bryos surrounded by thousands of trophoblast cells (*14*). The fact that hypoxia stimulates cytotrophoblasts but not most other cells (*15*) to undergo mitosis could help account for the discrepancy in size between the embryo and the placenta, which continues well into the second trimester of pregnancy (*16*). Although we do not as yet understand this phenomenon at a mechanistic level, we know that cytotrophoblasts within the uterine wall mimic a vascular adhesion molecule phenotype (*17*). In other tissues, hypoxia induces the production of vascular endothelial growth factor, which stimulates endothelial cell proliferation (*18*), raising the possibility that similar regulatory pathways operate during placental development.

We suspect that the effects of oxygen tension on cytotrophoblast differentiation and invasion could have important implications. Relatively high oxygen tension promotes cytotrophoblast differentiation and could help explain why these cells extensively invade the arterial rather than the venous side of the uterine circulation. Conversely, if cytotrophoblasts do not gain access to an adequate supply of maternal arterial blood, their ability to differentiate into fully invasive cells may be impaired. We suggest that the latter scenario could be a contributing factor to pregnancy-associated diseases, such as preeclampsia, that are associated with abnormally shallow cytotrophoblast invasion and faulty differentiation, as evidenced by their inability to up-regulate integrin α1 expression (*19*).

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9. After 48 hours, the medium was aspirated, and fresh medium with 5 μCi/ml [^{3}H]thymidine (44 Ci/mM; Amersham) was added for 24 hours. The villi were then washed with phosphate-buffered saline (PBS) containing unlabeled thymidine (5 μg/ml), treated with Dispase (Collaborative Biomedical Research, Bedford, MA), and lysed; radioactivity was measured after trichloroacetic acid precipitation.
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11. After 72 hours, the cultures were washed in PBS, and the villus explants (n = 6 per group) were homogenized in 10 mM tris (pH 7.2) containing 1% Nonidet P-40, 0.15 M NaCl, 1 mM EDTA, 50 μM Na_2MoO_4, 1 mM Na_3VO_4, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, 25 μg/ml leupeptin, and 10% (w/v) glycerol. Samples containing 100 μg of protein, determined as described by M. M. Bradford [*Anal. Biochem.* **72**, 248 (1976)], were separated by SDS–10% polyacrylamide gel electrophoresis [U. K. Laemmli, *Nature* **227**, 680 (1970)]. The separated proteins were transferred to nitrocellulose paper [H. Towbin, T. Staehelin, J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350 (1979)]. Blots were blocked in TBST [20 mM tris-HCl (pH 8.0), 120 mM NaCl, 0.1% Tween-20] containing 4% nonfat dry milk. The replicas were incubated overnight with primary antibodies in TBST containing 2% nonfat dry milk. Anti–cyclin B (Pharmingen, San Diego, CA) was used at 2.5 μg/ml and anti-p21 (Oncogene Research Products, Cambridge, MA) at 2 μg/ml. Antibody binding was assessed with the use of horseradish peroxidase–conjugated secondary antibodies and enhanced chemiluminescence detection (Amersham). Densitometric analysis was performed using the NIH Image program.
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20. Placental-bed biopsy specimens were obtained as previously described (*5, 17*). Sections of these specimens were incubated with anti-Ki67 (Dako, Carpinteria, CA) and anti–human cytokeratin (7D3) [S. J. Fisher *et al.*, *J. Cell Biol.* **109**, 891 (1989)]. Antibody binding was detected with fluorescein-conjugated rabbit anti–mouse immunoglobulin G (IgG) and rhodamine-conjugated goat anti-rat IgG (Jackson Immunoresearch). Tissue blocks of cultured anchoring villi were prepared like the biopsy specimens. Sections cut from these blocks were double stained with 7D3 and an anti–integrin α1 mouse IgG (T Cell Diagnostics, Woburn, MA). Different sections were stained with an anti–HLA-G mouse monoclonal antibody produced in our laboratory (*5*), anti–integrin α5 (*3*), anti-αv/β3 (LM609, D. Cherish, Scripps Research Foundation), anti–human placental lactogen (Harlan Bioproducts, Indianapolis, IN), anti–cyclin B, and anti-p21 (both from Santa Cruz Biotechnology, Santa Cruz, CA). Antibody binding was detected as described above.
21. Anchoring villi were dissected from 6- to 8-week-old human placentas and plated on Matrigel substrates (Collaborative Biomedical Research) [O. Genbacev, S. Schubach, R. K. Miller, *Placenta* **13**, 439 (1993)]. Control and hypoxic cultures were maintained for 3 days as previously described (*8*). Dissolved O_2 at the cell-medium interface, measured using a micro-oxygen electrode (MI-730; Microelectrodes, Inc., Londonderry, NH), was 20% (98 mm Hg) under standard tissue culture conditions, and either 6% (40 mm Hg) or 2% (14 mm Hg) in hypoxic conditions.
22. After 48 hours, the culture medium was aspirated, and medium containing 1 μM BrdU (Sigma) was added. After 24 hours, villi on filter substrates were washed with PBS for 20 min, fixed for 1 hour (4°C) in 4% paraformaldehyde, embedded in optimal–cutting-temperature medium, and frozen in liquid nitrogen (*5, 17*). Sections cut from these blocks were incubated with a fluorescence-labeled antibody to BrdU (Boehringer Mannheim, Indianapolis, IN). Adjacent sections were stained with 7D3.

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Proteolysis and DNA Replication: The *CDC34* Requirement in the *Xenopus* Egg Cell Cycle

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The cell division cycle gene, *CDC34*, is required for ubiquitin-mediated degradation of G_1 regulators and cell cycle progression through the transition from G_1 to S phase in budding yeast. A *CDC34* requirement for S phase onset in higher eukaryotes has not been established. Studies of the simple embryonic cell cycle of *Xenopus laevis* eggs demonstrated that Cdc34p in a large molecular size complex was required in the initiation of DNA replication. Cdc34p appears to regulate the initiation function of Cdk2–cyclin E, perhaps through the degradation of the *Xenopus* cdk inhibitor, Xic1.

Protein ubiquitination and degradation were linked to cell cycle control by the discovery that Cdc34p, an essential G_1- to S-phase regulator in budding yeast, is a ubiquitin conjugating enzyme (UBC3) (*1*). The Cdc34p-dependent degradation of a single substrate, p40sic1p [an inhibitor of cyclin-dependent kinase (cdk) Cdc28p complexed with cyclins Clb5p or Clb6p], appears to be sufficient to trigger the transition from G_1 to S phase (*2*). This degradation requirement in yeast is a key element coupling extrinsic control of cell proliferation to the cell cycle. The human homolog of *CDC34* can complement the yeast *cdc34-2* temperature-sensitive strain (*3*). Cdc34p in mammalian cells may also function to couple extrinsic control by growth factors to cell cycle progression. However, Cdc34p may play a more basic role in the intrinsic control of each cell cycle. A requirement for Cdc34p in the early embryonic cell cycle of the frog *Xenopus laevis*, where cell division is independent of extrinsic controls, may reveal a more fundamental role for protein degradation in regulating the initiation of DNA replication.

We tested whether Cdc34p is required for the onset of DNA replication in higher eukaryotes and examined the nature of such a requirement using an in vitro DNA rep-

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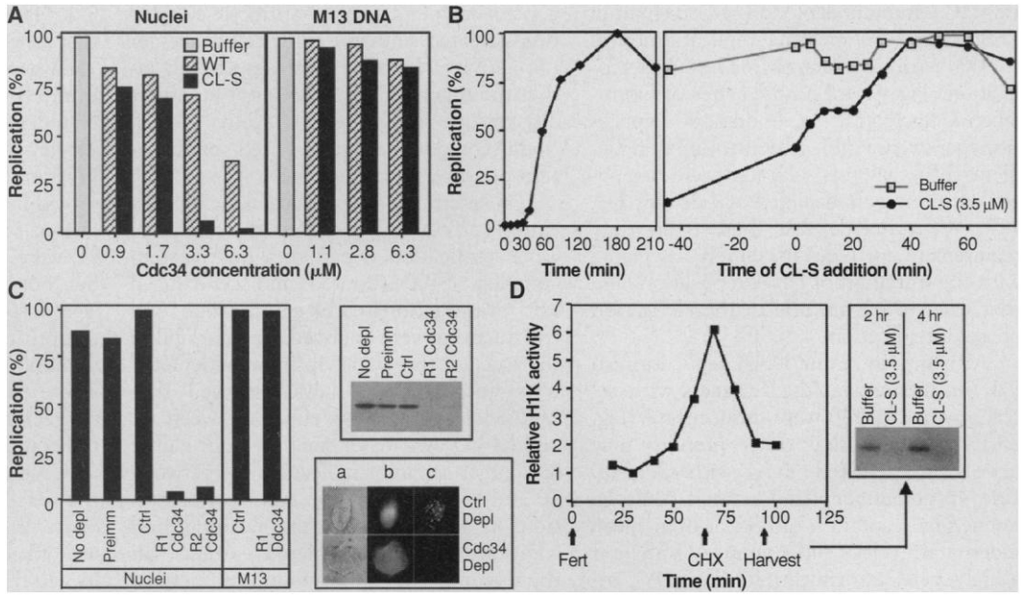

Fig. 1. Requirement of Cdc34p for initiation of chromosomal replication. (**A**) WT and CL→S hCdc34p (*8*) were added to LSS (*16, 17*) to concentrations of 0.9, 1.7, 3.3, or 6.3 μM and incubated for 20 min at 23°C before the addition of sperm nuclei (left panel) (*18*). WT and CL→S were also added to LSS to concentrations of 1.1, 2.8, or 6.3 μM 20 min before the addition of ssM13 DNA (*13*) (right panel). DNA replication was normalized to 100% of that in the buffer control (Buffer) (*18*). (**B**) Replication of nuclei was measured over 240 min in LSS with no additions (left) or after 240 min with XB– (*23*) (□) or 3.5 μM CL→S (●) (right). (Right panel) XB– or CL→S was added at time points between −45 and +70 min. Samples were incubated at 0°C (−45 min) or at 23°C (all other samples), and nuclei were added at time zero. (**C**) Left panel: LSS was not depleted (No depl) or depleted with pA-seph coupled to preimmune Ig (Preimm), rabbit Ig (Rlg) (Ctrl), rabbit 1 anti-hCdc34p Ig (R1 Cdc34), or rabbit 2 anti-hCdc34p Ig (R2 Cdc34), and either sperm nuclei or ssM13 DNA was added (*18, 19*). Replication is normalized to 100% of that in the control-depleted sample (Ctrl). (Upper right) Anti-Cdc34p immunoblot of depleted LSS (*20*). (Lower right) Sperm nuclei visualized by phase contrast (a), Hoechst staining (b), or rhodamine-strepavidin staining of biotinylated deoxyuridine triphosphate incorporation (c) after incubation in LSS depleted with pA-Seph beads coupled to Rlg (Rlg pA-Seph) (Ctrl depl) or anti-hCdc34p Ig (Cdc34 depl) (*21*). Images were acquired on a Zeiss Axiophot with an integrating color video camera, using a Northern Exposure Software Phase 3 Imaging System (Milford, Massachusetts). (**D**) (Left) Relative histone H1 kinase activity (*16*) of fertilized embryos over time where embryos were fertilized at time zero (Fert), placed into cycloheximide buffer at 68 min (CHX), and harvested at 95 min (Harvest). (Right) DNA replication in extracts prepared from fertilized embryos with sperm nuclei and added XB– (Buffer) or CL→S to 3.5 μM (CL-S 3.5 μM) after 2 or 4 hours (*18*).

lication system derived from *Xenopus* eggs (*4*). When nuclei are added to *Xenopus* interphase egg extracts, they faithfully reproduce initiation events preceding DNA replication and undergo semi-conservative chromosomal replication, using all of the known eukaryotic replication initiation factors (*5*). Despite a lack of G_1 control before the midblastula transition (MBT), *Xenopus* embryos may still share a core regulatory requirement for DNA replication, and hence, the same Cdc34p requirement.

To test whether general proteolysis via the proteasome is required for DNA replication, we measured chromosomal replication in interphase egg extracts in the presence of inhibitors of proteasome function. Methyl ubiquitin inhibited DNA replication of added sperm nuclei by 80% at 1 mg/ml, whereas the peptide inhibitor *N*-acetyl-leu-leu-norleucinal inhibited DNA replication by 70% at 0.3 μM (*6*). This suggests that degradation via ubiquitination and the proteasome may be required for efficient DNA replication in *Xenopus*.

We cloned the *Xenopus* and mouse homologs of the human *CDC34* gene and found that human, *Xenopus*, and mouse Cdc34p shared >92% amino acid identity (*6*). In budding yeast, a mutant yeast Cdc34p, in which the active site cysteine and downstream leucine are changed to serine, blocks cell growth and in vitro ubiquitination of Cln2p (*7*). We generated an analogous pair of mutations in human Cdc34p (hCdc34p). This mutant hCdc34p (CL→S) inhibits the in vitro stability and ubiquitination of the mammalian cdk inhibitor, p27, and thus acts as a dominant negative mutant (*8*).

To determine whether Cdc34p is required for the onset of S phase, recombinant CL→S was added to *Xenopus* interphase egg extracts (also referred to as low-speed supernatant or LSS), and DNA replication of added sperm nuclei was measured. The addition of CL→S to LSS inhibited chromosomal DNA replication by ~90% at 3.3 μM (Fig. 1A). This concentration is approximately eight times higher than the endogenous Cdc34p concentration (400 nM) in LSS. At 3.3 μM, the wild-type (WT) hCdc34p showed little inhibition of DNA replication. Addition of CL→S and WT hCdc34p had little effect on DNA replication when an M13 single-stranded DNA (ssDNA) template was used (Fig. 1A). M13 ssDNA replication is considered to be a model for the elongation step in DNA replication because it requires neither nuclear formation around the DNA nor initiation events (*9*).

To further distinguish a requirement for Cdc34p in the initiation of replication from a requirement in elongation, we varied the time of addition of CL→S. The CL→S mutant was added to LSS to a concentration of 3.5 μM before and after the addition of sperm nuclei (time zero). We began to observe incorporation of [α-^{32}P]deoxyadenosine triphosphate (dATP) into DNA 30 min after the addition of sperm nuclei, and by 40 min, about 7% of the total [α-^{32}P]dATP was incorporated. When the CL→S was added after 30 min, it did not inhibit DNA replication, despite the completion of only a small fraction of the total replication (Fig. 1B).

Because LSS is prepared from eggs released from metaphase arrest, they are in the interphase of the first embryonic cell cycle which might exhibit special requirements for replication. We prepared extract from fertilized eggs in the interphase of the second cell cycle to test whether they too exhibited a Cdc34p requirement for DNA replication. Results showed that addition of 3.5 μM CL→S to this extract inhibited DNA replication (Fig. 1D), indicating that the requirement for Cdc34p is not limited to the first *Xenopus* cell cycle.

To further investigate the Cdc34p requirement for the onset of DNA replication, we attempted to immunodeplete egg extracts of Cdc34p. Antibodies generated against hCdc34p recognized a single protein band in extracts from mammalian cells and *Xenopus*. Protein immunoblotting of the depleted extracts showed >95% removal of Cdc34p

(Fig. 1C). Immunodepletion of Cdc34p from LSS reduced chromosomal replication by 90 to 95% with no effect on M13 ssDNA replication (Fig. 1C). Localized sites of biotin-labeled nucleotide incorporation (replication foci) were visible in control-immunodepleted LSS, whereas such foci were not observed in extracts depleted of Cdc34p (Fig. 1C). We conclude that there is an early requirement for Cdc34p, closely associated with the initiation of DNA replication, and that Cdc34p has no role in the elongation phase of replication.

Attempts to rescue DNA replication in Cdc34p-immunodepleted extracts with recombinant Cdc34p were unsuccessful (Fig. 2A), indicating that other proteins may have been depleted along with Cdc34p. Cdc34p-immunodepleted extracts could be rescued by readdition of LSS or high-speed supernatant (HSS; supernatant of LSS after a high-speed centrifugation) (Fig. 2A), indicating that extracts were not irreversibly inhibited by the immunodepletion process. Anti-Cdc34p–coupled beads incubated in LSS, washed with 500 mM KCl buffer, and added to Cdc34p-immunodepleted extracts restored DNA replication to 60% of that in control samples. Control antibody-coupled beads treated similarly exhibited no rescuing activity. These results suggest that either the human Cdc34p does not functionally replace the *Xenopus* Cdc34p or additional components are required for initiation of DNA replication.

To determine whether the Cdc34p-rescuing activity required for the onset of DNA replication is part of a multiprotein complex, S100 (diluted and centrifuged LSS) was fractionated by gel filtration, and the fractions were analyzed for their ability to rescue the Cdc34p-immunodepleted LSS. Immunoblot analysis showed that >90% of the Cdc34p eluted between 42 and 64 kD, whereas a small amount eluted at a larger apparent molecular size between 70 and 450 kD (Fig. 2B). After concentration on anti-Cdc34p beads, washing, and addition to immunodepleted extracts, all the rescuing activity was found to fractionate between 320 and 440 kD. These fractions contained about 5% of the Cdc34p. No rescuing activity was detected in the fractions containing the bulk of the Cdc34p. Therefore, Cdc34p in a complex of large molecular size is the functional Cdc34p required for chromosomal replication in egg extracts.

When Cdc34p was immunopurified from LSS onto anti-CDC34 beads, washed, eluted, and examined by SDS–polyacrylamide gel electrophoresis (SDS-PAGE), a 34-kD band corresponding to Cdc34p was observed by immunoblotting (Fig. 2C). In addition, two other bands of ~180 and 220 kD were eluted from the anti-Cdc34p beads but not control beads. These two bands were also observed when S100 and gel filtration fractions 11 and 12 (Fig. 2B) were immunopurified on anti-Cdc34p beads (6). These two proteins copurify and coimmunoprecipitate with Cdc34p and are potential components of the complex.

Given that the p40sic1p inhibitor of the Cdc28-Clb5,6 kinase must be degraded to allow entry into S phase in yeast (*2*), we looked for evidence of a similar process in vertebrate cells. The analogous S phase regulator in *Xenopus* is probably the Cdk2–cyclin E kinase, which is required for the initiation of DNA replication in *Xenopus* egg extracts (*10*). When mutant hCdc34p was added to extracts to a concentration of 2.3 μM, DNA replication was reduced by ~80% and 92% in experiments 1 and 2, respectively (Fig. 3). Baculovirus-expressed and affinity-purified *Xenopus* Cdk2–cyclin E restored replication to 85% (experiment 1, 63 nM) and 75% (experiment 2, 53 nM) of the control amount (Fig. 3), whereas addition of kinase inactive *Xenopus* Cdk2 with or without cyclin E did not restore DNA replication activity. Control extract with added

