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14. ABSTRACT

Cyclin D is a positive regulator of the cell cycle and is found to be highly expressed in breast cancer cells. Cyclin D is post-transcriptionally regulated by the ubiquitin mediated protein degradation pathway. Cdc34 and SCF are postulated to be the specific E2 and E3 enzymes which target Cyclin D for ubiquitination. It is currently unclear how regulation of the Cdc34-SCF complex may modulate Cyclin Dproteolysis. In this regard, we have studied the regulation of Cdc34 by phosphorylation, by Cdc34-associated proteins and by the interaction with SCF components, Cul1 and Roc1. Our results suggest that the carboxyl-terminal acidic tail domain of Cdc34 is required for efficient in vitro polyubiquitination of p27kip1, though it is not critical for Cdc34 interactions with Cul1 and Roc1 in an in vitro binding assay. The role of Cdc34 phosphorylation on Cul1 and Roc1 binding is still unclear. Currently, our work has focused on identifying the proteins which tightly associate with Cdc34 and which are required for DNA replication initiation in Xenopus egg extracts. We are developing strategies to purify the Cdc34-associated proteins from HeLa cell extracts and from Xenopus egg extracts. We predict that these previously unidentified Cdc34-associated proteins will play an important role in regulating the ubiquitination of cyclin D in vivo.

15. SUBJECT TERMS
Cdc34-SCF, cyclin D, cell cycle, breast cancer

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The role of ubiquitin-mediated proteolysis of cyclin D in breast cancer

INTRODUCTION

Cyclin D is a positive regulator of the cell cycle and is found to be highly expressed in breast cancer cells (7). Cyclin D is post-transcriptionally regulated by the ubiquitin mediated protein degradation pathway. Cdc34, a ubiquitin conjugating enzyme, and SCF (Skp1, Cullin, F-box, ring protein), a ubiquitin ligase, are postulated to be the specific E2 and E3 enzymes which target Cyclin D for Ubiquitination (9). It is currently unclear how regulation of the Cdc34-SCF complex may modulate Cyclin D proteolysis. Currently we are studying the regulation of Cdc34 by phosphorylation, by interactions with the SCF components, Cul1 and Roc1 and by Cdc34-associated proteins. In an attempt to understand the biological significance of Cdc34 phosphorylation and cyclin D proteolysis, we have generated WT Cdc34, truncation mutants of Cdc34 and phosphomutants of Cdc34 in yeast expression plasmids, mammalian expression plasmids and bacterial expression plasmids as well as SCF components in mammalian expression vectors. These reagents have allowed us to examine the biological significance of Cdc34 phosphorylation *in vitro* and *in vivo*.

I am also developing strategies to identify the Cdc34-associated proteins which are required for DNA replication initiation in Xenopus egg extracts (8). We propose that these previously unidentified Cdc34-associated proteins play an important role in regulating the ubiquitination of cyclin D in vivo.

BODY

Task 1: Months 1-9

To study the regulation of Cdc34 function by phosphorylation in normal and transformed breast cells.

- **a.** To study how the phosphorylation of Cdc34, primarily by CK2, may regulate the function of Cdc34 during the G1/S phase transition.

- **b.** To examine the phosphorylation of Cdc34 and cyclin D half life in transformed cells and normal breast cancer cells.

a. To study the biological significance of Cdc34 phosphorylation at the G1/S transition, we transiently over expressed wild type human Cdc34 (WT), the non-phosphorylated Cdc34 mutant (5 PT MUT), or the carboxyl-terminal 36 amino acid truncation mutant of Cdc34 (1-200) into 10T-1/2 cells followed by flow cytometry and cell cycle analysis (**Figure 1** of Appendix). The results suggest that overexpression of hCdc34 WT and 1-200 mutant result in a significant increase in the G1 population and a concomitant decrease in the number of cells both in S and G2/M phases compared to cells transfected with the vector alone (VECTOR) (**Figure 1**, top panel). A similar, but

less pronounced effect was observed with the Cdc34 5 PT MUT (**Figure 1**, top panel). In addition to 10T-1/2 cells which are diploid murine fibroblasts, we also transfected U2OS human osteosarcoma cells with vector alone, Cdc34 WT, 5 PT MUT, 1-200, and the Cdc34 active site mutant with active site cysteine and downstream leucine altered to serine (CL-S). In U2OS cancer cells, the effect of Cdc34 overexpression results in an increase in G1 cells, a mild decrease in S phase cells for some samples and a decrease in the number of G2/M cells (**Figure 1**, bottom panel). Overall, the effects of Cdc34 overexpression were less pronounced in the U2OS cancer cell line. The preliminary results in 10T-1/2 cells have been repeated, but will be further studied. Thus far, the results suggest that overexpression of any Cdc34 protein results in a dominant negative effect on the entry into S and M phases. This implies that the overexpressed Cdc34 binds and perhaps titrates out a critical regulator of the cell cycle, making it difficult to use this approach to specifically study the Cdc34 phosphomutan. The experimental design of these past studies relied on transient transfection assays using Cdc34 expressed from mammalian expression plasmids which is limited in its ability to target a large population of cells. We have developed an alternative approach to perform these studies which uses recombinant Adenoviruses expressing human Cdc34. We have generated a recombinant Adenovirus which expresses a human tagged WT Cdc34 which allows for infection of 100% of mammalian cells and high expression of tagged human Cdc34 protein. We are currently planning on generating additional recombinant Adenoviruses which express different Cdc34 mutant proteins to further address the experiments of this specific aim.

b. To address this specific aim, we chose normal and breast tumor cell lines that were available at this Institute for our studies. Our published studies indicate that human Cdc34 is phosphorylated by casein kinase 2 (CK2) (3). We first measured the level of phosphorylation of endogenous Cdc34 by CK2 in normal or tumor breast cell lines. We performed an immunoprecipitation of cell lysates with affinity purified Cdc34 antibody (CDC34 Ig) or non-immune rabbit antibody (RIg) followed by an in vitro kinase reaction (**Figure 2** of Appendix).These studies did not show a consistent increase in Cdc34 phosphorylation in breast tumor cells over normal breast cells as we had expected. In continuing studies, we will examine other commercially available established breast cancer cell lines. We will also examine cells derived of other tissue types and we will first determine whether CK2 is elevated in the tumor cells before analyzing the phosphorylation status of endogenous Cdc34 by orthophosphate labeling and immunoprecipitation. If we observed an alteration in Cdc34 phosphorylation in breast cancer cells versus normal breast cells, then we would examine cyclin D turnover in these cells.

Task 2: Months 6-36
To study the regulation of Cdc34-SCF function by compartmentalization during the G1 to S phase transition.

a. To determine the cell localization of the cyclin D ubiquitinating activity mediated by Cdc34-SCF in normal and breast cancer cells.

b. To determine the mechanism of Cdc34 compartmentalization through protein-protein interactions with known Cdc34-interacting proteins and to characterize how phosphorylation may regulate the interaction of Cdc34 with other proteins.

a. Previous studies have shown that Cdc34 in a large molecular weight complex is required for initiation of DNA replication in *Xenopus laevis* egg extracts (8). Immunodepletion of Cdc34 from Xenopus egg extracts inhibits DNA replication, implying a requirement for Cdc34 in the degradation of critical G1 to S phase regulators prior to onset of S phase. Recombinant Cdc34 cannot restore DNA replication in Cdc34 immunodepleted extracts while a Cdc34 protein complex can. This suggested that there were Cdc34 associated proteins which played an important role in the initiation of DNA replication. We postulate that the Cdc34-associated proteins play an important role in regulating the ubiquitination function of Cdc34. These as yet unidentified proteins may regulate the compartmentalization or post-translational modifications of Cdc34. These proteins may also be altered in breast cancer cells and may play a role in altering the ubiquitination of Cdc34 substrates such as cyclin D during the development of breast cancer.

We have focused on purifying the Cdc34-associated proteins from Xenopus egg extracts, also called "Low Speed Supernatant" (LSS) which are readily available and which contain robust activity that restores DNA replication to Cdc34-immunodepleted egg extracts. When beads coupled to Cdc34 antibody were incubated in S100 (cytosolic fraction of Xenopus egg extracts), washed extensively with 500 mM KCl and eluted with glycine, a number of bands corresponding to approximate molecular weights of 70, 90, 180 and 220 Kd were observed on a silver stained gel (**Figure 3** of Appendix).These bands were specific to anti-Cdc34 beads and were not observed on control beads coupled to control antibody. These Cdc34 associated proteins are not yet identified.

In order to identify the proteins which tightly associate with Cdc34 and are required for DNA replication, I have taken a biochemical approach. As a first biochemical step, I have studied the use of ammonium sulfate precipitation and found that most of the monomer form of Cdc34 (inactive form) can be separated from the functional Cdc34 in a high molecular weight complex using a low percentage of ammonium sulfate. The functional Cdc34 complex containing all of the replication rescue activity precipitates at a concentration of 20% ammonium sulfate (**Figure 4** of Appendix). Using this one step, I have attained a 20-fold purification and a 90% recovery of rescue activity. As a second step, I have utilized ion exchange chromatography. I have used the anion exchanger, DEAE sepharose. I have found that replication rescue activity elutes exclusively in the 150mM salt elution while the high salt elutions and the unbound fractions do not contain any rescuing activity (**Figure 5** of Appendix). My results indicate that biochemical fractionation using DEAE yields a 20-fold purification of Cdc34-rescuing activity and at least a 30% recovery of activity. My final biochemical

purification step is to use immunoaffinity chromatography using a Cdc34 affinity purified polyclonal rabbit antibody. I am currently increasing the scale of my purification and combining all three purification steps to isolate enough material for Mass spectrometry analysis. The strategy for purification and identification of Cdc34 associated proteins in Xenopus egg extracts is outlined in **Figure 6** of Appendix.

