Active APC/C together with its cofactor cell division cycle 20 (Cdc20; APC/$C^{Cdc20}$) targets the anaphase inhibitor securin and the Cdk1 (cyclin-dependent kinase 1)-activating cyclin B for proteasome-dependent destruction resulting in exit from meiosis. Recently, XErp1/Emi2 was identified as the molecular nature of CSF in *Xenopus* eggs that directly inhibits the APC/C (Schmidt *et al*, 2005; Tung *et al*, 2005). Fertilization of the egg causes the activation of calmodulin/calcium-dependent kinase II (CaMKII). CaMKII, together with Polo-like kinase 1 (Plx1), then targets XErp1 for degradation, resulting in APC/C activation and the consequent completion of meiosis II (Liu & Maller, 2005; Rauh *et al*, 2005). The activity of XErp1 during CSF arrest is negatively regulated by Cdk1 and positively controlled by $p90^{RSK}$ kinase (Nishiyama *et al*, 2007; Wu *et al*, 2007a, b). Specifically, phosphorylation of XErp1 by Cdk1 decreases both its half-life and its affinity for the APC/C. These phosphorylations are removed by the protein phosphatase 2A, which binds to XErp1 upon phosphorylation of XErp1 by $p90^{RSK}$. Thus, by alternate activation of APC/C or CSF (XErp1), these cybernetic mechanisms ensure constant Cdk1 activity at metaphase II, despite continuous cyclin B synthesis.

A similar mechanism controls the association between Cdc20 and components of the spindle assembly checkpoint (SAC) by a dynamic balance of ubiquitylation and deubiquitylation. Activation of the SAC by unattached kinetochores leads to the association of Cdc20 with the SAC proteins Mad2 and BubR1, which prevent Cdc20 from activating the APC/C. Addition of the E2 enzyme UbcH10 to SAC-arrested cell extract triggers the APC/$C^{Cdc20}$-dependent multi-ubiquitylation of Cdc20, and possibly other components of the APC/C–Cdc20–SAC complex, resulting in the release of Mad2 and BubR1 from Cdc20 (Reddy *et al*, 2007; Ge *et al*, 2009). Although in checkpoint arrest conditions this ubiquitylation reaction is antagonized by the activity of the ubiquitin hydrolase USP44 (Stegmeier *et al*, 2007), it rapidly activates APC/C in a switch-like manner as soon as the last kinetochore is properly attached. In this study, we show that ectopic UbcX—the *Xenopus* orthologue of UbcH10 (Yu *et al*, 1996)—induces release from CSF arrest. In the presence of elevated levels of active UbcX, XErp1 is ubiquitylated and dissociated from the APC/C, suggesting that the APC/C inhibitory activity of XErp1 in CSF arrest can be modulated in an UbcX-dependent manner.

## RESULTS AND DISCUSSION

The finding that the APC/C can liberate itself from inhibition by the SAC prompted us to test whether a similar mechanism controls CSF activity. First, we analysed whether UbcX can suppress SAC activity in *Xenopus* egg extract. Reportedly, egg extract with an artificially high ratio of nucleus to cytoplasm activates the SAC on treatment with spindle poisons (Minshull *et al*, 1994). We consistently observed that *in vitro* translated $^{35}$S-labelled securin ($^{35}$S-securin) remained stable when calcium was added to the extract containing high concentrations of sperm nuclei and

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nocodazole (Fig 1A, panel 1). Western blot analyses showed that calcium triggered XErp1 destruction, demonstrating that APC/C inhibition was maintained by SAC activity, but not by CSF activity (Fig 1A, panel 1). Importantly, addition of wild-type UbcX ($UbcX^{wt}$), but not the catalytically inactive form ($UbcX^{ci}$), to SAC-arrested extract induced the destruction of $^{35}$S-securin (Fig 1A), suggesting that the mechanism of UbcH10-mediated SAC inactivation is conserved between humans and *Xenopus*.

Next, we analysed whether recombinant $UbcX^{wt}$ is able to release CSF arrest. Addition of calcium to CSF extract caused the degradation of XErp1, $^{35}$S-securin and cyclin B2 and the consequent decondensation of chromatin (Fig 1B,C). Intriguingly, $UbcX^{wt}$, but not $UbcX^{ci}$, triggered calcium-independent exit from meiosis, as indicated by $UbcX^{wt}$-mediated destruction of APC/C substrates and chromatin decondensation (Fig 1B,C). Notably, in these conditions XErp1 was dephosphorylated but not degraded on exit from meiosis, as indicated by the shift in its electrophoretic mobility, suggesting that $UbcX^{wt}$ neutralizes CSF activity by a different means than XErp1 degradation. Like UbcX, catalytically active UbcH10 triggered premature CSF release (supplementary Fig S1A online), demonstrating that UbcX and UbcH10 are equivalent in their ability to override the CSF arrest.