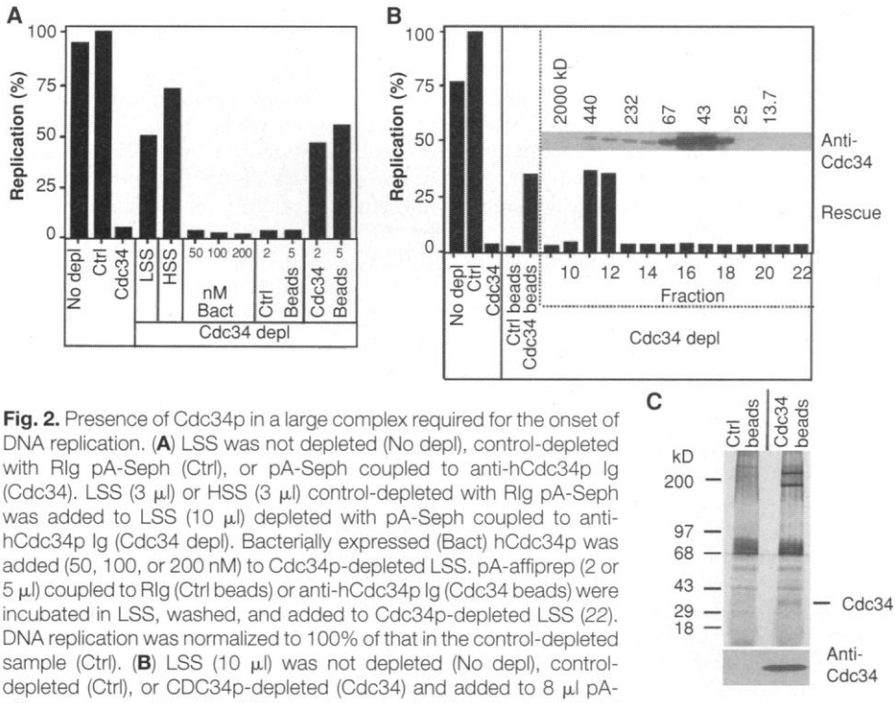

Fig. 2. Presence of Cdc34p in a large complex required for the onset of DNA replication. (**A**) LSS was not depleted (No depl), control-depleted with Rlg pA-Seph (Ctrl), or pA-Seph coupled to anti-hCdc34p Ig (Cdc34). LSS (3 μl) or HSS (3 μl) control-depleted with Rlg pA-Seph was added to LSS (10 μl) depleted with pA-Seph coupled to anti-hCdc34p Ig (Cdc34 depl). Bacterially expressed (Bact) hCdc34p was added (50, 100, or 200 nM) to Cdc34p-depleted LSS. pA-affiprep (2 or 5 μl) coupled to Rlg (Ctrl beads) or anti-hCdc34p Ig (Cdc34 beads) were incubated in LSS, washed, and added to Cdc34p-depleted LSS (*22*). DNA replication was normalized to 100% of that in the control-depleted sample (Ctrl). (**B**) LSS (10 μl) was not depleted (No depl), control-depleted (Ctrl), or CDC34p-depleted (Cdc34) and added to 8 μl pA-affiprep coupled to Rlg (Ctrl beads) or anti-hCdc34p Ig (Cdc34 beads), incubated in LSS, and washed (*22, 23*). Cdc34p-depleted LSS (Cdc34 depl) was also incubated with pA-affiprep coupled to anti-hCdc34p Ig and incubated with column fractions (*23*). DNA replication was normalized to 100% of that in the control-depleted sample (Ctrl). Photographed gel strip (Anti-Cdc34) represents fractions immunoblotted with anti-Cdc34p Ig. The molecular sizes listed above represent the elution of standards used to calibrate the column. (**C**) LSS was incubated with pA-affiprep coupled to either Rlg (Ctrl beads) or anti-hCdc34p Ig (Cdc34 beads), and the beads were washed, eluted, subjected to SDS-PAGE, and silver stained (*22*). Sizes of protein standards are listed to the left and Cdc34p is indicated. (Bottom panel) Immunoblot of eluted material with anti-CDC34 Ig.

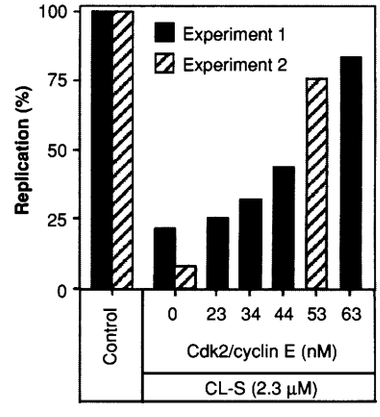

Fig. 3. DNA replication inhibited by mutant Cdc34p and restored by Cdk2–cyclin E. CL→S was added to a concentration of 2.3 μM, and baculovirus-expressed Cdk2–cyclin E (500 nM) (*11, 24*) was added to a concentration of 23, 34, 44, 53, and 63 nM. DNA replication was normalized to 100% of that in the control sample, which contained no CL→S and was equalized to the same final volume as all other samples with XB– (*23*).

