Supplemental Figure 1

HEK 293

ex

B56δ

WT

kd

anti-B56δ

62

83
Supplemental Figure 2
Supplemental figure 3
Supplemental figure 4
Supplemental figure 5

A

Time (min) 0 10 20 30 40 50 60 70 80 90 100 110 120
+GST (4 µg)
anti-pY15 Cdc2
anti-PSTAIRED
+14-3-3 (7 µg)
anti-pY15 Cdc2
anti-PSTAIRED
+14-3-3 (4 µg)
anti-pY15 Cdc2
anti-PSTAIRED

B

GST 4 µg 4 µg - -
14-3-3 - - 7 µg 4 µg
OA - + + +

anti-GST
anti-14-3-3
Purification of 14-3-3 release factor

1. **Xenopus laevis eggs**
   - 10,000 rpm

2. **Crude**
   - 200,000 x g
   - Isolate Pellet
   - Isolate Cytoplasm
   - Biotinylate Pellet
   - Add Pellet to Cytoplasm
   - Incubate +/- OA
   - Pellet at 200,000 x g

3. **Ultra**
   - Incubate with GST-14-3-3
   - Incubate with GST

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Retrieves bound proteins on glutathione beads, elute with SDS buffer and run on SDS-PAGE to visualize biotinylated proteins by western using HRP-streptavidin.

Supplemental figure 6
Supplemental figure 7

Phospho-Site

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P | L | L | S | P | | | | |
Supplemental Figure 1: Lentiviral shRNA vectors targeting PP2A B56δ (pMKO.1shB56δ) were produced by the RNAi Consortium (TRC, Moffat et al., 2006). Interphase Xenopus egg extract and lysates prepared from WT HEK 293 and B56δ knockdown cells were immunoblotted with anti-B56δ antibody.

Supplemental Figure 2: Protein sequence alignment of human (accession number AAH10692) and Xenopus B56δ protein used in this study. The underlined sequences and arrows indicate previously identified phosphorylated residues. The asterisk indicates Xenopus phospho-S37 and human phospho-S60 sites.

Supplemental Figure 3: GST-Cdc25 was treated with or without Chk1 and/or Cdk2/CyclinE in vitro in the presence of ATP at 30°C for 1 hr. Samples were washed and incubated with bacterially expressed and purified 35S-labeled Histagged 14-3-3 (1, 2.5 and 7.5 μg) in buffer for 1 hr at 4°C. Samples were washed in ELB plus NaCl and Triton X-100. Samples were resolved by SDS-PAGE and the amount of 35S-labeled 14-3-3 bound to GST-Cdc25 was quantified using a phosphorimager.

Supplemental Figure 4: A. Crude interphase Xenopus egg extract was treated with DMSO, OA, or Cyclin B1 to drive extract into mitosis. Treated extracts were spun at 200,000 x g for 1.5 hrs and cytosolic fraction was isolated. 14-3-3 bound GST-Cdc25 was added to each extract and incubated for 1 hr at 4°C. Samples
were washed with ELB plus 300 mM NaCl and 0.1% Triton X-100 and immunoblotted with anti-GST and anti-14-3-3 antibodies. B. HeLa cells were incubated +/- 1 μM OA for 1 hr, harvested and fixed with 1% formaldehyde and 70% ethanol in PBS. Cells were incubated with phospho-S33 keratin 18 antibody (Abcam) in PBS with 5% goat serum and subsequently stained with Alexa-488 conjugated goat anti-mouse secondary antibody (Molecular Probes). Cells were visualized by confocal microscopy (left panel) or analyzed with a BD FacScan Analyzer (right panel). C. HeLa cells were synchronized by a double thymidine block, released into the cell cycle and then harvested in triplicate at the indicated times. Cells were fixed and stained for pS33 keratin 18 as described in (B). The % increase in mean fluorescence pS33 keratin 18 staining represents the increase in mean fluorescence as compared to an asynchronous population (upper panel). DNA content was measured by propidium iodide staining of cells fixed in methanol and treated with 20 ug/mL RNAse A (Roche) (lower panel). All samples were analyzed with a BD FacScan Analyzer. Data was analyzed using CellQuest and WinMDI 2.8. Cdc2 activity was monitored by immunoblotting for pHH3 in lysates prepared from the cells (middle panel).

**Supplemental Figure 5:** A. *Xenopus* cycling extract was incubated with the indicated amounts of recombinant GST or His-tagged 14-3-3. Samples were collected at the indicated time points and resolved by SDS-PAGE for western blotting with anti-pTyr15 Cdc2 and anti-PSTAIRE for total Cdc2 levels. B. Crude interphase *Xenopus* egg extract was incubated with GST or His-tagged 14-3-3 at
the concentrations indicated. Extracts were treated with DMSO or OA. 14-3-3 bound GST-Cdc25 was then added to each extract for 1 hr at 4°C. Samples were washed with ELB plus 300 mM NaCl and 0.1% Triton X-100 and were resolved by SDS-PAGE for western blotting with anti-GST and anti-14-3-3.

**Supplemental Figure 6:** Schematic of 14-3-3 release factor identification. *Xenopus laevis* eggs were pelleted at 10,000 rpm to generate a crude egg extract. Crude extract was spun at 200,000 x g to separate out pellet, membrane and cytosol. The pellet and cytosol were isolated. Pelleted proteins were modified using a succinimide ester of biotin. Biotinylated pellet proteins were added to cytosol and incubated with DMSO or OA for 1 hr and re-pelleted at 200,000 x g. Cytosol was then resolved by SDS-PAGE and probed with HRP-streptavidin.

**Supplemental Figure 7:** Protein sequence alignment of the Cdk2/14-3-3 release phospho-site (Thr 138 in *Xenopus* Cdc25C; Thr 130 in human Cdc25C) in human Cdc25 A, B and C, mouse Cdc25 A and C, hamster Cdc25, pig Cdc25, rat Cdc25, *Xenopus* Cdc25 A and C. The arrow indicates the potential phosphorylated site homologous to Thr 138 in other Cdc25 molecules.

**Supplemental Figure 8.** During interphase, S287 of Cdc25 is phosphorylated and 14-3-3 is bound. As long as replication is ongoing, checkpoint kinases phosphorylate S37 of B56δ, thereby maintaining active T138 dephosphorylation
by PP2A. Upon completion of DNA replication, the checkpoint signal is lost and checkpoint kinases are turned off. Once B56δ is no longer phosphorylated, phospho-T138 accumulates through the continued constitutive action of Cdk2, reducing the affinity of 14-3-3 for Cdc25. Concomitantly, the accumulation of phosphorylated keratin proteins creates a 14-3-3 “sink.” Together, these activities promote the release of 14-3-3 from Cdc25, allowing PP1 to dephosphorylate S287 and thereby activate Cdc25. Once active, Cdc25 activates Cdc2/Cyclin B, which is then further activated by Cdc2-mediated positive feedback. Collectively, these events promote the full activation Cdc25.

References: