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13. However, the possibility exists that Kcv functions as a subunit, which up-regulates the activity of endogenous K^+ channels in oocytes. To exclude this possibility, we constructed a site-specific mutation in the selectivity filter sequence of Kcv by replacing Phe^{66} (F66) with Ala (A). If Kcv is a channel protein, the mutant protein should, by analogy to the Shaker channel, form a channel unable to conduct K^+ currents (*1*). Using standard voltage-clamp assays, nine oocytes expressing KcvF66A had currents similar in kinetics to those of H_2O-injected oocytes with no additional K^+ conductance compared to the H_2O-injected control cells. Hence, the absence of a prominent K^+-selective current in KcvF66A-expressing oocytes confirms that Kcv functions as a channel protein in oocytes and that the observed currents are not due to activation of endogenous channel proteins.
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18. Voltage-dependency of inhibition by Ba^{2+} was analyzed quantitatively based on a Woodhull block model [A. M. Woodhull, *J. Gen. Physiol.* **61**, 687 (1973)] by fitting data of relative block to

$$\left(1 - \frac{I}{I_o}\right) = \frac{b_{max}}{1 + \frac{k^0}{B_a} e^{(z\delta F/RT)}}$$

where I_0 is control and I blocked current, b_{max} the maximal block, B_a the concentration of Ba^{2+}, k^0 the dissociation constant of the blocking reaction at voltage = 0 mV, δ the fraction of the electrical field crossed by Ba^{2+}, and z = 2 the valence of the blocking ion. R, T, and F have their usual thermodynamic meaning. Fitting yields for 1 mM Ba^{2+}: b_{max} = 0.94, δ = 0.9 ± 0.03, and k^0 = 660 ± 12 μM (n = 3 oocytes).
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REPORTS

Requirement of the Prolyl Isomerase Pin1 for the Replication Checkpoint

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The peptidyl-prolyl isomerase Pin1 has been implicated in regulating cell cycle progression. Pin1 was found to be required for the DNA replication checkpoint in *Xenopus laevis*. Egg extracts depleted of Pin1 inappropriately transited from the G_2 to the M phase of the cell cycle in the presence of the DNA replication inhibitor aphidicolin. This defect in replication checkpoint function was reversed after the addition of recombinant wild-type Pin1, but not an isomerase-inactive mutant, to the depleted extract. Premature mitotic entry in the absence of Pin1 was accompanied by hyperphosphorylation of Cdc25, activation of Cdc2/cyclin B, and generation of epitopes recognized by the mitotic phosphoprotein antibody, MPM-2. Therefore, Pin1 appears to be required for the checkpoint delaying the onset of mitosis in response to incomplete replication.

The peptidyl-prolyl isomerase (PPIase) Pin1 affects cell cycle transitions. Originally identified in yeast–two hybrid screens as a protein that binds to and suppresses the toxicity of the fungal mitotic kinase Never In Mitosis A (NIMA), Pin1 is present in all eukaryotic cells examined (*1–4*). Although Pin1 is an abundant protein, the expression of which does not change during the cell cycle (Fig. 1), it clearly influences cell cycle dynamics. Overexpression of Pin1 is deleterious in the budding yeast *Saccharomyces cerevisiae* and causes a G_2 arrest in HeLa cells and in *Xenopus laevis* egg extracts, suggesting that the protein negatively regulates the initiation of mitosis (*1, 3*). The budding yeast Pin1 homolog ESS1 is encoded by an essential gene; *ess1* deletion mutants exhibit terminal mitotic arrest, suggesting a requirement for Pin1 in mitotic exit (*4, 5*). In contrast, Pin1 is not critical for any readily observable function in *Drosophila melanogaster* (*2*) or mouse (*6*).