A disadvantage of purifying the Cdc34-associated proteins from Xenopus extracts is that the Xenopus sequence database is not complete which makes it difficult to identify proteins based on Mass Spectrometry of biochemically purified proteins. An alternative approach is to use extracts from human cells since the human genome has been fully sequenced. We have shown that extracts from HeLa cells can complement Cdc34-immunodepleted egg extracts to restore DNA replication activity. These studies are described in the accompanying manuscript in preparation (2). In summary, these studies demonstrate that fractionated HeLa cell nuclear extracts can restore DNA replication to Cdc34-immunodepleted egg extracts and the studies go on to describe a strategy for the purification of the Cdc34 complex required for this activity. Once the Cdc34-associated proteins are identified, I will determine the role of these proteins on regulating the function of Cdc34, in particular, their role on regulating the ubiquitination of cyclin D. I will also determine how these proteins may be altered in breast cancer cells and how this may correlate with cyclin D expression.

b. The *CDC34* gene in budding yeast is essential and the *cdc34* temperature sensitive (ts) mutant strain arrests in G1 at the non-permissive temperature with a phenotype of multiple buds (5). In order to determine whether the phosphorylation of sites within the human Cdc34 (hCdc34) acidic tail may be required for functional complementation of the budding yeast ts strain, *cdc34-2*, several human Cdc34 clones were used to perform complementation studies in budding yeast. Our published results indicated that the phosphorylation of sites within the hCdc34 acidic tail were not required for complementation, however we found that the tail domain was essential for complementation, as hCdc34 1-200, the tail deletion mutant, like the active site mutant hCdc34 CL-S, could not complement the ts growth defect of *cdc34-2* (1, *included in the appendix*). To study the role of the hCdc34 tail domain, we collaborated with Michele Pagano and Joanna Bloom of New York University School of Medicine. We demonstrated that the Cdc34 1-200 mutant is efficiently charged with ubiquitin by E1 and readily associates with Cul1-Roc1, yet fails to support the ubiquitination of CDK inhibitor, $p27^{Kip1}$ *in vitro* (1). Studies in budding yeast suggest that both ubiquitin charged and uncharged Cdc34 associates with SCF^{Cdc4}, but only the charged Cdc34 properly dissociates from the SCF, a step that was shown to be essential for the efficient ubiquitination of budding yeast CDK inhibitor, $p40^{Sic1}$ (4). We postulated that the tail domain of hCdc34 might be important for its proper dissociation dynamics from the human SCF and lack of such proper dissociation could be the reason for failure to efficiently polyubiquitinate $p27^{Kip1}$.

To study the association-dissociation dynamics between human Cdc34 and Cul1-Roc1, I performed a modified "binding and release assay" based on the assay initially described by Deffenbaugh et al., 2003 (4). My results indicated that uncharged hCdc34 WT (**Figure 7** of Appendix) could bind to the Cul1-Roc1 complex, consistent with studies in budding yeast. However, contrary to the findings in budding yeast, my studies suggested that uncharged Cdc34 WT could efficiently dissociate (release) from the Cul1-Roc1 complex. I observed similar dissociation with charged hCdc34 WT (**Figure 8** of Appendix) and charged hCdc34 1-200 mutant protein (**Figure 9** of Appendix). The dissociation of hCdc34 WT and 1-200 proteins was specific because FLAG-Cul1 was not detected in the released fractions. The graphs comparing the dissociation of uncharged vs. charged hCdc34 WT show no statistically significant difference in their dissociation dynamics (**Figure 10** of Appendix). The percent release at the 90 minute time point indicates that uncharged (4.9 %) and charged Cdc34 WT (6.1 %) differ slightly in their dissociation from the Cul1-Roc1 complex, however the difference is not as pronounced as observed for budding yeast Cdc34, where 10 % of charged budding yeast Cdc34 was released compared to less than 1 % of uncharged Cdc34 (4). This preliminary result indicates that the interaction dynamics of human Cdc34 and the SCF may differ between budding yeast and humans. The graphs comparing the dissociation of charged hCdc34 WT (6.1 % release at 90 min) with charged hCdc34 1-200 (5.8 % release at 90 min) show similar dissociation dynamics for the two proteins (**Figure 11** of Appendix).These results suggest that the C-terminal tail domain of human Cdc34 is not critical for proper interaction dynamics with the SCF and the defect of the Cdc34 1-200 mutant may lay more directly in a late step of substrate ubiquitination. A study in budding yeast showed that the acidic loop of yeast Cdc34 was dispensable for the attachment of the first ubiquitin onto Sic1, but was required for subsequent steps of ubiquitin chain synthesis (6). A similar role for the human Cdc34 tail domain is possible, which we plan to examine using $p27^{Kip1}$ as a substrate. Other Cdc34 substrates such as cyclin D will also be studied using the Cdc34 1-200 to determine whether the lack of $p27^{Kip1}$ polyubiquitination by Cdc34 1-200 is a general characteristic of Cdc34 1-200 or whether this effect is specific only to the $p27^{Kip1}$ substrate.

Our previous work has shown that mammalian Cdc34 is phosphorylated by CK2 (3).We focused further studies on trying to understand how Cdc34 phosphorylation may regulate its association with Cul1 and Roc1. To study this, I performed in vitro co-immunoprecipitation assays in the presence or absence of CK2 enzyme along with co-translated Cul1-Roc1 proteins and bacterially-expressed recombinant 6His-tagged WT Cdc34. My preliminary results indicated similar levels of binding of Cdc34 and Cul1-Roc1 in the presence and absence of CK2 (**Fig. 12** of Appendix), suggesting that phosphorylation has no effect on the *in vitro* interactions of Cdc34 with Cul1 and Roc1. However, there are caveats to this experiment. It was not possible to determine the percentage of Cdc34 that was phosphorylated in the binding assay and we expect that not all the Cdc34 was phosphorylated under the conditions utilized. Future studies will determine the exact conditions that are required to ensure that the majority of Cdc34 is phosphorylated by CK2 in the binding assay. Additionally, further experiments indicated

that in the presence of rabbit reticulocyte lysate (as included in the binding assay to provide the co-in vitro translated Cul1-Roc1 proteins); Cdc34 can be phosphorylated even in the absence of any added CK2 enzyme. Therefore, at this time, no conclusions can be drawn from this result and further experiments are required. I also performed further binding studies using WT Cdc34, Cdc34 1-200 (acidic tail deletion mutant), Cdc34 5 PT A (phosphomutant which alters all five Cdc34 phosphorylation sites to alanine), and Cdc34 5 PT E (mutant which alters all five Cdc34 phosphorylation sites to glutamic acid to mimic constitutive phosphorylation). Our published results on these studies show no significant difference in the ability of WT and 1-200 Cdc34 proteins to bind Cul1 or Roc1 implying that the carboxyl-terminal acidic tail of Cdc34 may not play a significant role in the association with Cul1 and Roc1 in an in vitro binding assay (1). However, I did observe that the Cdc34 5 PT A mutant was reduced in its ability to bind to both Cul1 and Roc1 compared to the Cdc34 WT and the Cdc34 5 PT E mutant, suggesting that the phosphorylation of sites within the acidic tail may play some role in the interaction between Cdc34 and Cul1-Roc1. Because these in vitro binding results are somewhat contradictory, in the future we will perform *in vivo* binding studies to better examine the role of Cdc34 phosphorylation on its association with SCF components.

TRAINING

This traineeship has helped me gain valuable research experience and develop important skills to pursue a career in academics. During the duration of this fellowship, interactions with prominent researchers in the field of breast cancer biology have been both informational and inspirational. Listening to presentations given by the participants of our departmental seminar series has kept me informed with the latest in the field. I have also kept myself informed by reading the literature and discussing important publications in our weekly lab meetings. Data presentations during these lab meetings have helped me critically analyze my work and develop important presentation skills. My presentation during the 2005 annual departmental retreat was judged best amongst the talks given by the fourth year graduate students.

In the recent months I have attended conferences on "Ubiquitin and Cancer" and I also had an opportunity to present a poster during one of these meetings. During these meetings, I could discuss my work with prominent researchers in the field of ubiquitin and cancer biology. In particular, discussions with Dr. Ning Zheng and Dr. Brenda Schulman have provided me with new ideas on my project. Dr. Ning Zheng is a potential collaborator, with whom we plan to study different Cdc34 substrates in an *in vitro* ubiquitination assay using Cdc34 WT and the tail deletion mutant, Cdc34 1-200. Hopefully we will determine the role of Cdc34 tail domain in substrate ubiquitination. I also look forward to the identification of Cdc34 associated proteins. These proteins may play a role in altering the ubiquitination of Cdc34 substrates such as cyclin D during the development of breast cancer. In summary, this traineeship has given me invaluable training in the area of breast cancer and has solidified my commitment to pursue breast cancer research as a future independent researcher.

KEY RESEARCH ACCOMPLISHMENTS

- Development of biochemical purification strategy for Cdc34 high molecular weight complex from Xenopus egg and HeLa cell extracts.
- Generation of recombinant Adenovirus expressing wild type human Cdc34.

REPORTABLE OUTCOMES

Abstracts:

Appikonda Srikanth and P. Renee Yew. 2006. Interaction Dynamics between Human Cdc34 and the SCF. An AACR special conference in cancer research. Ubiquitin and cancer: From molecular targets and mechanisms to the clinic. Lake Buena Vista, Florida. (Poster presentation)

Appikonda Srikanth and P. Renee Yew. 2006. Interaction Dynamics between Human Cdc34 and the SCF. Graduate Student Association, The University of Texas Health Science Center at San Antonio, Texas. (Abstract submission)

Manuscripts:

Block K, Appikonda S, Lin HR, Bloom J, Pagano M, Yew PR. 2005. The acidic tail domain of human Cdc34 is required for p27Kip1 ubiquitination and complementation of a cdc34 temperature sensitive yeast strain. Cell Cycle.4 (10):1421-7.

Awards:

Karen Block. Ph.D. Degree in Molecular Medicine conferred. 2003. Cdc34 regulation and its role in vertebrate DNA replication initiation. Current position: Postdoctoral fellow in the laboratory of Dr. Hanna Abboud, Department of Nephrology, The University of Texas Health Science Center at San Antonio, Texas.

Srikanth Appikonda. First place (4^{th} year graduate students). Annual Retreat 2005, Department of Molecular Medicine, The University of Texas Health Science Center at San Antonio, Texas.

LIST OF PERSONNEL RECEIVING SUPPORT

Karen Block, Graduate Student
Appikonda Srikanth, Graduate Student

CONCLUSIONS

We have generated a recombinant Adenovirus expressing human tagged wildtype Cdc34 which will enable us to study the regulation of Cdc34 by phosphorylation in normal and breast cancer cells. To further address this aim we need to generate additional recombinant Adenoviruses which would express different Cdc34 mutant proteins. Our preliminary results on effect of phosphorylation on human Cdc34 interactions with Cul1 and Roc1 are currently inconclusive. We have observed that the Carboxy-terminal acidic tail domain of Cdc34 is not required for *in vitro* interactions with Cul1 and Roc1 but phosphorylation of sites within the tail may be required. Further *in vivo* studies will be necessary to clearly understand these interactions. We have shown that the tail domain of hCdc34 is required for efficient *in vitro* polyubiquitination of $p27^{Kip1}$. This defect of hCdc34 1-200 is not due to a lack of enzymatic activity or due to improper association –dissociation dynamics with the SCF. Further studies focusing on transfer of ubiquitin from Cdc34 to the substrates may reveal the role of Cdc34 acidic tail domain in substrate ubiquitination. We have developed biochemical purification strategies to purify Cdc34 high molecular weight complexes from Xenopus egg and HeLa cell extracts. Now we are focusing on purifying these complexes to identify the Cdc34 associated proteins. We postulate that these yet unidentified proteins may be altered in breast cancer cells causing up regulation of cyclin D. We hope that our research will help understand mechanisms of Cyclin D regulation and identify new proteins of therapeutic value.