In conditions in which the SAC is active, ubiquitylation of Cdc20 is antagonized by the ubiquitin hydrolase USP44 (Stegmeier *et al*, 2007). However, we could not deplete USP44 from CSF extract using any of the three antibodies we raised against Xenopus USP44, or efficiently inhibit hydrolases in CSF extract using ubiquitin aldehyde, a competitive pan-ubiquitin hydrolase inhibitor (Hershko & Rose, 1987; data not shown). Therefore, for technical reasons, we were not able to determine whether USP44 is involved in the regulation of CSF arrest. To exclude the possibility that the $UbcX^{wt}$-induced CSF release was due to an unspecified overload of the system, we analysed the amount of $UbcX^{wt}$ required for this release. Quantitative western blot analyses determined the concentration of endogenous UbcX in CSF extract to be approximately 0.8 μM (Fig 1D). Titration experiments showed that a twofold excess of $UbcX^{wt}$ (approximately 1.6 μM) was sufficient to trigger partial destabilization of $^{35}$S-securin, whereas efficient APC/C activation was mediated by an eightfold excess of $UbcX^{wt}$ (Fig 1E). To further analyse whether elevated levels of UbcX interfere with the function of XErp1 during meiotic progression of *Xenopus* oocytes, we injected recombinant $UbcX^{wt}$ into stage VI oocytes and induced meiotic maturation by progesterone treatment. Consistent with the idea that the APC/C-inhibitory function of XErp1 is essential for the transition from meiosis I to meiosis II (Ohe *et al*, 2007), we observed that injection of $UbcX^{wt}$ into oocytes prevented entry into meiosis II, as indicated by the failure to reaccumulate cyclin B2 after meiosis I (Fig 1F). In summary, these data—together with the observation that $UbcX^{ci}$ did not affect the CSF arrest (Fig 2B)—demonstrate that the APC/C-inhibitory activity of XErp1 is sensitive to subtle changes in E2 activity.

As $UbcX^{wt}$/$UbcH10^{wt}$ triggered CSF release but not XErp1 destruction, we investigated whether APC/C activity is required for the exit from meiosis, by Cdc20 immunodepletion (Fig 2A). As expected, addition of calcium to Cdc20-depleted extract did not induce $^{35}$S-securin destruction (Fig 2B). Similarly, addition of UbcH10 to the extract depleted of Cdc20 or the core APC/C-subunit Cdc27 did not mediate $^{35}$S-securin degradation (Fig 2B,C). Add-back of recombinant Cdc20 confirmed the specificity of the Cdc20 depletion (supplementary Fig S1C,D online). Thus, although ectopic E2 enzyme does not destabilize XErp1, $APC/C^{Cdc20}$-dependent ubiquitylation is still essential for this calcium-independent abrogation of the meiosis II arrest.

Recently, the model that suggests that the APC/C can liberate itself from SAC inhibition has been challenged. Specifically, it has been postulated that in the presence of Mad2/BubR1/Bub3, Cdc20 is turned into an APC/C substrate resulting in its ubiquitylation and degradation (Nilsson *et al*, 2008). Thus, although the 'activation model' proposes that ubiquitylation of Cdc20 results in APC/C activation, the 'inactivation model' proposes that it induces Cdc20 degradation, that is, APC/C inactivation. In support of the latter, it has been reported that budding yeast Cdc20 is destabilized in response to SAC activation (Pan & Chen, 2004; King *et al*, 2007). The controversy about these models prompted us to determine whether CSF arrest is mediated by the destabilization of Cdc20. As shown in Fig 3A, inhibition of protein synthesis by cycloheximide (CHX) did not significantly reduce Cdc20 levels in CSF extract. To corroborate this finding, we analysed the levels of Cdc20 when protein destruction was blocked. Addition of MG262 to CSF extract did not affect Cdc20 levels (Fig 3B), but it completely inhibited calcium-induced degradation of cyclin B (data not shown) confirming efficient inhibition of the proteasome by MG262. Thus, *Xenopus* Cdc20 is not a short-lived protein in egg extract. Moreover, as CHX-treated extract was able to trigger calcium-induced degradation of cyclin B (Fig 3A), our data demonstrate that Cdc20 levels are sufficient to effectively target APC/C substrates for destruction even when its resynthesis is inhibited. These results—together with our observation that exogenous UbcX induces premature CSF release, rather than an enhancement of the arrest—indicate that a stable CSF state is not aided by the destabilization of Cdc20.