In vitro, Pin1 binds a subset of mitotic proteins containing a motif composed of a phosphoserine or phosphothreonine residue followed by a proline residue (*3, 7–9*) that is also recognized by the MPM-2 monoclonal antibody (*10, 11*). Among these potential cell cycle targets, only substoichiometric interaction of Pin1 with the mitotic phosphatase Cdc25C has been demonstrated in vivo (*12*); thus, it is unclear whether the numerous phosphoproteins associated with Pin1 in vitro are biologically relevant targets for Pin1 in vivo. Endogenous Pin1 protein has been implicated in transcriptional regulation and RNA processing in yeast (*5, 13–16*) and in mediating the association of phosphorylated tau with microtubules in brain extracts (*17*). The relation of these functions to control of the cell cycle remains unclear, and events regulated by Pin1 that influence the cell cycle have yet to be defined. We examined Pin1 function in *Xenopus* egg extracts that are transcriptionally inactive, thus allowing us to avoid possible effects of Pin1 on transcriptional events that might affect cell cycle progression. This model system provided the opportunity to focus on specific cell cycle transitions and thereby evaluate the contribution of Pin1 protein to each transition.

The *Xenopus* Pin1 homolog was isolated by low-stringency hybridization screening of a *Xenopus* gastrula cDNA library with a human Pin1 probe (*18*). The inserts of three independently isolated clones each encoded an identical open reading frame (*xPin1*). The predicted polypeptide sequence shared 89% identity with human Pin1 and >45% identity with each of the eukaryotic parvulins over its full length of 159 residues. Recombinant xPin1 was purified from bacteria (Fig. 1A) and used to generate polyclonal antiserum that recognized a single protein of 18 kD in *Xenopus* egg extracts (Fig. 1B). The concentration of Pin1 in egg extracts was estimated to be 20 ng/μl, or ~1 μM, and this did not change throughout the cell cycle (Fig. 1C).

The mitotic arrest observed in yeast lacking ESS1 suggested a function for the protein in mitotic exit. To test this directly, we used cytostatic factor–arrested egg extracts (CSF extracts) (*19*) to examine the consequences of the removal of Pin1 on mitotic exit and DNA replication. CSF extracts, generated in the presence of EGTA to prevent calcium-dependent degradation of cyclin B, exhibit high H1 kinase activity and other hallmarks of normal M phase arrest. Calcium addition, which recapitulates a physiological consequence of fertilization, causes the extracts to proceed into interphase, characterized by nuclear envelope formation, chromatin

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decondensation, and initiation of replication. When CSF extracts were immunodepleted of Pin1 (*20*), we found that Pin1-depleted and mock-depleted extracts were equally capable of exiting the M phase (Fig. 2, A and B). Furthermore, Pin1 depletion had no effect on DNA replication (Fig. 2C). Thus, in this cell-free system, Pin1 appears not to be required for either S phase or the M to G_1 transition.

Mitotic arrest observed in *ess1* mutant yeast could be the consequence of a premature mitotic entry triggered in the absence of Pin1. To examine the effects of Pin1 depletion on the isolated G_2 to M transition, we used both interphase extracts that were induced to enter M phase by the addition of exogenous cyclin B protein, and cycling extracts, which intrinsically oscillate between S and M phases of the cell cycle (*21*). Microscopic examination of nuclei, coupled with the measurement of Cdc2-cyclin B1–catalyzed histone H1 phosphorylation, revealed that both types of extract, when depleted of Pin1, entered mitosis more rapidly than did control extracts (Fig. 3, A through D). Although the absolute timing of mitotic entry varied from extract to extract, removal of Pin1 consistently accelerated the transition into M phase.

We explored the possibility that the premature mitosis in Pin1-depleted extract was due to the failure of a negative regulatory influence at the G_2 to M transition. The duration of interphase in *Xenopus* extracts can be prolonged by supplementing extracts with high concentrations of sperm chromatin, which increases the time required for DNA synthesis (*22*). The presence of unreplicated sperm DNA triggers the G_2 replication checkpoint that delays mitotic initiation by preventing activation of Cdc2 (*22–24*). The effects of low and high concentrations of sperm in Pin1-depleted or mock-depleted extracts were compared (Fig. 3D). The higher DNA concentration caused a G_2 delay in mock-depleted extracts. However, this delay was greatly reduced in Pin1-depleted extracts. Thus, the difference in timing of mitotic entry observed between mock-depleted and Pin1-depleted extracts may reflect the inability of Pin1-depleted extracts to halt mitotic entry in the presence of unreplicated DNA. Notably, when the concentration of DNA was low, the transition out of mitosis into interphase occurred normally, even without Pin1. In contrast, the Pin1-depleted extract that was supplemented with DNA to achieve a high concentration of chromatin failed to exit mitosis. It is possible that the M phase arrest in these extracts occurs because mitosis is initiated in the presence of unreplicated DNA, and therefore, M phase fail-safe mechanisms are triggered to prevent segregation of damaged chromosomes.

To test the hypothesis that the operation of the replication checkpoint requires Pin1, we suspended replication with the DNA polymerase inhibitor aphidicolin. In mock-depleted extracts, aphidicolin treatment postponed mitotic entry as expected (Fig. 4A). Depletion of Pin1 from extracts or addition of caffeine [a treatment that disables the replication checkpoint (*22, 25–27*)] prevented the aphidicolin-induced cell cycle delay. Supplementation of Pin1-depleted extracts with recombinant xPin1 restored the G_2 delay elicited by aphidicolin (Fig. 4B), and the delay remained caffeine sensitive in the reconstituted extract (*28*). This indicated that Pin1 itself is an essential component of the replication checkpoint in *Xenopus*.

Pin1's effects on cell cycle kinetics might be mediated through its established association with mitotic phosphoproteins (*3, 12*). For this reason, we examined the status of several Pin1-binding proteins in extracts depleted of Pin1. In all of our assays, the premature mitosis observed in Pin1-depleted extract was indistinguishable from that occurring in the presence of caffeine. Hyperphosphorylation of Cdc25, increased H1 kinase activity, and the appearance of MPM-2 epitopes (Figs. 3A and 4C) accompanied microscopically observed mitotic entry in both cases. Therefore, Pin1 is not required for MPM-2 epitope generation or for the ability of these phosphoproteins to regulate mitotic progression. Instead, precocious activation of Cdc25 may be the direct consequence of Pin1 removal.

Recently, it was reported that Pin1 antagonizes in vitro phosphorylation of the mitotic regulators Cdc25, Myt1, and Wee1 by Cdc2/cyclin B (*29*). Although the ability of Pin1 to bind mitotic phosphoproteins appears to be important for this inhibition, association alone is not sufficient for endogenous Pin1 function. We introduced a point mutation [Cys^{109} → Ala^{109} (C109A)] into *Xenopus*

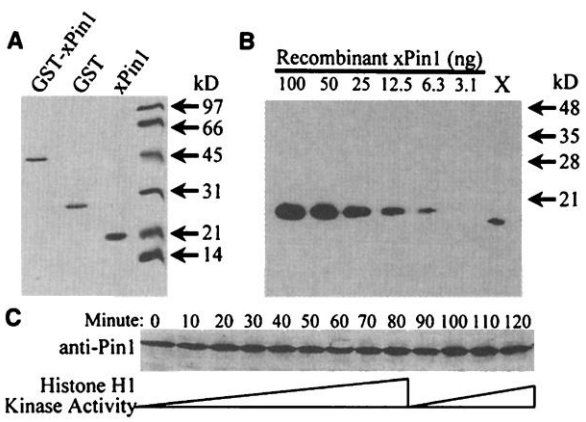

Fig 1. Identification of the *Xenopus laevis* Pin1 homolog. (**A**) Recombinant xPin1 was purified from bacteria as a glutathione *S*-transferase (GST)–xPin1 fusion protein and eluted by cleavage from the GST with thrombin. Coomassie blue staining of 1 μg of each indicated protein separated by SDS–polyacrylamide gel electrophoresis is shown. (**B**) Using antiserum to xPin1 (diluted 5000-fold), we recognized a single protein in *Xenopus* egg extract; 0.5 μl of extract was loaded in lane X. (**C**) The Pin1 content in *Xenopus* extracts (1 μl extract per lane) was visualized by immunoblot over the course of two cell cycles, as assessed by histone H1 kinase activity (*35*) and indicated schematically below the gel lanes.

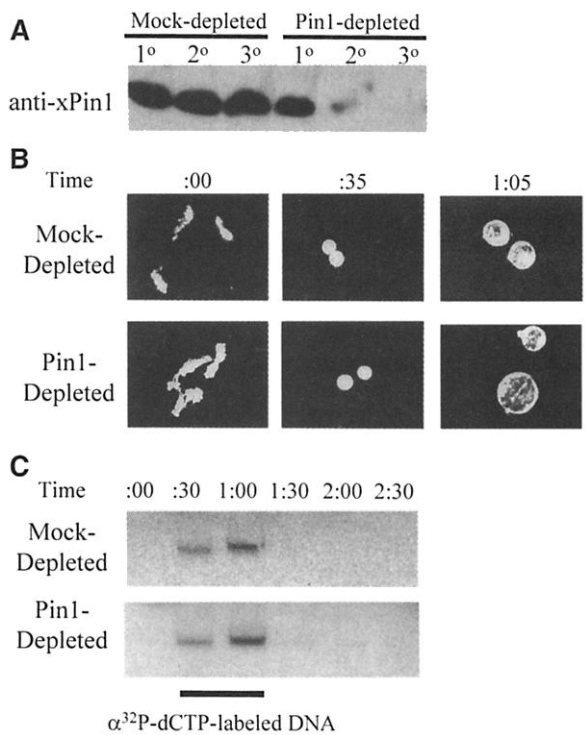

Fig. 2. Exit from mitosis in *Xenopus* extracts lacking Pin1. (**A**) Immunoblot of Pin1 remaining in CSF extracts (*33, 36*) after each stage of immunodepletion. Three successive treatments (1°, 2°, and 3°) removed >95% of Pin1 (*20*). (**B**) 3° depleted extracts were supplemented with demembranated sperm chromatin (*37*) and adenosine 5′-triphosphate (ATP) regenerating mix (*32*) and released from CSF arrest with 400 μM $CaCl_2$. At various times, measured with respect to the time of $CaCl_2$ addition in hours and minutes, portions of the extract were withdrawn, diluted 1:1 with Hoechst 33258 [10 μg/ml in 26% formaldehyde, 0.2 M sucrose, and 10 mM Hepes (pH 8.0)], and examined by fluorescence microscopy. (**C**) DNA replication was detected by pulse labeling of DNA in extracts with α^{32}P–deoxycytidine 5′-triphosphate, agarose gel electrophoresis, and autoradiography, as described (*22*).

Pin1 that compromised the prolyl isomerase activity of the enzyme by >90% (*30*) without diminishing its protein binding avidity (Fig. 4D). This mutant was incapable of restoring the checkpoint response in Pin1-depleted extracts when added to achieve concentrations sufficient for complementation of the checkpoint defect by wild-type xPin1 (Fig. 4B). Furthermore, *Xenopus* Pin1 complements the