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1. **Block K, Appikonda S, Lin HR, Bloom J, Pagano M, Yew PR**. 2005. The acidic tail domain of human Cdc34 is required for p27Kip1 ubiquitination and complementation of a cdc34 temperature sensitive yeast strain. Cell Cycle.4 (10):1421-7. ***Attached as an appendix***

2. **Block K, Boyer TG, Bloom J, Pagano M, Yew PR**. Biochemical characterization of a human Cdc34 complex that rescues DNA replication in Cdc34-deficient Xenopus egg extracts. (Manuscript in preparation). ***Attached as an appendix***

3. **Block K, Boyer TG, Yew PR**. 2001. Phosphorylation of the human ubiquitin-conjugating enzyme, Cdc34, by casein kinase 2. J Biol Chem.276(44):41049-58

4. **Deffenbaugh AE, Scaglione KM, Zhang L, Moore JM, Buranda T, Sklar LA, Skowyra D**. 2003. Release of ubiquitin-charged Cdc34-S - Ub from the RING domain is essential for ubiquitination of the SCF (Cdc4)-bound substrate Sic1. Cell.114(5):611-22.

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Appendix

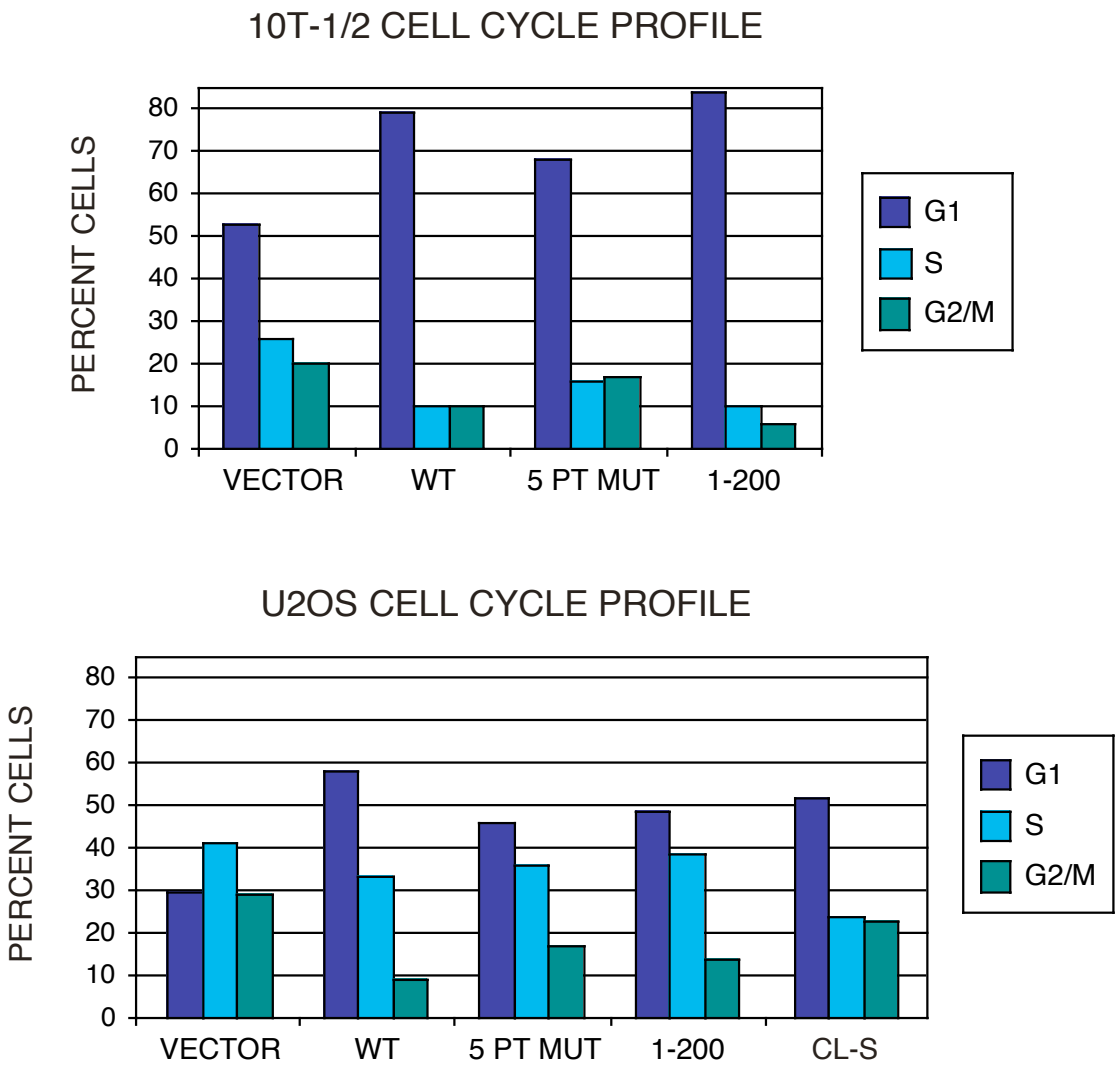

Figure 1. Transient transfection of Cdc34 into mammalian cells causes an increase of cells in the G1 phase. (Top panel) pCS2+ (VECTOR), wildtype human Cdc34 (WT), non-phosphorylated Cdc34 mutant (5 PT MUT) and the carboxyl-terminal 36 amino acid truncation mutant of Cdc34 (1-200) were transfected into 10T-1/2 cells followed by flow cytometry and cell cycle analysis. (Bottom panel) VECTOR, WT, 5 PT MUT, 1-200 and Cdc34 active site mutant (CL-S) were transfected into U2OS cells followed by flow cytometry and cell cycle analysis.

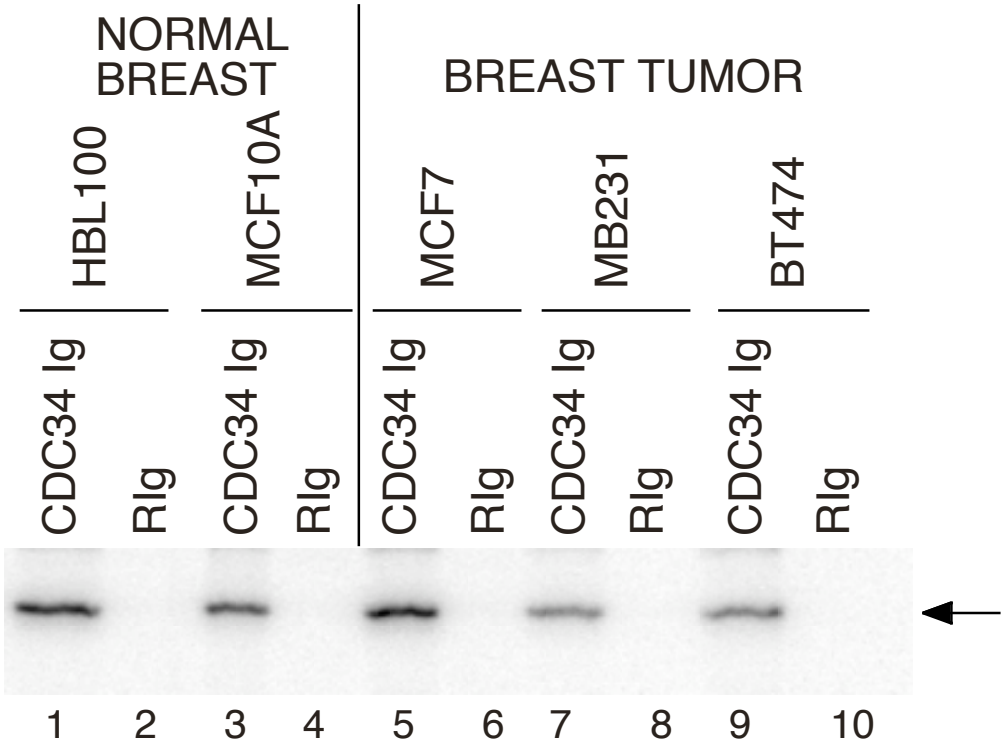

Figure 2. Immunopurified Cdc34 from breast cancer cell lines does not show an increased phosphorylation. Cdc34 was immunoprecipitated with affinity purified Cdc34 antibody (CDC34 Ig) or non-immune rabbit antibody (RIg) followed by an in vitro kinase assay, followed by another immunoprecipitation (IP-kinase-IP) in normal breast cell lysates (lanes 1-4) or established breast cancer cell lysates (lanes 5-10). The arrow indicates phosphorylated Cdc34 protein.

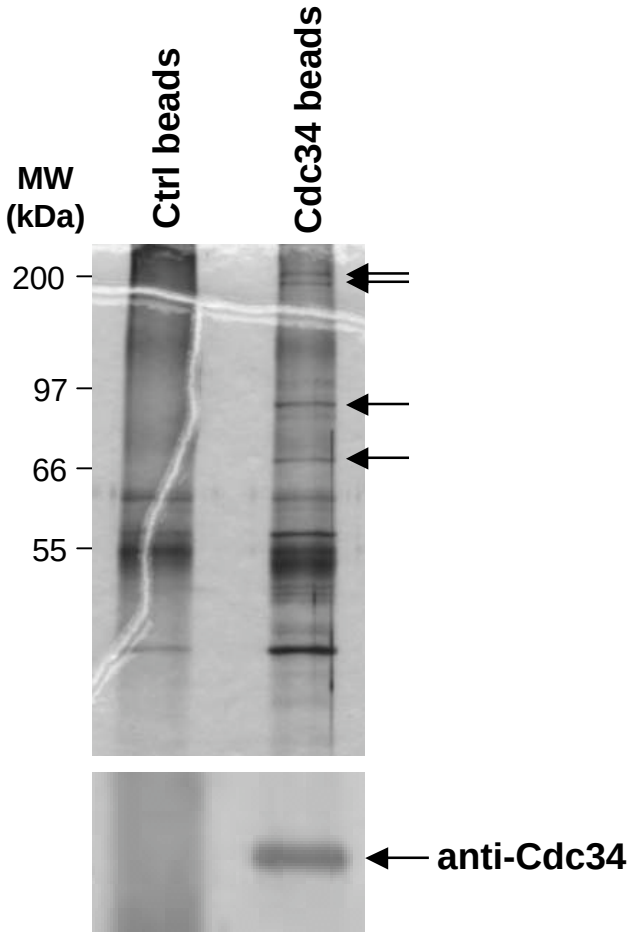

Figure 3. Immunoprecipitation of Cdc34 from S100 (cytosolic fraction of Xenopus egg extract) indicating putative Cdc34 associated proteins. (Top panel) Normal rabbit serum coupled beads (Ctrl beads) or affinity purified Cdc34 antibody coupled beads (Cdc34 beads) were incubated in S100, washed with 500mM KCl buffer and then eluted with glycine. Samples were resolved on SDS-PAGE and silver stained. Arrows indicate the putative Cdc34 associated proteins. (Bottom panel) Anti-Cdc34 western blot of the eluted material with arrow indicating the Cdc34 protein band.

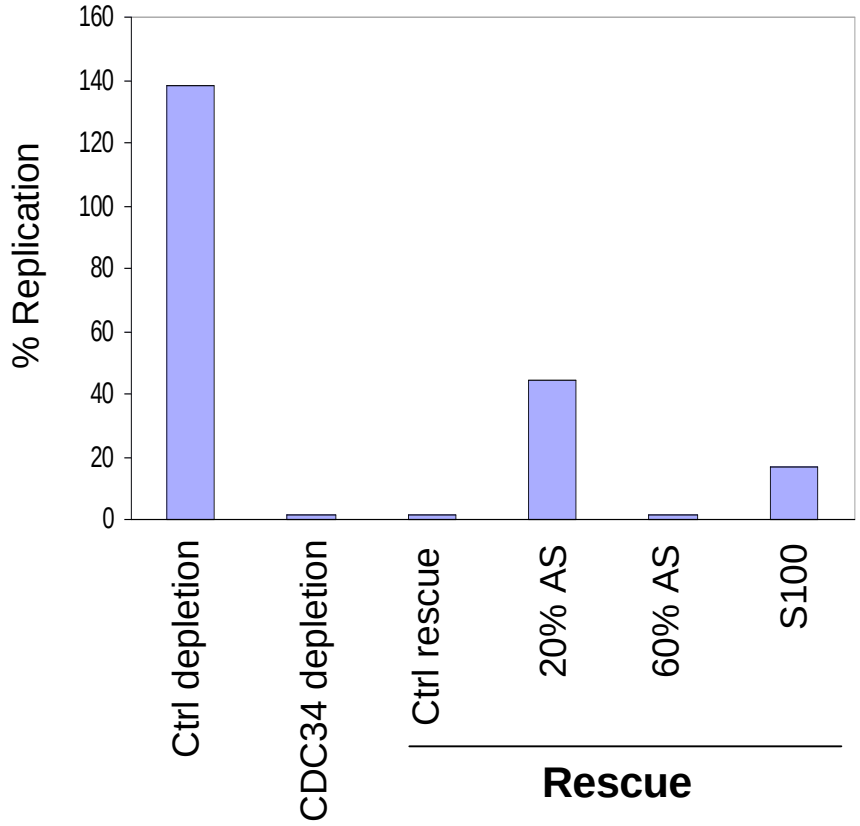

Figure 4. Replication rescue activity is observed in 20% ammonium sulfate precipitate. Depletion-rescue assay where Xenopus egg extracts (LSS) were either control depleted (ctrl depletion) or depleted with anti-Cdc34 beads (CDC34 depletion). Xenopus Sperm Chromatin (XSC) was added to the depleted LSS and allowed to replicate with values indicated in % replication of the input template. For rescue, anti-Cdc34 beads were incubated with 20 % ammonium sulfate (AS), 60 % ammonium sulfate (AS) and S100 (starting material). The beads were washed, mixed with depleted LSS and XSC, and allowed to replicate. For control (ctrl) rescue, control beads were incubated with LSS.

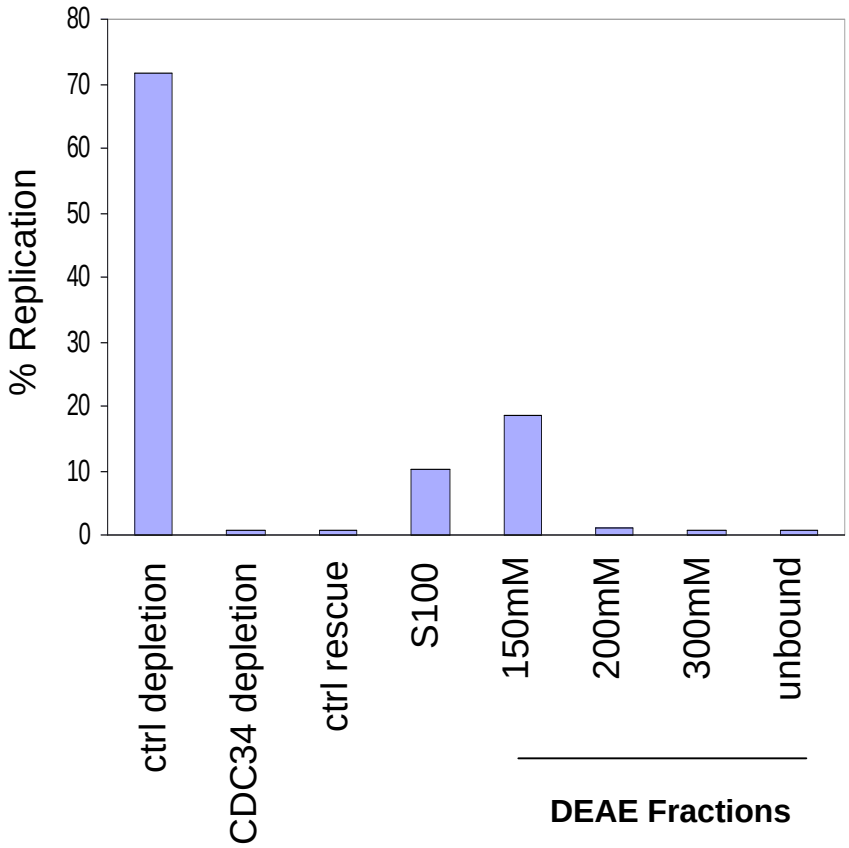

Figure 5. Replication rescue activity is observed in low salt elution of DEAE column purification. S100 was applied over a DEAE column at a salt concentration of 50mM potassium chloride (KCl) and the flow through was collected as unbound fraction. The proteins were then eluted using buffers with 150, 200, and 300mM KCl.The collected fractions were then used in a depletion-rescue assay where Xenopus egg extract (LSS) was either control depleted (ctrl depletion) or depleted with anti-Cdc34 beads (CDC34 depletion). Xenopus Sperm Chromatin (XSC) was added to the depleted LSS and allowed to replicate with values indicated in % replication. For rescue, anti-Cdc34 beads were incubated with S100, 150mM KCl elution, 200mM KCl elution, 300mM KCl elution, and the unbound fraction. The beads were washed and to them depleted LSS and XSC were added and allowed to replicate. For the control (ctrl) rescue, control beads were incubated with LSS. Rescue activity was seen in the starting material (S100) and in the 150mM KCl elution. No activity was detected in the high salt elutions or in the unbound fraction.

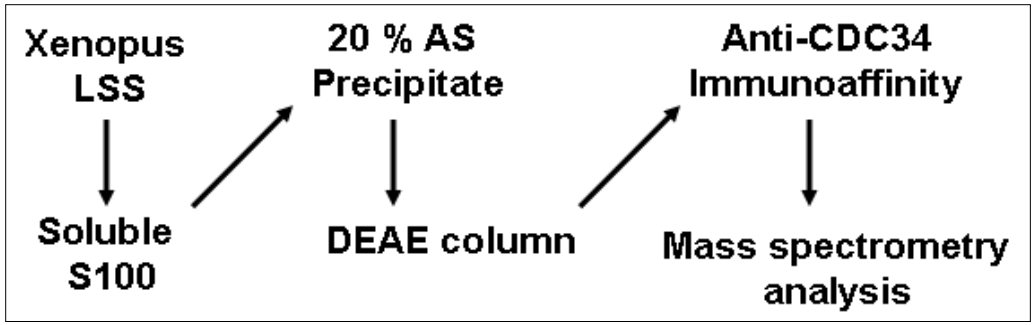

Figure 6. Cdc34 complex purification procedure. Purification scheme to purify Cdc34 high molecular weight complex required for initiation of DNA replication in Xenopus Interphase egg extracts.

