Supplementary information

Material and Methods

Plasmids

H.s. UbcH10, X.l. UbcX and X.l. Cdc20 were amplified from cDNA libraries. Catalytically inactive UbcH10^{ci} and UbcX^{ci} were generated by mutating cysteine 114 of UbcH10^{wt} and cysteine 114 of UbcX^{wt} respectively, to serine. Securin constructs were as described (Schmidt et al, 2005). 6xhis-Ubiquitin and 6xhis-Ubiquitin$^{\Delta GG}$ constructs were a generous gift of M. Scheffner.

Proteins and Antibodies

6xhis-tagged UbcX, UbcH10 and ubiquitin were purified from BL21pRIL bacteria and 10xhis-2xTEV-Cdc20 from SF9 cells by Ni^{2+}-NTA agarose chromatography. UbcX and UbcH10 proteins were dialysed into buffer containing 20mM K-HEPES pH 7.7, 150mM KCl, 10% Glycerol and 1mM DTT. Human E1 enzyme was purchased from Boston Biochem.

Antibodies against Cdc20 and CyclinB2 were purchased from Abcam, against Cdc27 and α-tubulin from Sigma. Antibodies against βTRCP were a kind gift of T. Lorca. Anti-XErp1 antibodies were described previously (Schmidt et al, 2005). Anti-UbcX antibodies were generated by immunizing rabbits with full-length 6xhis-UbcX and affinitiy purification of the anti-UbcX antibodies from the obtained serum. Anti-USP44 antibodies were generated by immunizing one rabbit with 6xhis-USP44 aa 1-200 and two rabbits with a mix of USP44-232 peptide (KMNQKNSPTTKQKTPAPTSDKAC) and USP44-CT peptide (C-ENGHLSDTLPVHGSPQSPPR). Antibodies were affinity purified using 6xhis-USP441-200, USP44-232 peptide or USP44-CT peptide.

Xenopus extracts

Xenopus CSF egg extracts were prepared and DNA morphology was examined as described (Murray, 1991). Incubations were done at 20°C. When indicated, $CaCl_2$ was added to 600μM, cycloheximide to 350μM, MG262 to 100μM, and nocodazole to 33μM. "High sperm" and "low sperm" extract contained $11{,}5x10^6$ and $1x10^6$, respectively, sperm nuclei per ml extract. In general, 6xhis-UbcH10^{wt}, 6xhis-UbcH10^{ci} were added to a final concentration of 12μM, 6xhis-UbcX^{wt} or 6xhis-UbcX^{ci} to 6μM (unless explicitly mentioned) and a corresponding volume of dialysis

buffer was used as buffer control. To dephosphorylate extract proteins, samples were diluted with 9 volumes of 1x CIP-buffer (NEB) containing 10 units CIP (NEB) and incubated for 30 min at 25°C. For glycerol-gradient centrifugation, extracts were cleared by centrifugation at 186,000 g and resolved on 10–40% glycerol gradient by centrifugation at 150,000 g. 0.5-ml fractions were collected and analyzed by SDS-PAGE and immunoblotting.

***Xenopus* oocyte injections**

Oocytes were obtained, cultured and injected as described previously (Ohe et al. 2006). Briefly, *Xenopus* stage VI oocytes were injected with 10nL of a 8mg/mL UbcX protein solution or buffer and maturation was induced with progesterone (5 µg/ml; SIGMA) for 1 h. Oocytes undergoing GVBD in a time window of 15 min were pooled and at the indicated timepoints after GVBD collected and snap frozen in liquid N_2. Oocytes were homogenized in 1x CIP-buffer (NEB) containing complete protease inhibitors (Roche) and incubated with 10 units CIP (NEB) for 30' at 20°C. An equal volume of 3x laemmli buffer was added and the samples were analyzed by WB.