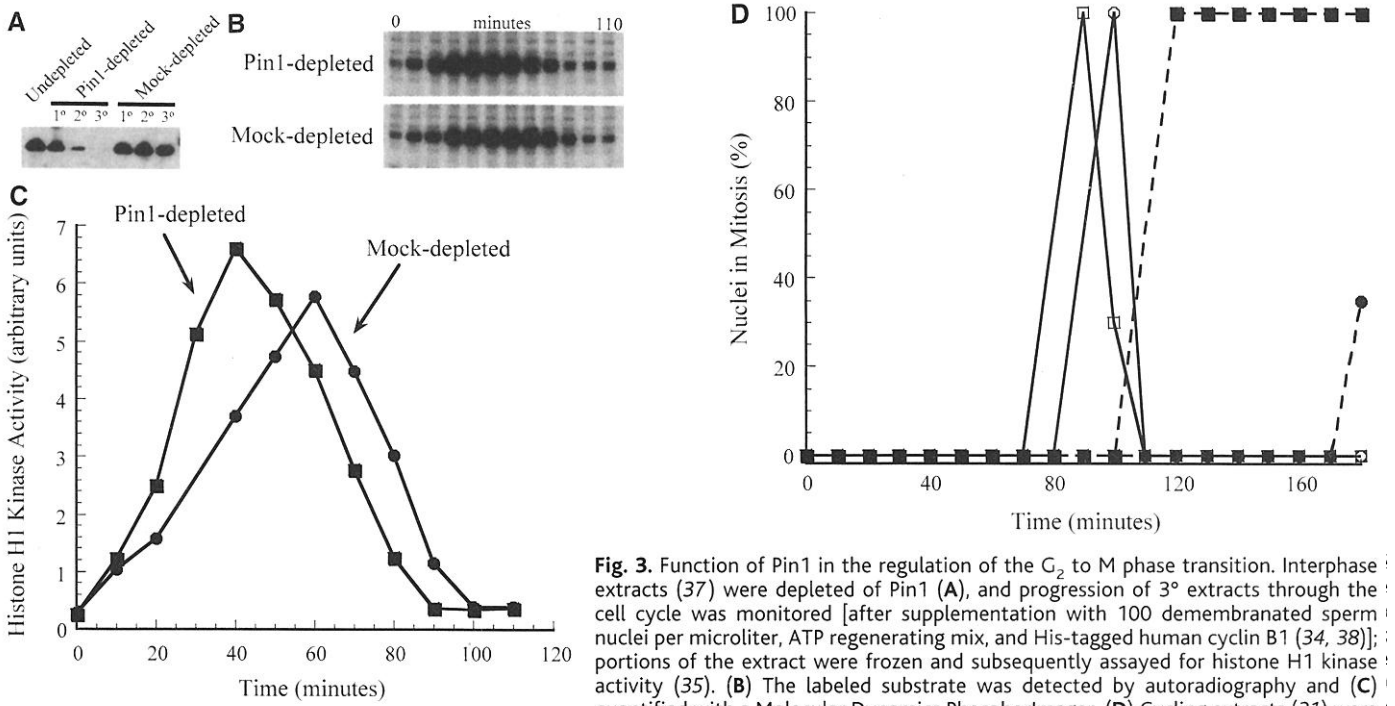

Fig. 3. Function of Pin1 in the regulation of the G_2 to M phase transition. Interphase extracts (*37*) were depleted of Pin1 (**A**), and progression of 3° extracts through the cell cycle was monitored [after supplementation with 100 demembranated sperm nuclei per microliter, ATP regenerating mix, and His-tagged human cyclin B1 (*34, 38*)]; portions of the extract were frozen and subsequently assayed for histone H1 kinase activity (*35*). (**B**) The labeled substrate was detected by autoradiography and (**C**) quantified with a Molecular Dynamics PhosphorImager. (**D**) Cycling extracts (*21*) were depleted of Pin1 (squares) or mock depleted (circles) and supplemented with ATP regenerating mix and 100 demembranated sperm nuclei per microliter (open symbols) or 500 nuclei per microliter (solid symbols). The mitotic index was monitored by fluorescence microscopy. Each of these experiments is representative of our observations in several extracts.