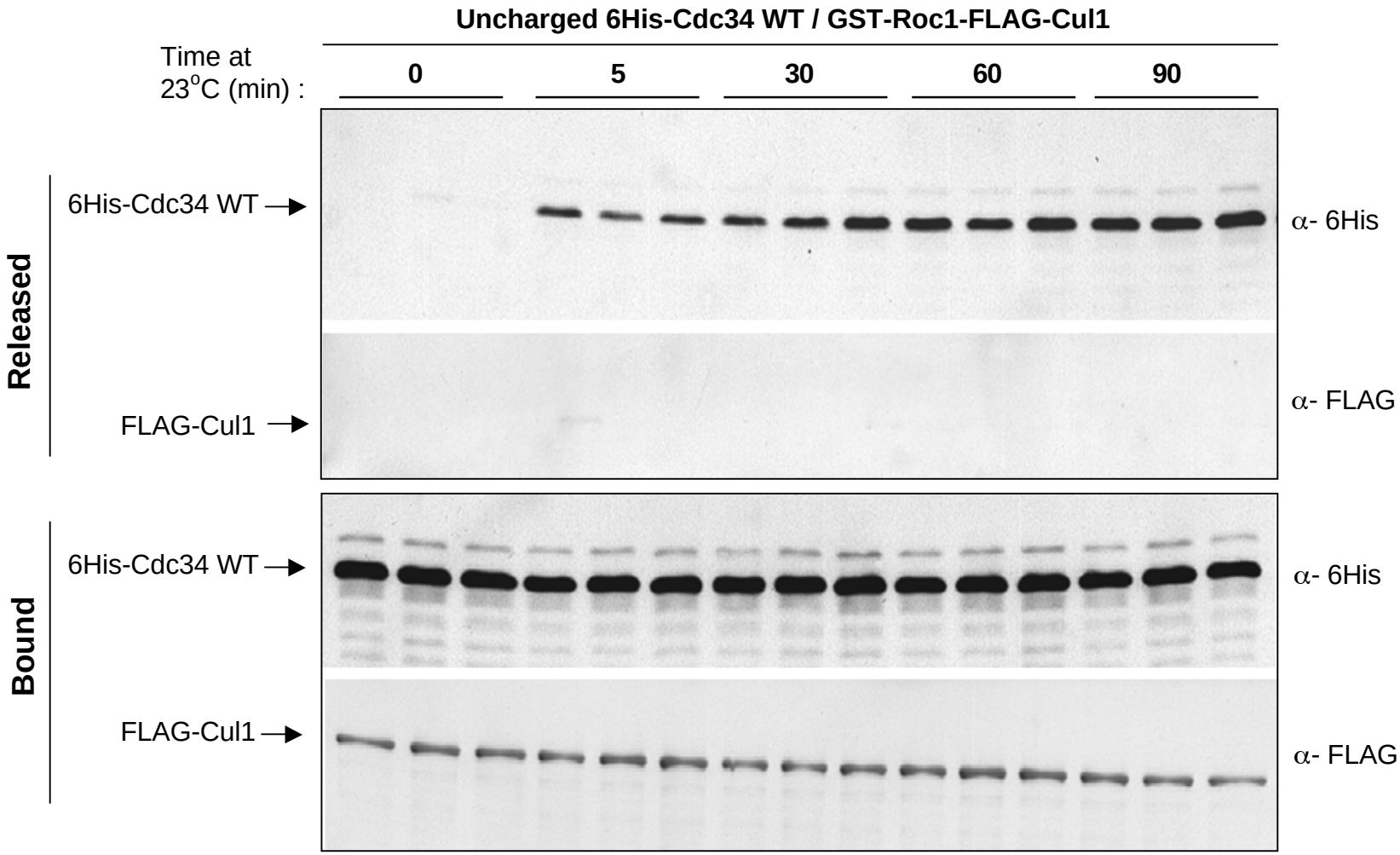

Figure 7. Uncharged human Cdc34 WT can dissociate from Cul1-Roc1 complex. 6His-Cdc34 WT in the absence of Ub_{mix} (E1, ATP and ubiquitin) was bound to Flag-Cul1-GST-Roc1 immobilized on GST Sepaharose 4B beads at 23° C. The beads were washed and incubated in buffer without the Ub_{mix} for indicated times. The beads (bottom panel, Bound) were separated from the supernatants (top panel, Released) followed by SDS-PAGE and immunoblotting for 6His and FLAG tags on Cdc34 and Cul1 respectively. For released fractions, 75 % of total supernatant and for bound fractions, 10 % of total bead bound sample were loaded. The assay was performed in triplicate for each time point.

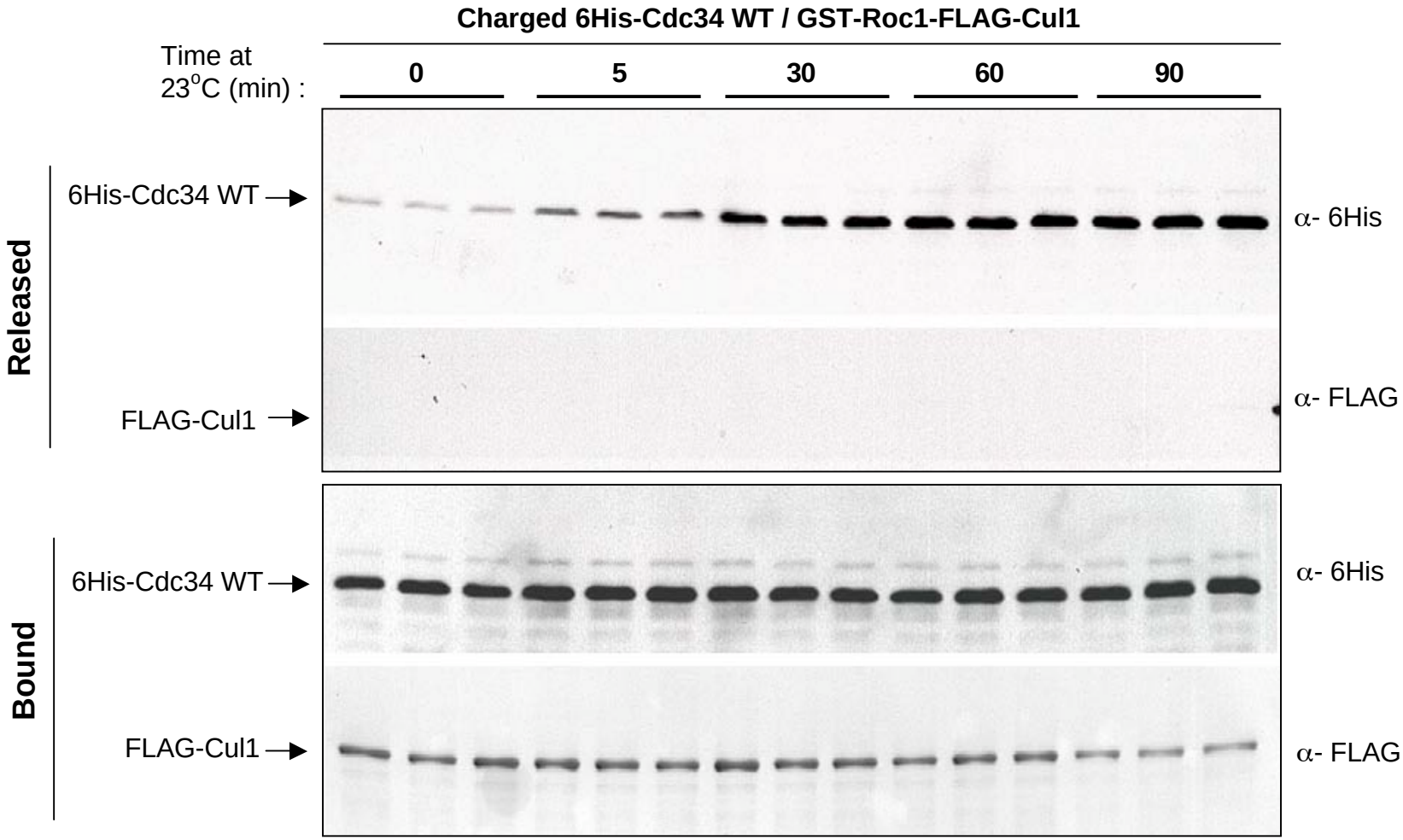

Figure 8. Charged human Cdc34 WT can dissociate from Cul1-Roc1 complex. 6His-Cdc34 WT in the presence of Ub_{mix} (E1, ATP and ubiquitin) was bound to Flag-Cul1-GST-Roc1 immobilized on GST Sepaharose 4B beads at 23° C. The beads were washed and incubated in buffer with the Ub_{mix} for indicated times. The beads (bottom panel,Bound) were separated from the supernatants (top panel,Released) followed by SDS-PAGE and immunoblotting for 6His and FLAG tags on Cdc34 and Cul1 respectively. For released fractions, 75% of total supernatant and for bound fractions, 10% of total bead bound sample was loaded. The assay was performed with triplicates for each time point.

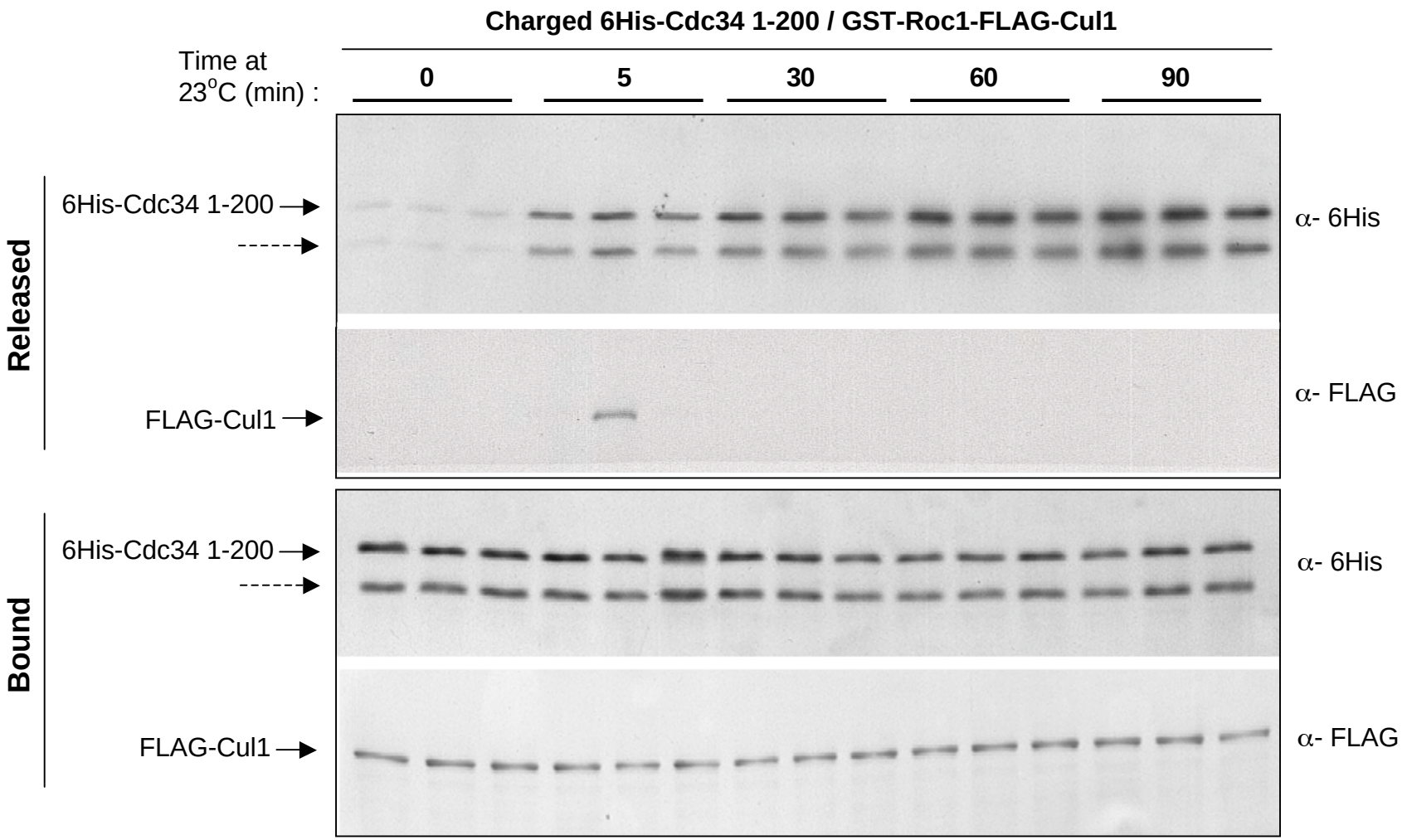

Figure 9. Charged human Cdc34 1-200 can dissociate from Cul1-Roc1 complex. 6His-Cdc34 1-200 in the presence of Ub_{mix} (E1, ATP and ubiquitin) was bound to Flag-Cul1-GST-Roc1 immobilized on GST Sepaharose 4B beads at 23° C. The beads were washed and incubated in buffer with the Ub_{mix} for indicated times. The beads (bottom panel, Bound) were separated from the supernatants (top panel, Released) followed by SDS-PAGE and immunoblotting for 6His and FLAG tags on Cdc34 1-200 and Cul1 respectively. For released fractions, 75% of total supernatant and for bound fractions 10% of total bead bound sample were loaded. The assay was performed in triplicate for each time point. The broken arrows indicate a degradation product observed in our purification of Cdc34 1-200 from bacteria.