Next, we addressed the mechanism of UbcX/UbcH10-mediated CSF release. As addition of these E2 enzymes did not induce XErp1 destruction, we investigated whether $UbcH10^{wt}$ interferes with the APC/C-inhibitory function of XErp1. First, we used sedimentation velocity centrifugation to analyse the interaction between XErp1 and the APC/C in extract. Consistent with their effect on the CSF arrest, $UbcH10^{wt}$ but not $UbcH10^{ci}$ caused the dissociation of XErp1 from the APC/C (Fig 3C). To investigate more directly whether $UbcH10^{wt}$ affects the interaction between XErp1 and the APC/C, we analysed the amount of XErp1 coprecipitating with Cdc27 from the extract treated with UbcH10 and MG262 to prevent exit from meiosis. Intriguingly, a significantly reduced amount of XErp1 was detected when Cdc27 was purified from the extract supplemented with $UbcH10^{wt}$ than from the extract containing control buffer or $UbcH10^{ci}$ (Fig 3E). Equal amounts of XErp1 in the input samples confirmed that this effect was due to reduced binding of XErp1 to the APC/C (Fig 3D). In summary, addition of $UbcH10^{wt}$ causes premature CSF release, by interfering with the ability of XErp1 to bind to the APC/C.

Elevated levels of UbcH10 drive SAC inactivation by APC/C-dependent ubiquitylation of Cdc20 or other components of the APC/C–Cdc20 checkpoint complex (Reddy *et al*, 2007). However, when we affinity-purified Cdc20 from CSF extract supplemented with $UbcX^{wt}$, no slower-migrating forms of Cdc20, indicative of ubiquitylated Cdc20, could be detected (Fig 4A). By contrast, a