Cdk2–cyclin E (34 to 63 nM) exhibited no increase in replication activity. When DNA replication was inhibited by 95% or more by immunodepletion of Cdc34p, addition of Cdk2–cyclin E only partially rescued replication activity. From these results, it appears likely that at least one function of Cdc34p is to directly or indirectly regulate Cdk2–cyclin E activity, perhaps by targeting an inhibitor of Cdk2–cyclin E for destruction.

The *Xenopus* cdk inhibitor Xic1 is related to both p21 and p27 in mammals, inhibits Cdk2–cyclin E activity, and inhibits DNA replication when added to *Xenopus* egg extracts (*11*). To test whether Xic1 may be a substrate of Cdc34p, Xic1 was in vitro translated (ivt) and added to LSS. Contrary to expectations, ivt Xic1 was stable in both control- and Cdc34p-depleted extracts (Fig. 4A). However, when sperm nuclei (2500 nuclei/μl) were added to extracts, ivt Xic1 was efficiently degraded in control-depleted extracts, with a half-life of 60 to 90 min (Fig. 4, A and B). Xic1 was not degraded in similar extracts that had been depleted of Cdc34p (Fig. 4A). The half-life of ivt Xic1 decreased with increasing numbers of nuclei. When 5000 nuclei/μl were added (the approximate concentration at the MBT), the half-life of total ivt Xic1 was 45 min (Fig. 4B). This result suggests that Xic1 may be a bona fide substrate of Cdc34p in *Xenopus* eggs, but that its degradation may occur in a nucleus-dependent manner, perhaps requiring localization to the nucleus for Cdc34p-dependent degradation. We postulate that localized nuclear degradation by Cdc34p may target specific regulatory proteins for degradation at sites of initiation. Permeabilization of the nucleus during each mitosis could allow a pool of Cdc34p substrates to reenter the nucleus after each division without a requirement for protein synthesis.

The degradation of ivt Xic1 in LSS was inhibited by methyl ubiquitin and reversed by the addition of ubiquitin (Fig. 4C), confirming that ivt Xic1 is degraded by the ubiquitin degradation pathway. The degradation of ivt Xic1 appeared to be inhibited by 6-dimethylaminopurine (6-DMAP), an inhibitor of Cdk's and the initiation of DNA replication (*12*), but not by aphidicolin, an inhibitor of the elongation phase of DNA replication (Fig. 4C). This suggests that Cdk2 activity or initiation events, or both, may be required for Xic1 degradation, whereas elongation is not. Ivt Xic1 was not degraded in mitotic extracts with or without nuclei, indicating that its degradation may be cell cycle regulated (Fig. 4C). Ivt Xic1 from mitotic extracts had a lower mobility during SDS-PAGE which was reversed by treatment with calf intestinal phosphatase (CIP) (Fig. 4C).

These studies indicate that Cdc34p in a large molecular size complex is required for events involved in the initiation of DNA replication and that localized degradation of an inhibitor of Cdk2–cyclin E such as Xic1 and perhaps other S phase regulators may be required in each *Xenopus* cell cycle. We do not rule out the possibility that Cdc34p may also regulate the function of Cdk2–cyclin A2 in *Xenopus* interphase egg extracts. Genetic studies in yeast suggest that Cdc34p function may require a complex of Cdc34p, Cdc53p, Skp1p, and Cdc4p, although biochemical characterization is still incomplete (*13*). However, all the yeast components thus far identified are smaller than the polypeptides associated with *Xenopus* Cdc34p.

In the early *Xenopus* embryo, the cell cycle is specifically modified such that materials are stockpiled for at least 12 divisions and there is no G_1 or G_2 phase and no growth (*14*). Between fertilization and late cleavage stages, there is no external control of the cell cycle by mitogens and the pace is set by the complete degradation and resynthesis of cyclin B (*15*). Under these circumstances, a Cdc34p degradation requirement for DNA replication was not necessarily expected. Our results indicate that there are one or more proteolytic steps in the intrinsic control of DNA replication and that degradation is not simply a feature of extrinsic control, but that in higher eukaryotes it may be an obligatory step in each round of initiation of DNA replication.