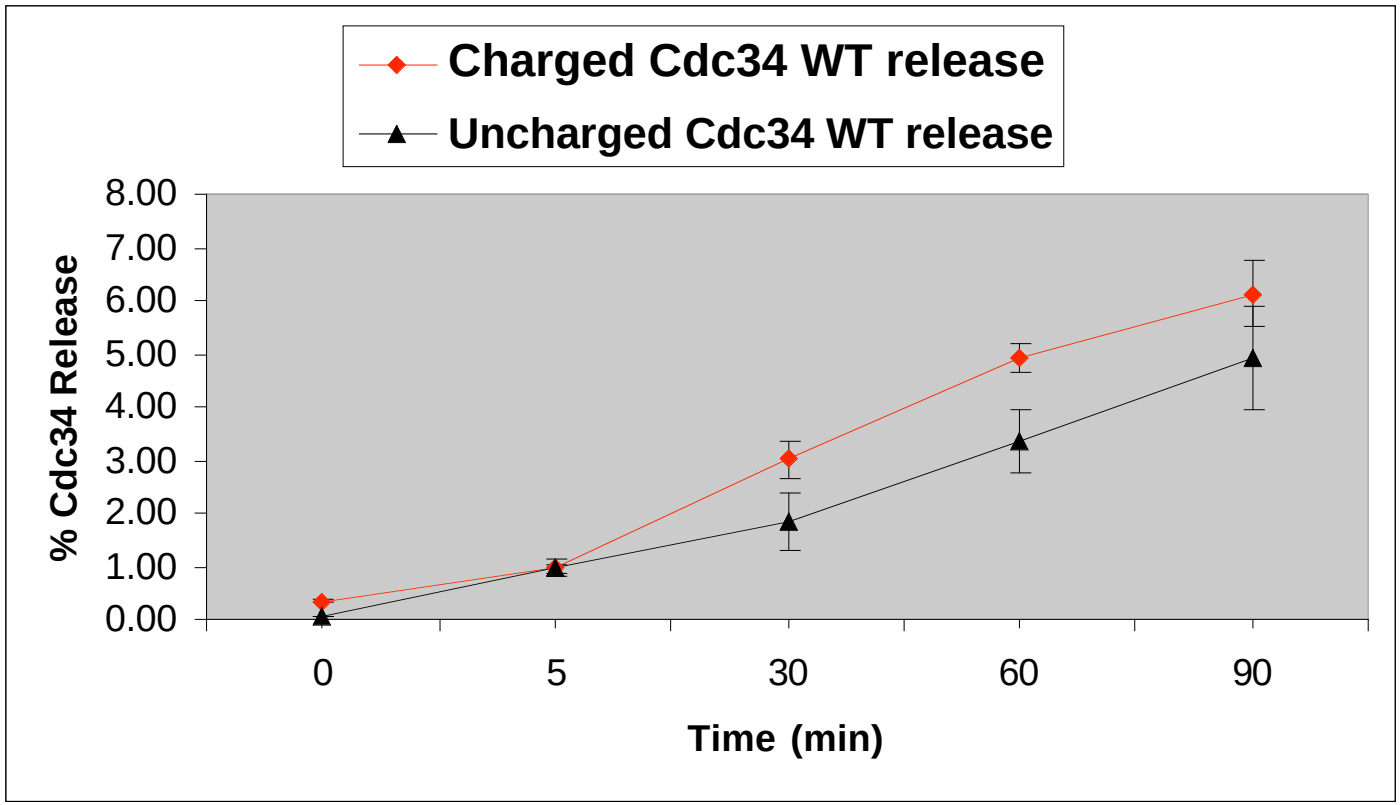

Figure 10. Uncharged and charged human Cdc34 WT do not differ significantly in their dissociation from Cul1-Roc1 complex. Images from α-6His chemiluminescence blots (Figures 7 and 8) were acquired on Kodak Image Station 2000R. Analysis was done by quantitating the net intensities of released uncharged Cdc34 WT (triangles) and charged Cdc34 WT bands (diamonds). The percent Cdc34 released was determined relative to the 0 minute bound Cdc34 which was normalized to 100%.

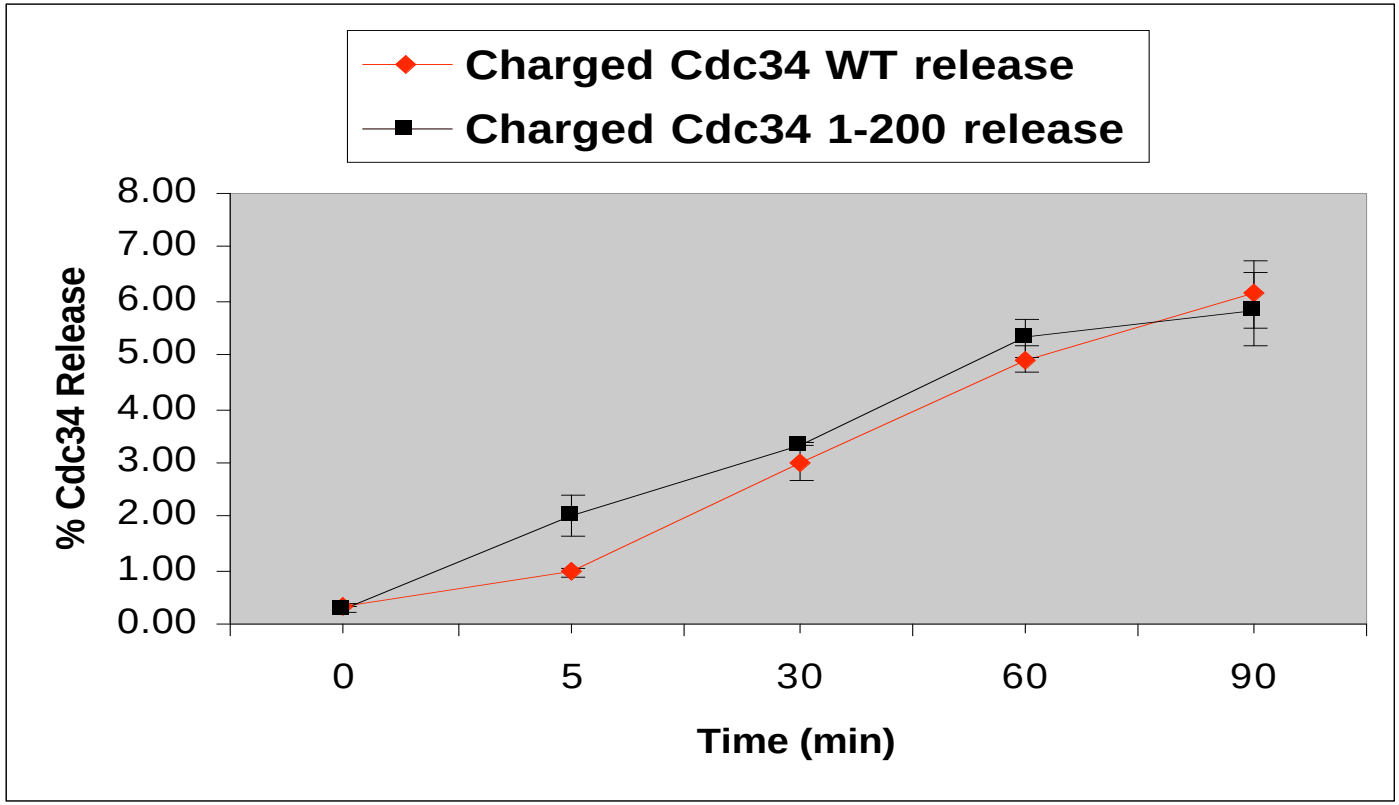

Figure 11. Charged human Cdc34 WT and human Cdc34 1-200 have similar dissociation dynamics from Cul1-Roc1 complex. Images from α-6His chemiluminescence blots (Figures 8 and 9) were acquired on Kodak Image Station 2000R. Analysis was done by quantitating the net intensities of released charged Cdc34 WT (diamonds) and charged Cdc34 1-200 bands (squares). The percent Cdc34 released was determined relative to the 0 minute bound Cdc34 which was normalized to 100%.