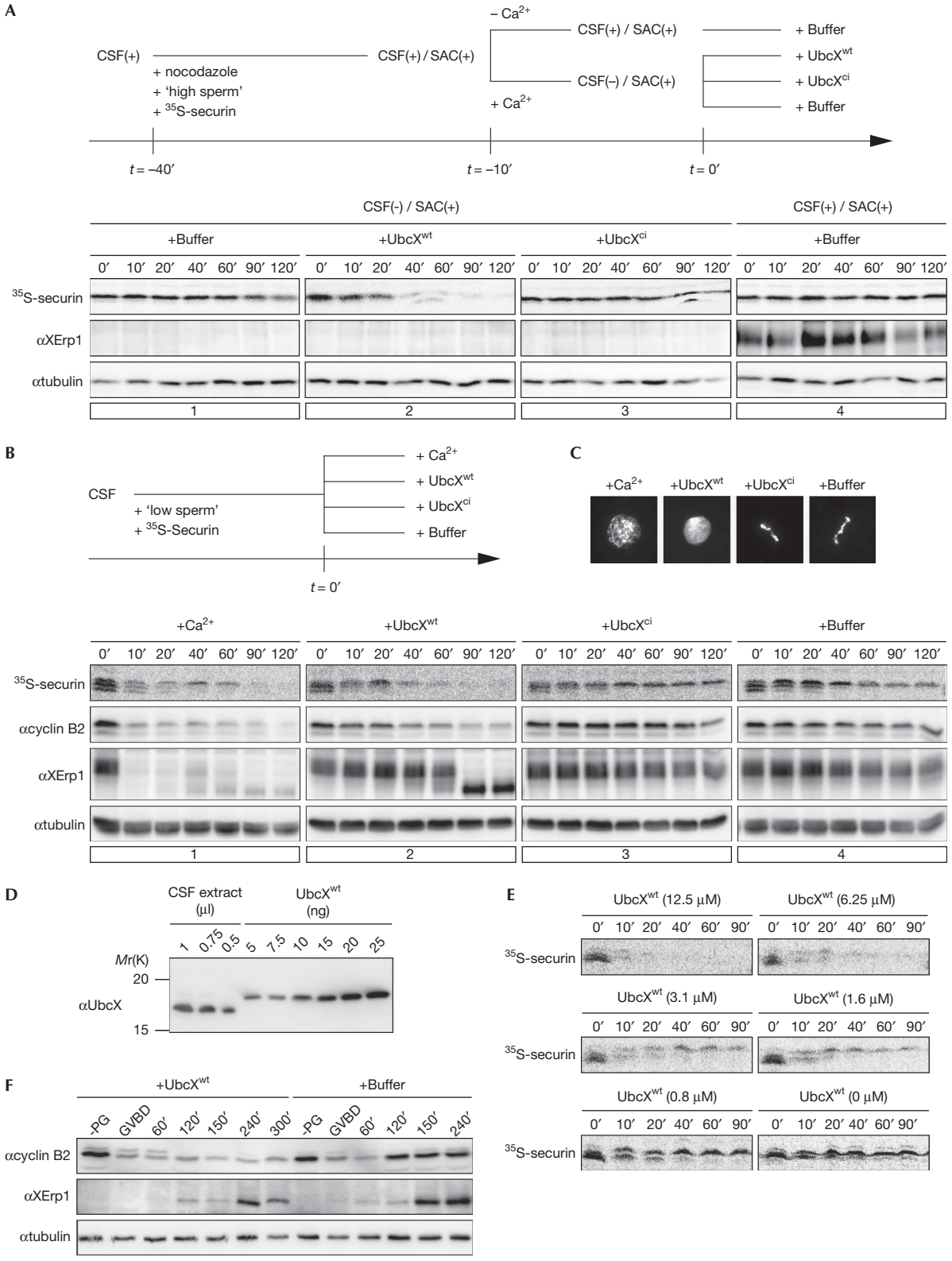


**Fig 1** | Ectopic UbcX$^{wt}$ overrides spindle assembly checkpoint and cytostatic factor arrest in *Xenopus* egg extract. (**A**) CSF extract containing $^{35}$S-securin was supplemented with nocodazole and high concentrations of sperm to activate the SAC in the CSF arrest. CSF arrest was released by the addition of calcium. Samples were taken at the indicated time points after the addition of the specified reagents, $^{35}$S-securin was detected by autoradiography and XErp1 and α-tubulin by WB. (**B**) The indicated reagents were added to CSF extract, and the stabilities of $^{35}$S-securin, XErp1 and cyclin B2 were analysed as before. (**C**) The extract was treated as in (**B**) and chromatin structures were analysed after 90 mins. (**D**) The amount of UbcX in CSF extract was determined by WB, using recombinant UbcX$^{wt}$ as standard. (**E**) CSF extract was supplemented with the indicated concentrations of UbcX$^{wt}$ and CSF release was monitored by autoradiographic analysis of $^{35}$S-securin. (**F**) Stage VI oocytes were injected with buffer or with 80 ng UbcX$^{wt}$, resulting in 8.9 μM exogenous UbcX, which is approximately 11-fold more than levels of the endogenous protein. Maturation was induced by progesterone treatment and samples were taken for WB analysis at the indicated time points after GVBD. ci, catalytically inactive; CSF, cytostatic factor; GVBD, germinal vesicle breakdown; PG, progesterone; SAC, spindle assembly checkpoint; $^{35}$S-securin, *in vitro* translated $^{35}$S-labelled securin; WB, western blotting; wt, wild type.