Immunodepletion, immunoprecipitation

Immunodepletion experiments were performed as described (Schmidt et al, 2005). To quantify immunoprecipitated Cdc27 and XErp1, immunoblots were visualized with ECL reagent. Signal intensities were quantified using Image J software. Anti-Cdc27 immunoprecipitates were used as loading controls and the relative amounts of copurified XErp1 were calculated. To XErp1 and Cdc20 immunoprecipitation assays (Fig.5), 125 µM N-ethylmaleimide was added. For the purification of his-tagged ubiquitin conjugates a protocol described in (Glockzin et al, 2003) was modified with the help of Alejandro Rojas. Briefly, to CSF extract 12,5 µM 6xhis-Ubiquitin or 6xhis-Ubiquitin$^{\Delta GG}$ was added and incubated with 6xhis-UbcX^{wt}, 6xhis-UbcX^{ci} or buffer at 20°C for 30'. The extract was denatured at 4°C by adding 9 volumes of denaturation buffer (6M guanidinium-HCl, 100 mM Na_2HPO_4/NaH_2PO_4 pH 8.0, 10 mM imidazole, 10 mM β-mercaptoethanol and complete protease inhibitors (Roche)), cleared by centrifugation and incubated with Ni^{2+}-NTA beads (Quiagen). The beads were washed, the bound proteins were re-natured, eluted into 3x laemmli buffer containing 200mM Imidazole and analyzed by Western blotting

In vitro ubiquitylation assays

APC/C was immunoprecipitated from 2 ml CSF extract using 25 µg Cdc27 antibodies immobilized on protein G dynabeads (Invitrogen). Assays were done as described previously (Schmidt et al. 2005), except that 27µM 6xhis-UbcX was used and no exogenous Cdc20 or XErp1 was added: The beads were washed once in QA and twice in CSF-XB and equally divided into 10 reaction tubes. A mix containing E1, Ubiquitin and an energy regeneration system in CSF-XB buffer was added and the reaction was started by the addition of 6xhis-UbcX. For Cdc20 ubiquitylation assays, an equal volume of 3x laemmli buffer was added to the reaction at the indicated timepoints. To assay XErp1 ubiquitylation and release from the APC/C, the beads were separated from the supernatant on a magnet and the supernatant was added to an equal amount of 3x laemmli buffer. Samples were directly processed and analyzed by WB.

Legend to Supplementary Figure 1

Fig S1 (**A**) UbcH10 overrides CSF-arrest in *Xenopus* egg extract. To CSF-extract the indicated reagents were added and the stabilities of ^{35}S-securin, XErp1, and cyclinB2 were analyzed by WB. (**B**) UbcX accumulates during oocyte maturation. Stage VI oocytes were obtained and maturation was induced by progesterone treatment. At the indicated time points after GVBD samples were taken for WB analysis. (**C**) UbcX^{wt}-induced CSF-release requires APC/C^{Cdc20} activity. Cdc20 was depleted from CSF-extracts in three rounds of immunodepletion and recombinant Cdc20 was added to a final concentration of 170nM as indicated. Cdc20 was detected by WB in the input, in the control, in the extract after the third round of Cdc20-depletion (ΔCdc20) as well as in the depletion/add-back sample. Note that recombinant Cdc20 is 8 kDa bigger than endogenous due to the 10xhis-2xTEV-tag for purification. (**D**) Cdc20-depleted or Cdc20 depletion/add-back extract was supplemented with calcium, buffer or UbcX^{wt} and the stability of ^{35}S-securin was analyzed by autoradiography. (**E**) Cdc20 ubiquitylation. CSF-extract containing his-Ub^{wt} or his-Ub$^{\Delta GG}$ were incubated with buffer, UbcX^{wt} or UbcX^{ci} for 30 min and processed for WB (input) or his-ubiquitin modified proteins were purified with Ni^{2+}NTA beads under denaturating conditions (his-Pulldown). Cdc20 was detected by WB.

Supplementary Figure 1