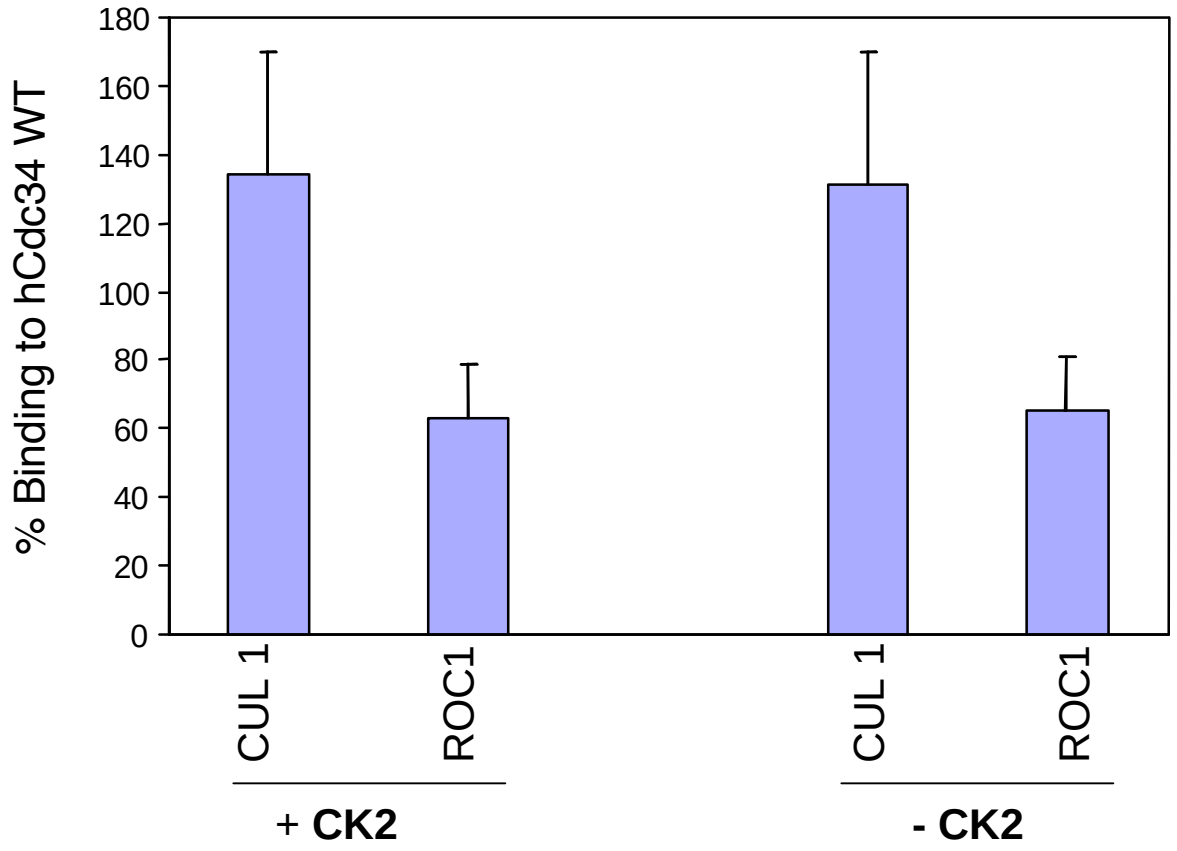

Figure 12. Phosphorylation of Cdc34 by CK2 does not appear to influence the interaction of Cdc34 with CUL1 and ROC1. *In vitro* Kinase and Co-Immunoprecipitation assay. Purified 6His human Cdc34 wild type (hCdc34 WT) was incubated first with Casein Kinase 2 (CK2) (left) or with control buffer (right) and then with co-in vitro translated ^{35}S methionine labeled CUL1-ROC1. The samples were then immunoprecipitated with Cdc34 antibody. Samples were analyzed by SDS-PAGE and phosphoimager analysis. Results are shown as percentage of Cdc34 bound to CUL1 or to ROC1.

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Report

The Acidic Tail Domain of Human Cdc34 is Required for $p27^{Kip1}$ Ubiquitination and Complementation of a cdc34 Temperature Sensitive Yeast Strain

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ABSTRACT

Human Cdc34 is an ubiquitin conjugating enzyme or E2 that ubiquitinates substrates including $p27^{Kip1}$, IκBα, Wee1, and MyoD. Cdc34 possesses a core catalytic domain encoding the active site cysteine and an acidic tail domain within the carboxyl terminal 36 amino acids. Studies suggest that Cdc34 is phosphorylated in mammalian cells at 5 potential residues within the tail domain. In order to study the biological significance of the Cdc34 acidic tail domain and the possible significance of phosphorylation within this region, we tested the ability of human Cdc34 mutants to complement the *cdc34–2* temperature sensitive (ts) strain of *Saccharomyces cerevisiae*. Our studies indicated that complementation of the *cdc34–2* ts strain was critically dependent upon the carboxyl-terminal 36 amino acids of human Cdc34, but did not require phosphorylation of human Cdc34 residues S203, S222, S231, T233, and S236. Further studies demonstrated that although a Cdc34 mutant bearing a deletion of the C-terminal 36 amino acids (Cdc34 1–200) was efficiently charged with ubiquitin by E1, it was severely reduced for the ability to ubiquitinate $p27^{Kip1}$ in vitro compared to wildtype Cdc34. Both in vivo and in vitro binding studies indicated that Cdc34 1–200 bound to the E3-SCF components, Cul1 and Roc1, at levels comparable to the wildtype Cdc34. These studies suggest that the 36 amino acid acidic tail domain of human Cdc34 is critical for its ability to transfer ubiquitin to a substrate and is dispensable for the association of Cdc34 with Cul1 and Roc1. We postulate that the tail domain of Cdc34 may be important for its efficient dissociation from Cul1 and Roc1, an essential requirement for ubiquitination by the budding yeast Cdc34p, or it may be required more directly for ubiquitin transfer to the substrate.

INTRODUCTION

Eukaryotic cells require ubiquitin-mediated protein degradation to maintain proper cell cycle progression and homeostasis.[1,2] The regulated polyubiquitination of a substrate protein is dependent upon an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2 or UBC), and often, an ubiquitin protein ligase (E3).[3] Cdc34 is an ubiquitin conjugating enzyme (UBC3) that is required for the G_1 to S phase transition in *Saccharomyces cerevisae* and the initiation of DNA replication in *Xenopus laevis* interphase egg extracts.[4-6] Studies in budding yeast have demonstrated that the human *CDC34* gene can functionally complement a *cdc34* temperature sensitive (ts) strain at the non-permissive temperature.[7]

Structurally, human Cdc34 is comprised of an amino-terminal catalytic domain encoding the active site cysteine and a carboxyl-terminal acidic tail domain. In both budding yeast and in mammals, Cdc34 has been shown to function in association with the E3 called SCF.[8] The SCF is comprised of an F-box binding protein called Skp1, an F-box protein which binds the substrate, a cullin protein which serves as a scaffold, and a RING finger protein called Roc1/Rbx1/Hrt1 which helps to recruit the E2.[8-10] Cdc34 and SCF^{Skp2} have been shown to mediate the in vitro polyubiquitination of the mammalian cyclin-dependent kinase (CDK) inhibitor, $p27^{Kip1}$.[11-14] The modeled three dimensional crystal structure of the human SCF^{Skp2} complex suggests that Cul1 and Roc1 recruit the E2 and position its active site cysteine optimally for ubiquitin transfer to the Skp2-bound substrate.[10] In addition, past studies have indicated that residues 195–208 of human Cdc34 were required for a stable interaction with Cul-Roc1, while residues 209-236 enhanced this interaction significantly and were required for maximal binding to Cul1-Roc1.[15] These results were based on in vitro binding studies of human Cdc34 with Cul1-Roc1 and on in vitro substrate-independent polyubiquitin chain assembly (or

Cdc34 auto-ubiquitination) studies.[15] Recent studies in budding yeast have also demonstrated that the critical step in the ubiquitination of the yeast CDK inhibitor, $p40^{Sic1}$, is actually the efficient dissociation of yeast Cdc34p from SCF^{Cdc4}.[16] These studies indicated that both charged and uncharged Cdc34p efficiently associated with the SCF, but only charged Cdc34p efficiently dissociated from the SCF and supported the ubiquitination of $p40^{Sic1}$.[16]

Our past studies have suggested that human Cdc34 is a phosphoprotein in mammalian cells that is phosphorylated by Casein Kinase 2 (CK2).[17] CK2 is a constitutive serine-threonine kinase that is essential for cell cycle progression in both budding yeast and mammalian cells.[18-22] The in vivo phosphorylation sites of human Cdc34 are localized within the carboxyl-terminal 36 amino acids at five potential CK2 consensus sites (S203, S222, S231, T233, S236).[17] Alanine mutation of these five residues eliminated all in vivo phosphorylation of Cdc34 in mammalian cells and shifted the cellular localization of Cdc34 from the nucleus to the cytoplasm.[17] This suggested that Cdc34 phosphorylation by CK2 might play a role in modulating Cdc34 cell localization and function. Previous studies have demonstrated a role for CK2-mediated phosphorylation in promoting the nuclear localization of SV-40 large T antigen.[23] Presumably, the phosphorylation status of human Cdc34 may alter its association with proteins that regulate the subcellular localization of Cdc34. The subcellular localization of Cdc34 is normally predominantly nuclear, but the Cdc34 primary sequence does not reveal any obvious nuclear localization sequence consensus sites.[17]

Although the residues phosphorylated in human Cdc34 are not conserved in budding yeast Cdc34p, we wanted to address whether phosphorylation of C-terminal residues within human Cdc34 or the acidic tail domain in general are required for complementation of a *cdc34* ts budding yeast strain. Our results demonstrate that while phosphorylation at five CK2 consensus sites appears to be dispensable, the carboxyl-terminal 36 amino acids of human Cdc34 are essential for complementation of a *cdc34-2-ts* budding yeast strain. Importantly, this acidic tail domain of Cdc34 is also required for the efficient in vitro ubiquitination of $p27^{Kip1}$ by SCF^{Skp2}. In order to understand the defect of Cdc34 bearing a deletion of the carboxyl-terminal 36 amino acids (1–200), we examined its ability to be charged by E1 and to bind the SCF. Our results demonstrate that the Cdc34 1-200 mutant is charged by E1, and contrary to past studies, also efficiently interacts with the SCF components, Cul1 and Roc1, both in vivo and in vitro. This suggests that the ubiquitination defect of human Cdc34 deleted of the C-terminal tail domain functionally lies after its association with the SCF. We postulate that the C-terminal 36 amino acids of human Cdc34 may be required for its efficient dissociation from the SCF or may instead be more directly required for the transfer of ubiquitin from Cdc34 to the substrate.

MATERIALS AND METHODS

Cell culture, transfection and preparation of whole-cell extracts. Human osteosarcoma U2OS cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 units/ml of penicillin and 50 ug/ml streptomycin sulfate at 37°C with 5% CO_2. Cells were transiently cotransfected with either pCS2+/2HA-Cdc34 or pCS2+/2HA-Cdc34(1–200) as well as pcDNA3.1/Cul1-HA and pCS2+/Myc-Roc1 using the *N,N*-bis(2-hydroxyethyl)-2-amino-ethanesulfonic acid (BES)-based calcium phosphate precipitation method as previously described.[17] Forty hours post-transfection, untransfected or transfected whole-cell extracts were prepared as described previously.[24] Briefly, cells were resuspended in ice-cold NETN buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl pH 7.5, and 0.1 % NP40) supplemented with protease inhibitor cocktail (Sigma), and then subjected to three freeze/thaw cycles. Insoluble debris was removed by centrifugation at 14,000 x g for 15 min at 4°C. Total protein concentrations were determined by Bradford assay (Bio-Rad).