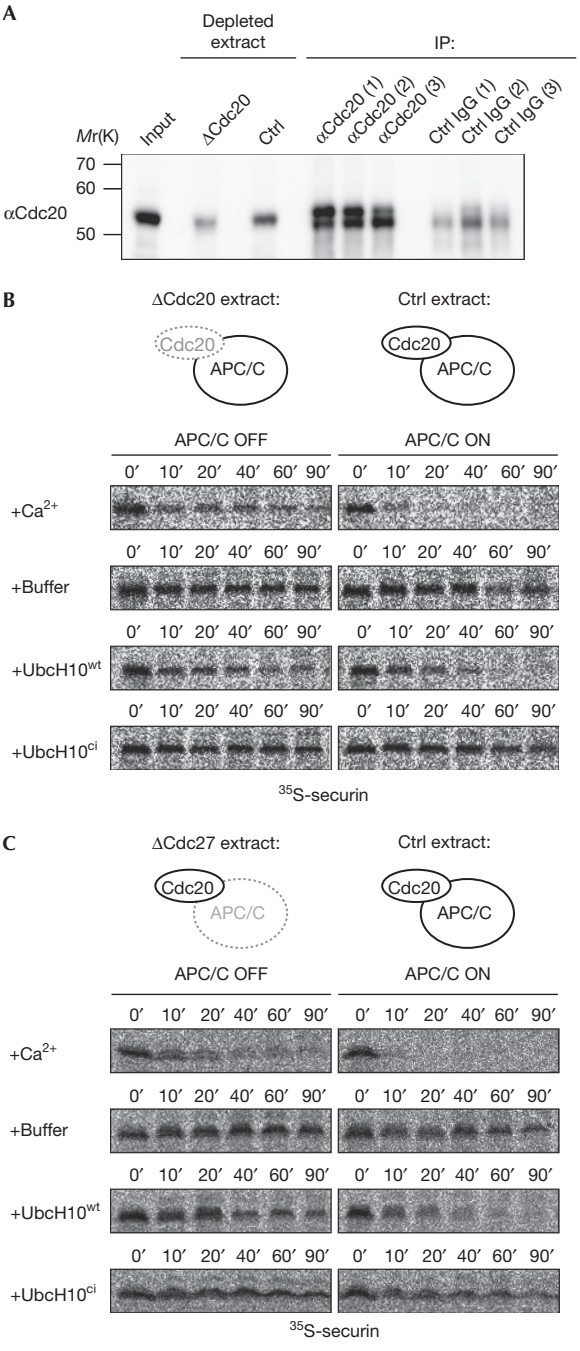
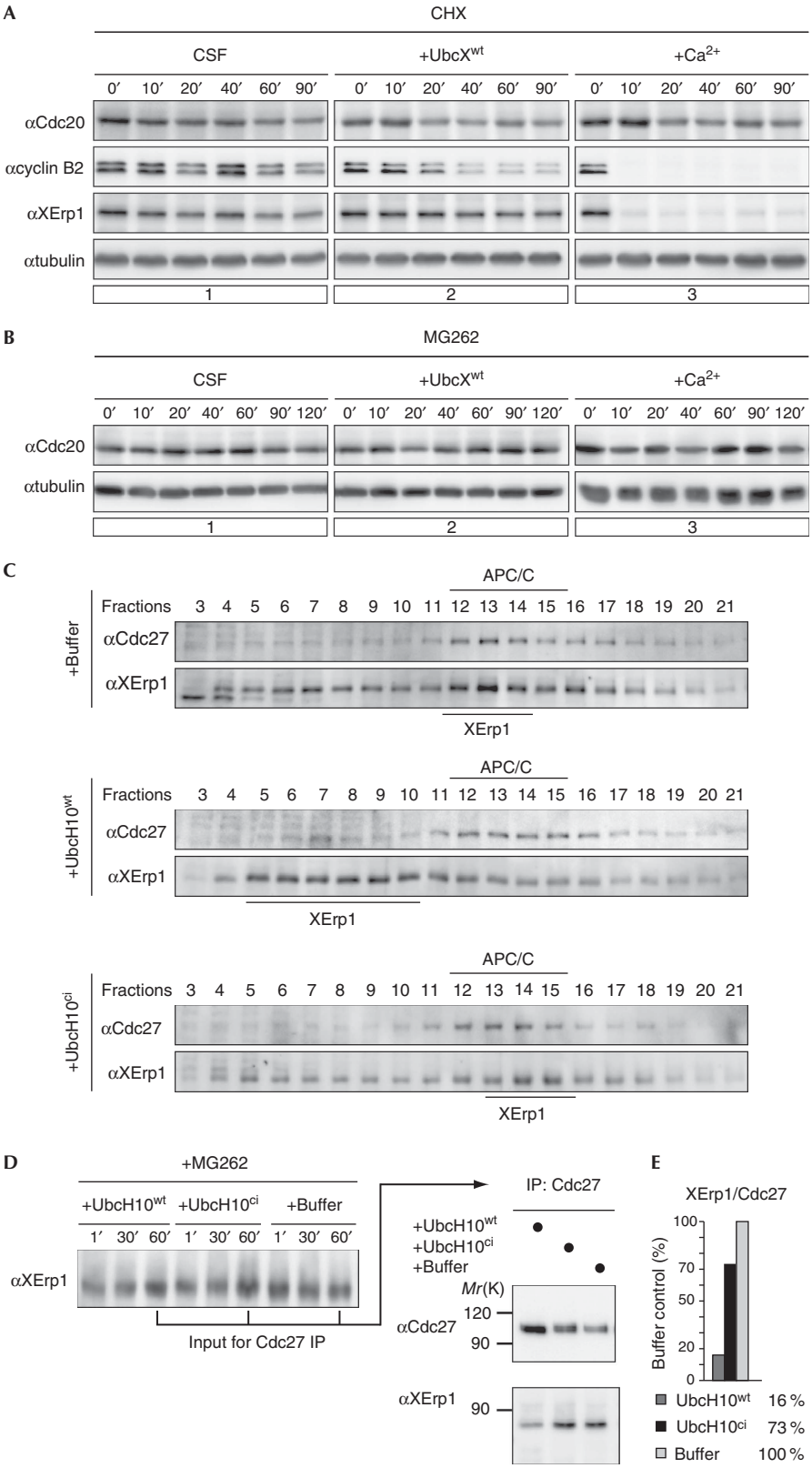



**Fig 2** | UbcH10$^{wt}$-induced cytostatic factor release requires anaphase-promoting complex/cyclosome activity. (**A**) Cdc20 was depleted from CSF extracts by three rounds of immunodepletion. Cdc20 was detected by WB analysis in the input fraction, in the extract after the third round of Cdc20 depletion (ΔCdc20) or control depletion, as well as on the α-Cdc20 and control beads (IP) after the indicated rounds of depletion. (**B**) Cdc20- or control-depleted extract was supplemented with calcium, buffer, UbcH10$^{wt}$ or UbcH10$^{ci}$ and the stability of $^{35}$S-securin was analysed by autoradiography. (**C**) CSF extract that was depleted of Cdc27 or control-depleted extract was supplemented with the indicated reagents and $^{35}$S-securin was detected at the indicated time points. APC/C, anaphase-promoting complex/cyclosome; Cdc, cell division cycle; ci, catalytically inactive; CSF, cytostatic factor; Ctrl, control; IgG, immunoglobulin G; IP, immunoprecipitation; $^{35}$S-securin, *in vitro* translated $^{35}$S-labelled securin; WB, western blotting; wt, wild type.