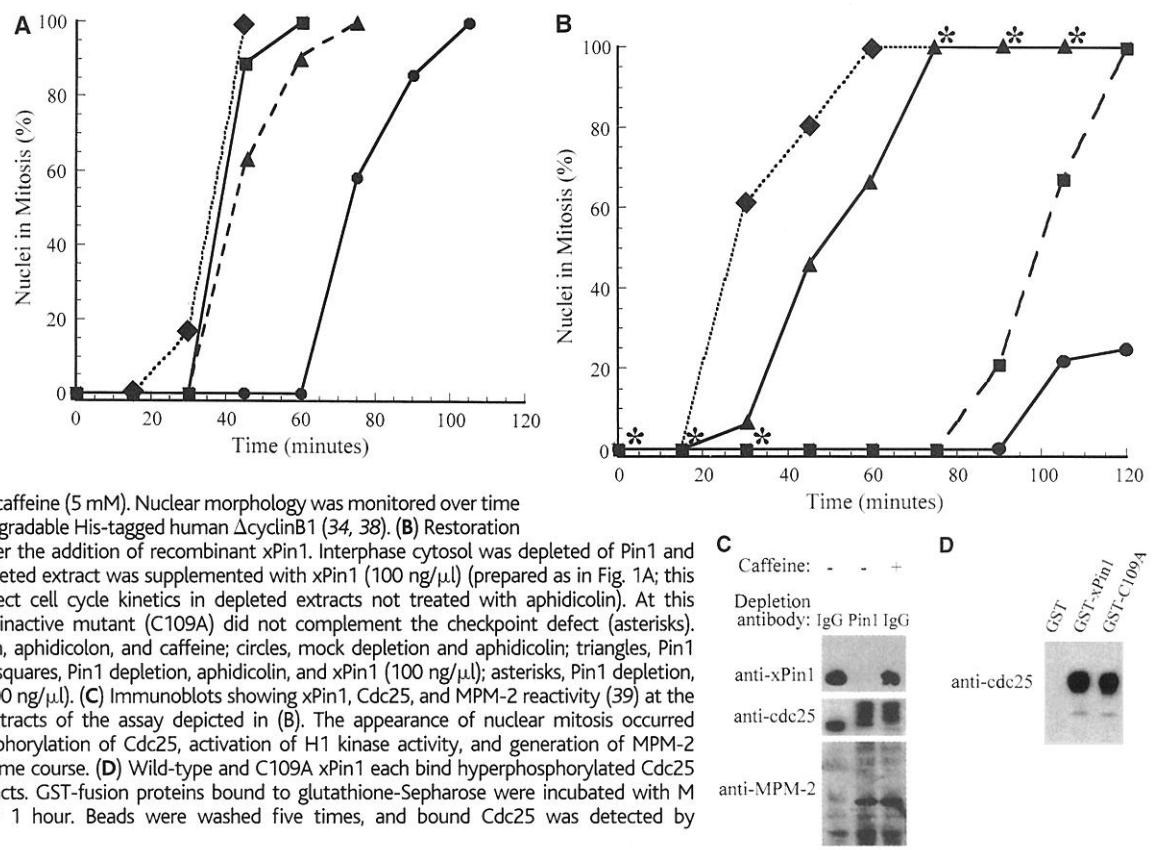

Fig. 4. Requirement of Pin1 for the checkpoint arrest in response to unreplicated DNA. (**A**) Depleted interphase cytosol (*37*) was supplemented with membranes isolated from cells in interphase (1:10), demembranated sperm nuclei (200 nuclei per microliter), and ATP regenerating mix. Dotted line with triangles, mock depletion; circles, mock depletion and aphidicolin (50 μg/μl); squares, Pin1 depletion and aphidicolin; dashed line with triangles, mock depletion, aphidicolin, and caffeine (5 mM). Nuclear morphology was monitored over time after the addition of nondegradable His-tagged human ΔcyclinB1 (*34, 38*). (**B**) Restoration of checkpoint function after the addition of recombinant xPin1. Interphase cytosol was depleted of Pin1 and treated as in (A). Pin1-depleted extract was supplemented with xPin1 (100 ng/μl) (prepared as in Fig. 1A; this concentration did not affect cell cycle kinetics in depleted extracts not treated with aphidicolin). At this concentration, the PPIase-inactive mutant (C109A) did not complement the checkpoint defect (asterisks). Diamonds, mock depletion, aphidicolon, and caffeine; circles, mock depletion and aphidicolin; triangles, Pin1 depletion and aphidicolin; squares, Pin1 depletion, aphidicolin, and xPin1 (100 ng/μl); asterisks, Pin1 depletion, aphidicolin, and C109A (100 ng/μl). (**C**) Immunoblots showing xPin1, Cdc25, and MPM-2 reactivity (*39*) at the 120-min time point in extracts of the assay depicted in (B). The appearance of nuclear mitosis occurred simultaneously with phosphorylation of Cdc25, activation of H1 kinase activity, and generation of MPM-2 epitopes throughout the time course. (**D**) Wild-type and C109A xPin1 each bind hyperphosphorylated Cdc25 in M phase *Xenopus* extracts. GST-fusion proteins bound to glutathione-Sepharose were incubated with M phase cytosol at 4°C for 1 hour. Beads were washed five times, and bound Cdc25 was detected by immunoblotting.

lethality of *ess1* mutants in budding yeast, but the C109A mutant is inactive in the complementation assay (*31*). Together, these observations indicate that Pin1 is functionally conserved and that its catalytic activity is required for its checkpoint role in *Xenopus* and its essential function in *S. cerevisiae*.