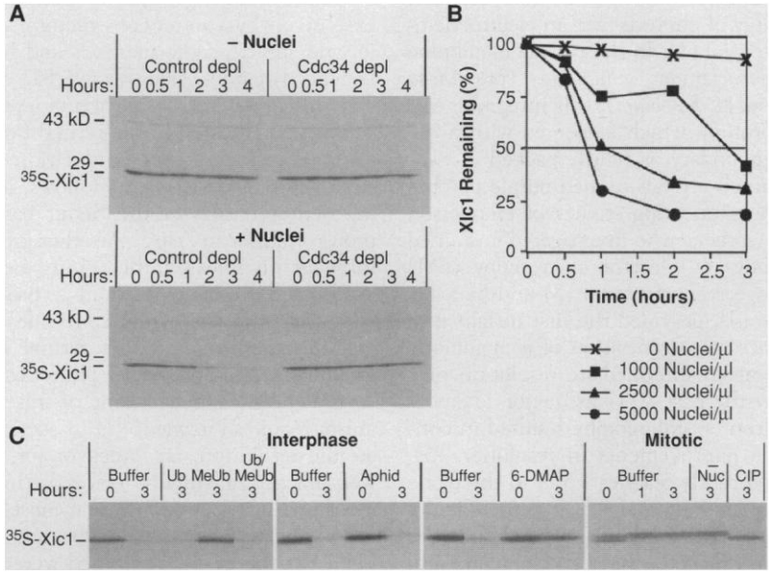

Fig. 4. Degradation of in vitro translated Xic1 in a Cdc34p- and nuclei-dependent manner. (**A**) LSS was either control-depleted with Rlg pA-Seph (Ctrl depl) or pA-seph coupled to anti-hCdc34p Ig (Cdc34 depl). Sperm nuclei were not added (–Nuclei) or added to a concentration of 2500 nuclei/μl (+Nuclei). Ivt Xic1 was added to LSS and samples were incubated for 0 to 4 hours (*25*). (**B**) LSS was incubated without nuclei (0 nuclei/μl) or with 1000, 2500, or 5000 nuclei/μl with ivt Xic1, and the relative amount of Xic1 remaining was determined by phosphoimager (*26*). (**C**) LSS (Interphase) was incubated with ivt Xic1 for 3 hours with 2500 nuclei/μl, in XB– (Buffer) (*23*), XB– with 1 mg/ml ubiquitin (Ub), 1 mg/ml methyl ubiquitin (MeUb), or a mixture of the two (*25*). Ivt Xic1 was also incubated in LSS with 2500 nuclei/μl with XB– with 10% ethanol (Buffer) or 50 μg/ml aphidicolin in XB– with 10% ethanol (Aphid) for 3 hours (middle left panel) or with XB– with 5% dimethylsulfoxide (DMSO) (Buffer) or 4 mM 6-DMAP in XB– with 5% DMSO for 3 hours (middle right panel). LSS was treated with cyclin Δ90 for 40 min (Mitotic), and ivt Xic1 was added without (–Nuc) or with nuclei to 2500 nuclei/μl (Buffer). The samples were incubated for 0 to 3 hours, and one sample was treated with CIP (right panel).

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17. LSS was generated as described (*16*). Extracts replicated 75 to 100% of the input template (5 ng/μl). Cycloheximide (0.1 mg/ml) was added to LSS before replication was assayed.
18. Demembranated sperm nuclei were prepared as described (*4*) and used at a final DNA concentration of 5 ng/μl. Plasmid template ssM13 DNA was added to a 10 ng/μl final concentration. DNA replication was measured by trichloroacetic acid precipitation of radiolabeled fragments (*4, 10*) and presented as a percent replication of the input template (5 ng/μl). Alternatively, samples were ethanol precipitated, run on agarose gels, and autoradiographed.
19. Antibodies were covalently cross-linked to protein A (pA)–Sepharose (Seph) or pA-affiprep beads as described [E. Harlow and D. Lane, *Antibodies: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988)]. Immunodepletion was performed by mixing 1 volume LSS to 1.2 volumes antibody-coupled pA-Seph beads for 2 hours at 0°C with resuspension every 10 min.
20. LSS, Superdex column fraction, or eluted affiprep material were analyzed by SDS-PAGE, transferred to nitrocellulose, incubated with affinity-purified anti-hCDC34 immunoglobulin (Ig), and analyzed by enhanced chemiluminescence (Amersham).
21. Replication foci were analyzed by stated methods (*10, 12*).
22. Five volumes of LSS were incubated with one volume of coupled pA-affiprep beads for 3 hours at 4°C. Beads were washed with 15 volumes each of XB– [extract buffer: 100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM Hepes (pH 7.7)] (one time), XB– with 500 mM KCl (three times), and 0.5× XB– (two times) (*23*). Immunodepleted LSS was added to the beads and assayed for replication. Alternatively, the washed beads were treated with 100 mM glycine (pH 2.5), and the eluate was neutralized with 1 M tris (pH 8). Samples were concentrated and analyzed by SDS-PAGE and silver stained or analyzed by immunoblotting.
23. S100 was generated by dilution of LSS with nine volumes 20 mM tris (pH 7.7), 100 mM KCl, 1 mM $MgCl_2$, and 1 mM dithiothreitol followed by centrifugation for 1 hour at 100,000*g*. S100 was concentrated to its original volume and loaded onto a HiLoad 16/60 Superdex 200 column (Pharmacia). Fractions were concentrated to one-tenth their volume and incubated with anti-hCDC34 affiprep beads for 2.5 hours at 0°C with resuspension every 10 min. Beads were washed with XB– supplemented with KCl to 500 mM (XB– with 500 mM KCl), added to Cdc34p-depleted LSS with sperm nuclei (5 ng/μl), and incubated at 23°C for 3 to 4 hours.
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25. In vitro Xic1 degradation reaction was performed by adding sperm nuclei to a concentration of 7.9 ng/μl in 5 μl LSS, with 0.3 μl ivt Xic1, 0.25 μl energy regeneration (ER) mix (*16*), 1.25 mg/ml ubiquitin, 0.1 mg/ml cycloheximide, and incubation at 23°C. Reactions were stopped by adding 2× SDS-PAGE sample buffer. Xic1 was in vitro transcribed from the SP6 promoter of Xic1/pCS2+ [D. L. Turner and H. Weintraub, *Genes Dev.* **8**, 1434 (1994)] (subcloned from Xic1/pBluescript) and translated with [^{35}S]methionine. Xic1 bands were quantitated by phosphorimager (Molecular Dynamics), and Xic1 levels were normalized to the percent Xic1 remaining to determine half-life values.
26. Xic1 degradation was assayed as before (*25*) except 0.14 μl ivt [^{35}S]met-labeled Xic1 was used, and sperm nuclei were added to a concentration of 3.2 ng/μl (1000 nuclei/μl), 7.9 ng/μl (2500 nuclei/μl), or 15.8 ng/μl (5000 nuclei/μl) and incubated for 3 hours at 23°C.
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X-ray Structure of Bacteriorhodopsin at 2.5 Angstroms from Microcrystals Grown in Lipidic Cubic Phases