smear characteristic of ubiquitylation was detected when XErp1 was immunopurified from UbcX$^{wt}$-containing extract (Fig 4B). Consistent with its inability to cause premature CSF release, UbcX$^{ci}$ did not change the electrophoretic mobility of XErp1 (Fig 4B). To confirm that ubiquitylation accounts for the high-molecular smear, we used his-tagged ubiquitin (his-Ub) to affinity-purify ubiquitylated proteins from CSF extract. Denaturing conditions during purification ensured that only proteins that were covalently attached to his-Ub were purified. As a control, CSF extract was supplemented with a truncated version of his-Ub (his-UbΔGG) that was unable to form isopeptide bonds with substrates. As shown in Fig 4C, XErp1 was ubiquitylated in a reaction dependent on active UbcX and conjugatable His-Ub$^{wt}$. As shown in Fig 4D,E, depletion of Cdc27 but not β-TRCP (transduction repeat-containing protein) abrogated the UbcX$^{wt}$-mediated ubiquitylation of XErp1, demonstrating that this reaction required APC/C activity but not SCF$^{β\text{-}TRCP}$ activity. To test this directly, we performed *in vitro* ubiquitylation assays using immunopurified APC/C. Addition of UbcX$^{wt}$ but not UbcX$^{ci}$ to immunopurified APC/C caused significant ubiquitylation of XErp1 (Fig 4F). Notably, we observed a small UbcX$^{wt}$-dependent increase in slower-migrating forms of Cdc20, indicating that the APC/C-associated fraction of Cdc20 is ubiquitylated by UbcX (Fig 4G). Importantly, when we performed *in vitro* APC/C-ubiquitylation assays using active UbcX$^{wt}$, and then separated the APC/C-bound fraction from the supernatant, we observed the dissociation of ubiquitylated forms of XErp1 from the APC/C (Fig 4F). Efficient dissociation of XErp1 was not detected when immunopurified APC/C was incubated with UbcX$^{ci}$ (Fig 4F).



◀ **Fig 3** | Cell division cycle 20 is stable in cytostatic factor arrest and UbcH10$^{wt}$ causes the dissociation of the anaphase-promoting complex/cyclosome–XErp1 complex. (**A**) CSF extract treated with CHX was supplemented with buffer control, UbcX$^{wt}$ or calcium. Samples were taken at the indicated time points, treated with phosphatase and analysed by WB for Cdc20, XErp1, cyclin B2 and α-tubulin. (**B**) CSF extract treated with MG262 was supplemented with buffer control, UbcX$^{wt}$, or calcium and samples were taken. After phosphatase treatment, Cdc20 and tubulin were analysed by WB. (**C**) CSF extracts were incubated with buffer, UbcH10$^{wt}$ or UbcH10$^{ci}$ and centrifuged through glycerol gradients. Fractions were taken from high (fraction 3) to low (fraction 21) glycerol concentrations. Cdc27 and XErp1 were detected by WB. (**D**) CSF extracts containing MG262 were treated as described in (**C**) and Cdc27 was immunoprecipitated. The immunoprecipitates were subjected to CIP phosphatase treatment and Cdc27 and XErp1 were detected by WB analysis. (**E**) Quantification of (**D**). The integrated intensities of Cdc27 and XErp1 signals were measured and the ratio of the signals of XErp1 to Cdc27 was calculated. Buffer control was arbitrarily set to 100. APC/C, anaphase-promoting complex/cyclosome; Cdc, cell division cycle; CHX, cycloheximide; ci, catalytically inactive; CIP, calf intestinal alkaline phosphatase; CSF, cytostatic factor; IP, immunoprecipitation; WB, western blotting; wt, wild type.

Collectively, these data suggest that UbcX triggers the ubiquitylation of XErp1 and, to a lesser extent, the ubiquitylation of Cdc20, resulting in the dissociation of the APC/C inhibitor XErp1 from the APC/C.

Thus, our studies identify a new mechanism for regulation of XErp1 activity in *Xenopus* egg extract. On fertilization, CaMKII and Plx1 cooperate to neutralize XErp1 activity by targeting it for rapid, SCF$^{\beta\text{-TRCP}}$-dependent destruction resulting in the irreversible exit from meiosis. During CSF arrest, continuous cyclin B synthesis (Yamamoto *et al*, 2005) is compensated for by transient Cdk1-dependent inactivation of XErp1. Here, we show that the APC/C can liberate itself from inhibition by CSF. Specifically, we observed that increasing the activity of the APC/C-cooperating E2 enzyme UbcX causes both the ubiquitylation of XErp1 and the dissociation of XErp1 from the APC/C, suggesting that the APC/C-mediated attachment of ubiquitin to XErp1 reduces its affinity for the APC/C. Although future studies are required to identify the acceptor lysine residue, the different consequences of APC/C$^{Cdc20}$- and SCF$^{\beta\text{-TRCP}}$-mediated ubiquitylation of XErp1 suggest that the two processes must differ in either the site of ubiquitin attachment or the type of ubiquitin linkages. Intriguingly, although we were unable to detect ubiquitylated Cdc20 in UbcX$^{wt}$-supplemented CSF extract, our *in vitro* APC/C assays showed that UbcX$^{wt}$ is able to ubiquitylate Cdc20, albeit inefficiently, indicating that only a small fraction of Cdc20 is modified and/or ubiquitylated Cdc20 is an efficient substrate of ubiquitin hydrolases. Thus, although we cannot rule out that the ubiquitylation of Cdc20 in addition to the one of XErp1 contributes to the reduced affinity of XErp1 for the APC/C$^{Cdc20}$, our data exclude the possibility that CSF-arrest is mediated by the destabilization of Cdc20. This conclusion is made on the basis of the observations that neither inhibition of protein translation or protein degradation detectably affected Cdc20 levels in CSF extract and, in conditions in which Cdc20 resynthesis is inhibited, the amount of Cdc20 in the extract is sufficient to mediate the destruction of APC/C substrates on calcium stimulus. As the second APC/C activator, Cdh1, is not expressed in *Xenopus* egg extract (Lorca *et al*, 1998), the proteolysis of APC/C substrates must be mediated by Cdc20.