Pin1 participates in the replication checkpoint in a manner requiring its catalytic activity. Target-specific inhibition of mitosis-promoting kinase activity provides a biochemical mechanism for Pin1's role in enabling the replication checkpoint. Pin1 could mediate inhibition of Cdc25 hyperphosphorylation and act in concert with 14-3-3–mediated cytoplasmic sequestration of the phosphatase to prolong the G_2 phase by preventing functional interaction of Cdc25 with Cdc2 under checkpoint conditions. This function is consistent with previous observations that the G_2 phase is prolonged when Pin1 is overexpressed. In the absence of DNA perturbation, consequences of Pin1 removal may not be manifested; this would explain the lack of apparent phenotype in Pin1-null metazoans. Our demonstration of the essential role of Pin1 in the replication checkpoint establishes a position for endogenous Pin1 in the eukaryotic cell cycle regulatory network.

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Requirement of the Inositol Trisphosphate Receptor for Activation of Store-Operated Ca^{2+} Channels

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The coupling mechanism between endoplasmic reticulum (ER) calcium ion (Ca^{2+}) stores and plasma membrane (PM) store-operated channels (SOCs) is crucial to Ca^{2+} signaling but has eluded detection. SOCs may be functionally related to the TRP family of receptor-operated channels. Direct comparison of endogenous SOCs with stably expressed TRP3 channels in human embryonic kidney (HEK293) cells revealed that TRP3 channels differ in being store independent. However, condensed cortical F-actin prevented activation of both SOC and TRP3 channels, which suggests that ER-PM interactions underlie coupling of both channels. A cell-permeant inhibitor of inositol trisphosphate receptor ($InsP_3R$) function, 2-aminoethoxydiphenyl borate, prevented both receptor-induced TRP3 activation and store-induced SOC activation. It is concluded that $InsP_3Rs$ mediate both SOC and TRP channel opening and that the $InsP_3R$ is essential for maintaining coupling between store emptying and physiological activation of SOCs.

Receptor-induced Ca^{2+} signals comprise two interdependent components—rapid Ca^{2+} release from Ca^{2+} stores in the ER and Ca^{2+} entry through slowly activating PM SOCs. The trigger for SOC activation is decreased Ca^{2+} in the ER lumen (*1, 2*). However, despite intense study, the ER-derived signal coupling store depletion with SOC activation remains unknown (*3*). Direct coupling between ER and PM has been hypothesized (*4, 5*), and evidence indicates that physical docking of ER with the PM is involved in SOC activation (*6–8*). The mammalian TRP family of receptor-operated ion channels has been suggested to share some operational

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