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Lipidic cubic phases provide a continuous three-dimensional bilayer matrix that facilitates nucleation and growth of bacteriorhodopsin microcrystals. The crystals diffract x-rays isotropically to 2.0 angstroms. The structure of this light-driven proton pump was solved at a resolution of 2.5 angstroms by molecular replacement, using previous results from electron crystallographic studies as a model. The earlier structure was generally confirmed, but several differences were found, including loop conformations and side chain residues. Eight water molecules are now identified experimentally in the proton pathway. These findings reveal the constituents of the proton translocation pathway in the ground state.

Bacteriorhodopsin (bR) is a light-driven proton-translocating pump that converts the energy of photons into an electrochemical potential (*1*). In the plasma membrane of *Halobacterium salinarium* (previously known as *H. halobium*), this integral membrane protein, which is located within the bilayer boundary, is tightly packed in two-dimensional crystals termed purple patches (*2*). The pioneering studies of Henderson and co-workers, who investigated unstained membranes by electron microscopy (EM) and image reconstruction (*3*) and by x-ray analyses (*4*), provided the first insight into the structural organization of a membrane protein at an intermediate resolution of 7 Å. Over the past 20 years, major advances in electron crystallography resulted in considerable improvements in resolution (*5*), which currently reaches 3.5 Å in the plane of the membrane and 4.3 Å perpendicular to it (*6*). These studies revealed that the structure of bR consists of seven membrane-spanning α helices that are connected by three external and three cytoplasmic loops (*7*). The pigment, retinal, is bound covalently (through a protonated Schiff base) to Lys^{216}. It is buried in the interior of the protein and is stabilized by the binding of the β-ionone ring in a hydrophobic pocket.

The function of bR has been studied extensively by a variety of structural, genetic, and spectroscopic methods and by molecular dynamics calculations (*6, 8–10*). The primary event, absorption of a photon, causes isomerization of the retinal from the all-trans to the 13-cis configuration. A series of intermediate events follows, including deprotonation of the Schiff base and proton transfer to Asp^{85}. Another proton is subsequently released, the Schiff base is reprotonated from Asp^{96}, and a proton is taken up from the cytoplasmic side. Thermal reisomerization of the retinal to the ground state completes the photocycle.

A detailed understanding of this mechanism requires knowledge of its structure at atomic resolution, an endeavor for which well-diffracting, highly ordered three-dimensional (3D) crystals appear most promising. Several attempts have been reported (*11*), but the crystals obtained were either too small or exhibited extensive mosaicity as a result of poor order along the *c* axis. To overcome these limitations, we devised a concept for the crystallization of membrane proteins by exploiting the properties of bicontinuous lipidic cubic phases (*12*). Membrane proteins, once inserted into this continuous, 3D curved lipid bilayer matrix, diffuse laterally to nucleate and eventually to yield well-ordered crystals. Our initial results with microcrystals of bR, which diffracted x-rays to 3.7 Å resolution (*12*), have

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