Our studies show that UbcX levels increase at the transition from meiosis I to meiosis II (supplementary Fig S1B online), but remain constant during meiosis II. Thus, it is tempting to speculate that the sudden increase in UbcX activity results in partial APC/C activation, resulting in exit from meiosis I. According to this hypothesis, the delayed accumulation of XErp1 (supplementary Fig S1B online) would reestablish APC/C inhibition, thus preventing entry into interphase and, finally, resulting in metaphase II-arrested mature eggs. As we have no evidence that UbcX is activated on fertilization, we favour the model that calcium-induced degradation of XErp1 is the initial trigger that partly activates the APC/C, which enables UbcX to facilitate the dissociation of XErp1 from the APC/C. This model implies that the robust, yet, rapidly switchable CSF arrest is mediated by a dynamic association of XErp1 with the APC/C, and that UbcX-mediated ubiquitylation helps to shift the balance towards the APC/C-unbound form of XErp1. Although under CSF arrest this mechanism would not be sufficient to result in APC/C activation, it could support the activation of the APC/C once fertilization induces the destruction of XErp1. Thus, the UbcX-mediated dissociation of XErp1 from the APC/C could provide a positive-feedback loop that contributes to an abrupt switch-like onset of anaphase on fertilization. In somatic cells, attempts of the APC/C to escape checkpoint inhibition in the presence of malattached chromosomes are constantly antagonized by the deubiquitylation of Cdc20 (Stegmeier *et al*, 2007). To fully understand how APC/C activity is regulated in CSF arrest, a future aim will be to identify the pathway that antagonizes UbcX-mediated ubiquitylation of XErp1.

## METHODS

**Plasmids.** UbcH10 and UbcX were amplified from complementary DNA. Catalytically inactive forms were generated by mutating Cys 114.

Cytostatic factor extracts were prepared as described previously (Murray, 1991). When indicated, $CaCl_2$ was added to a concentration of 600 μM, CHX to 350 μM, MG262 to 100 μM and nocodazole to 33 μM. 'High' and 'low' sperm extracts contained $1.2 \times 10^7$ and $1 \times 10^6$ sperm nuclei per ml extract, respectively. His-tagged UbcH10$^{wt/ci}$ and UbcX$^{wt/ci}$ were added to a final concentration of 12 and 6 μM, respectively. Proteins were dephosphorylated in 1 × calf intestinal alkaline phosphatase (CIP) buffer with 10 U of CIP (NEB).

*Xenopus* oocytes were obtained, cultured and injected as described previously (Ohe *et al*, 2007). Oocytes were homogenized in 1 × CIP buffer with 10 U of CIP (New England Biolabs) and complete protease inhibitors (Roche).

Immunodepletion experiments and purification of ubiquitin conjugates were performed as described (Glockzin *et al*, 2003; Schmidt *et al*, 2005).

*In vitro* ubiquitylation assays were carried out as described previously (Schmidt *et al*, 2005). To assay the ubiquitylation and release of XErp1 from the APC/C, magnetic beads were separated from the supernatant.

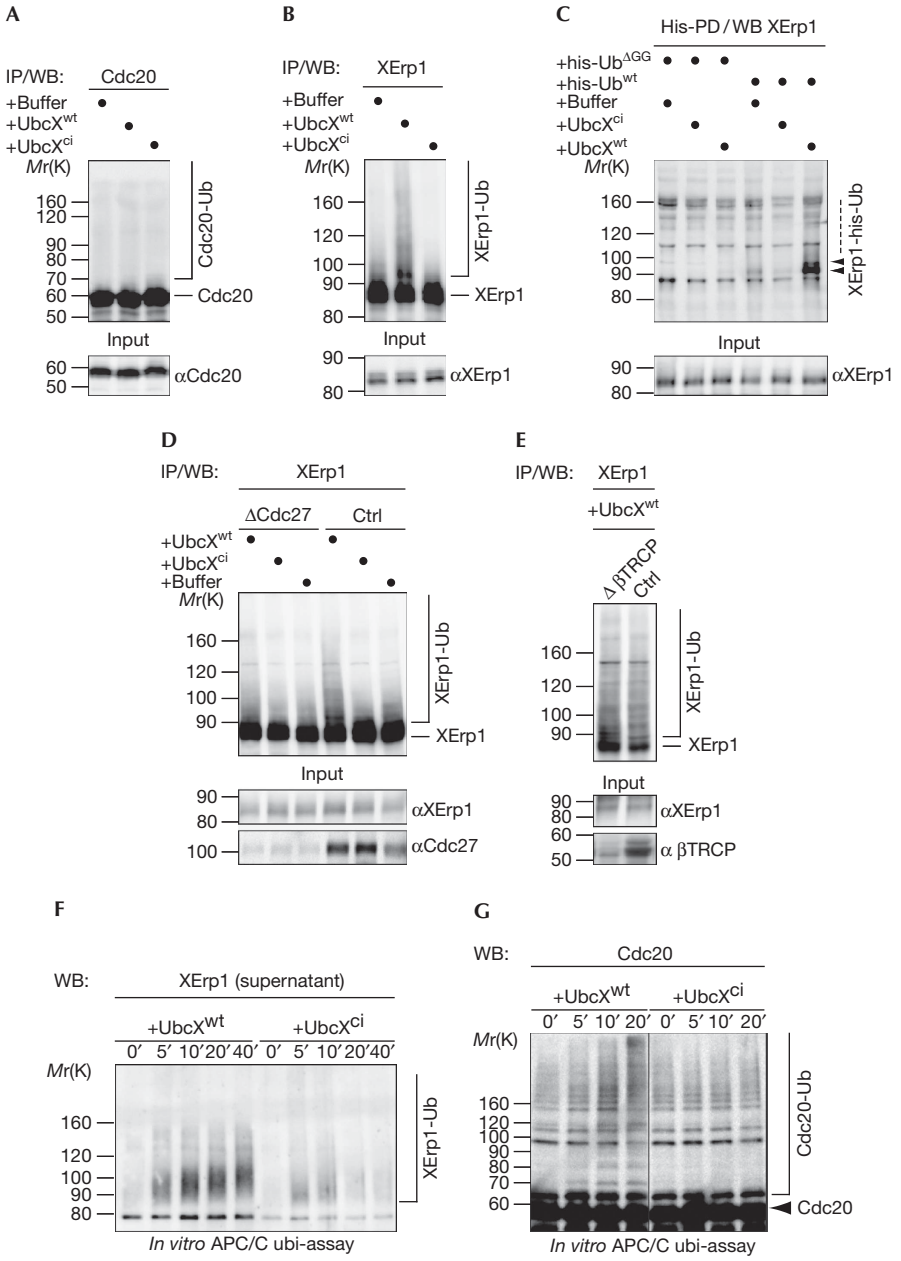



**Fig 4** | UbcX$^{wt}$ promotes XErp1 ubiquitylation. (**A**) CSF extract incubated with buffer, UbcX$^{wt}$ or UbcX$^{ci}$ for 30 min was processed for WB analysis (input) or used to IP Cdc20, followed by WB analysis. (**B**) CSF extract was treated as in (**A**) and XErp1 was immunoprecipitated and analysed by WB. (**C**) CSF extracts containing his-Ub$^{wt}$ or his-Ub$^{\Delta GG}$ were treated as in (**A**) and his-ubiquitin-modified proteins were purified with $Ni^{2+}$NTA beads under denaturating conditions (his-PD). XErp1 was detected by WB. (**D**) CSF extract that was depleted of Cdc27 or control-depleted extract was supplemented with the indicated reagent. Samples were taken and Cdc27 and XErp1 were detected by WB analysis (input), or XErp1 was immunoprecipitated and analysed by WB. (**E**) β-TRCP or control-depleted CSF extract was treated with UbcX$^{wt}$ and processed for WB analysis (input) or used to IP XErp1, followed by WB for XErp1. (**F**) APC/C was immunoprecipitated from CSF extract using magnetic beads coated with Cdc27 antibodies and treated with UbcX$^{wt}$ or UbcX$^{ci}$ for the indicated times. Then, the APC/C was removed from the reaction by the recapture of the beads and XErp1 in the supernatant was detected by WB. (**G**) APC/C was immunoprecipitated and treated as in (**F**) and the reactions were analysed by WB for Cdc20 at the indicated timepoints. Note: Samples in **A**, **B**, **D** and **E** were subjected to CIP phosphatase treatment before WB analysis. Arrowheads indicate modified XErp1. APC/C, anaphase-promoting complex/cyclosome; Cdc, cell division cycle; ci, catalytically inactive; CIP, calf intestinal alkaline phosphatase; CSF, cytostatic factor; his-Ub$^{\Delta GG}$, his-ubiquitin$^{\Delta GG}$; his-Ub$^{wt}$, his-ubiquitin; IP, immunoprecipitation; PD, pull-down; Ub, ubiquitin; WB, western blotting; wt, wild type; XErp1-Ub, ubiquitin-modified XErp1.

**Supplementary information** is available at EMBO *reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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