Plasmid construction. Cdc34 WT and Cdc34 mutants were cloned into pQE30 (Qiagen) for bacterial expression of amino-terminally tagged proteins as previously described.[6,17] pCS2+/Myc-Roc1 was generated by subcloning a BamHI-XbaI Myc-tagged Roc1 fragment into pCS2+. pcDNA3.1/Cul1-HA was a generous gift from A. Pause.

Recombinant protein expression and purification. Hexahistidine-tagged proteins were expressed from pQE30 and purified using Nickel-nitrilotriacedic acid Sepharose according to manufacturer's instructions (Qiagen) as previously described and were extensively dialyzed into 1X PBS before use in binding assays.[6,17] Purified SCF^{Skp2} and CDK2-cyclin E were generated as previously described.[12,14,25]

E2 charging reaction. The reaction mixture contained the following in a volume of 10 µl: 50 mM Tris-HCl pH 7.6, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 0.5 mM ATP, 5 µM biotinylated ubiquitin, 100 ng E1 and 500 ng Cdc34 (wildtype or mutants). After incubation at 37°C for 0, 15, 30 or 60 minutes, the reaction was stopped by the addition of sample buffer without β-mercaptoethanol. Samples were subjected to SDS-PAGE and analyzed by immunoblotting with Horseradish Peroxidase (HRP)-streptavidin.

p27 ubiquitination assay. The reaction mixture contained the following in a volume of 10 µl: 40 mM Tris-HCl pH 7.6, 5 mM $MgCl_2$, 1 mM DTT, 10% (*v*/*v*) glycerol, 2 µg/µl BSA, 10 mM phosphocreatine, 100 µg/µl creatine phosphokinase, 0.5 mM ATP, 1 µM ubiquitin aldehyde, 1 mg/ml methylated ubiquitin, 10 ng/µl E1, 150 ng/µl Cdc34 WT or mutants, 20 ng/µl SCF^{Skp2}, 4 ng/µl CDK2-cyclin E, and 0.3 µl [^{35}S]-p27 as described.[25] After incubation at 30°C for 0, 15, 30, or 60 minutes, samples were subjected to SDS-PAGE and autoradiography.

Antibodies, immunoblotting, in vitro coimmunoprecipitation assay, and immunoprecipitation (IP)-Western assay. Anti-Cdc34 antibody was generated as previously described and used following affinity purification for immunoblotting at a final concentration of 1.2 µg/ml.[6] Immunoblots were performed as previously described.[17] In vitro coimmunoprecipitation assay: In a 20 µl reaction volume, 5 µl of cotranslated [^{35}S]-methionine-labeled HA-Cul1/Roc1 (NEN, TNT Quick Coupled Kit, Promega) was added to 2.5 µg of recombinant 6xHis-tagged Cdc34 protein. Samples were allowed to bind at 30°C for 1 hr followed by a preclearing step with 20 µl protein A-sepharose CL-4B beads (Amersham Biosciences). The lysates were then immunoprecipitated with 2 µl anti-Cdc34 rabbit serum or normal rabbit serum (NRS) (Sigma) and 25 µl protein A sepharose beads. After incubation, the protein A sepharose beads were washed with RIPA buffer (100 mM Tris-Cl pH 8, 10 mM EDTA, 150 mM NaCl, 1% Nonidet P-40), the beads were aspirated and boiled in 1X sample buffer, and the samples were resolved by SDS-PAGE. Five percent ($1/20^{th}$) of the input [^{35}S]-labeled protein was included on the gel to aid in the quantitation of the bound fractions. Quantitation of the bound fractions was determined by Phosphorimager analysis using ImageQuant software (Molecular Dynamics). Background binding (binding to beads immunoprecipitated with NRS) was subtracted from the values for the bound fractions. The percentage of Cdc34 bound to Cul1 and Roc1 was then determined based on the 5% input value for Cul1 and Roc1. These values ranged between 0.76%-1.63% binding of WT Cdc34 to Cul1 and 0.42%-0.9% binding of WT Cdc34 to Roc1. The binding for WT Cdc34 was then normalized to 100% and the binding for the mutant Cdc34 samples was normalized relative to the WT Cdc34. The Standard Error of the Mean was then calculated for each set of samples and was included as error bars on the graph in Figure 3B. The recovery of equivalent amounts of Cdc34 protein (WT or mutants) was confirmed by examining a fraction of the total sample by coomassie blue staining. Data was used only from experiments where the Cdc34 samples were recovered equivalently. In Figure 3B, the data for Cul1 binding was the mean of eight experiments for WT Cdc34, eight experiments for Cdc34 1-200, seven experiments for 5 PT MUT A, and six experiments for 5 PT MUT E. The data for Roc1

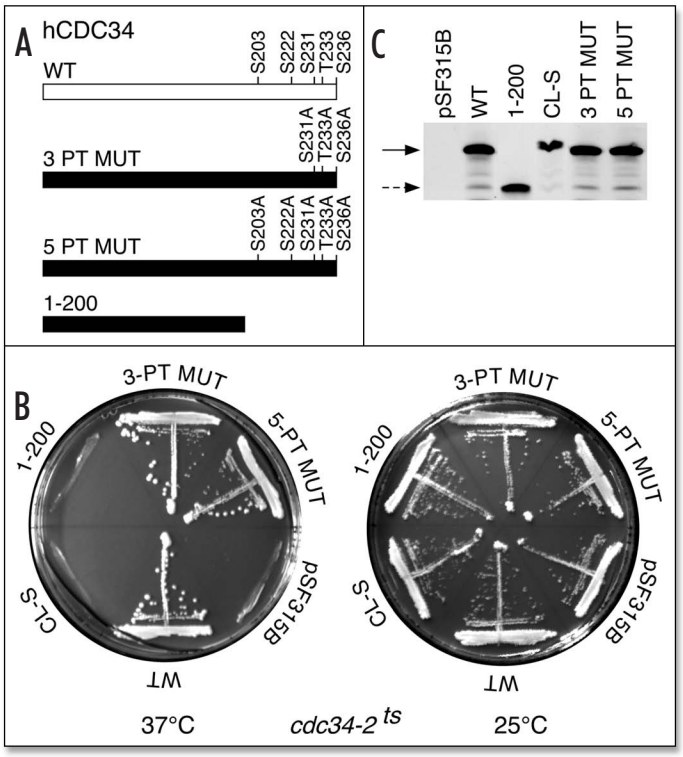

Figure 1. The C-terminal 36 amino acids of human Cdc34 are essential for complementation of the budding yeast *cdc34-2 ts* strain. (A) Schematic representation of the CK2 phosphorylation sites in the Cdc34 wild-type protein (WT), the point mutations of the Cdc34 3 point mutant (3 PT MUT), the point mutations of the Cdc34 5 point mutant (5 PT MUT), and the truncation mutation of Cdc34 that deletes the C-terminal 36 amino acids (1-200). (B) Complementation studies. Temperature sensitive *cdc34-2* strain was transformed with vector alone (pSF315B), wildtype human Cdc34 (WT), an active site cysteine mutant (C93S, L97S) of Cdc34 (CL-S), a deletion mutant of Cdc34 removing the C-terminal 36 amino acids (1-200), a triple point mutant (S231A, T233A, S236A) of Cdc34 (3-PT MUT), and a quintuple point mutant (S203A, S222A, S231A, T233A, S236A) of Cdc34 (5-PT MUT). Transformed yeast *cdc34-2* strains were streaked onto leucine (-), galactose (+) plates and grown at nonpermissive (37°C, left) and permissive (25°C, right) temperatures. (C) Immunoblot of human Cdc34 in yeast ts strain *cdc34-2*. Whole cell lysates from yeast ts strain *cdc34-2* transformed with vector (pSF315B), wildtype human Cdc34 (WT), Cdc34 1-200 mutant (1-200), Cdc34 active site cysteine mutant (CL-S), Cdc34 triple point mutant (3 PT MUT), or Cdc34 quintuple point mutant (5 PT MUT) were analyzed by SDS-PAGE followed by immunoblotting using an antibody against human Cdc34. The samples were normalized by measuring the O.D. of the yeast culture and loading equivalent O.D. units (volume of culture multiplied by O.D. of culture) for each sample. The solid arrow indicates the full-length human Cdc34 protein band and the broken arrow indicates the truncated Cdc34 protein band for mutant 1–200.

binding was the mean of seven experiments for WT Cdc34, seven experiments for Cdc34 1-200, six experiments for 5 PT MUT A, and five experiments for 5 PT MUT E.

IP-Western assay. Whole-cell extracts (1 mg/0.5 ml) from transfected U2OS cells were first precleared by incubation with 20 µl of protein A-Sepharose beads (Amersham Biosciences) at 4°C for 40 min. Supernatants were then incubated with 20 µl of anti-cMyc agarose beads (Sigma) at 4°C for 2 hr. Beads were washed seven times with ice-cold NETN buffer. The bead-bound immune complexes were eluted with 1X sample buffer and analyzed by SDS-PAGE. For Western blotting, proteins were transferred to a PVDF membrane (Millipore), probed with mouse anti-HA ascites (Covance) or mouse anti-Myc (9E10) antibodies (Santa Cruz), and detected by ECL (Amersham Biosciences).

Complementation studies in *Saccharomyces cerevisiae* and extract preparation. The human *CDC34* wildtype and mutants were cloned into pSF315B, a derivative of pRS315 for complementation studies using two different *cdc34-2* temperature sensitive strains which gave similar results: SJ1098-3d (MATa cdc34-2 leu2-3 ura3 trp1), a generous gift from S. Plon and B. Byers, and L6204 (MATa cdc34-2 leu2^2 ura3-52 gcn4^1 ade8-GCN4), a generous gift from G. Fink.[26] pSF315B plasmids were transformed into yeast strains using lithium acetate and then were analyzed for growth at 24°C, 30°C and 37°C on leucine-minus minimal plates supplemented with galactose (L6204) or galactose and raffinose (SJ1098-3d) as described.[27] For extract preparation, cells were collected by centrifugation, washed in water, and lysed using lysis buffer containing 50 mM NaOH, 2% SDS, 10% glycerol, 5% β-mercaptoethanol. After vortexing, the samples were boiled, titrated to pH 7.0 using HCl, and centrifuged at 14K rpm for 5 min.

RESULTS

The carboxyl-terminal 36 amino acids of human Cdc34 are essential for complementation of a temperature sensitive budding yeast strain. Cdc34 protein from budding yeast and humans share ~41% amino acid identity with the highest homology exhibited within the amino-terminal catalytic domain surrounding the active site cysteine residue. Although little homology is observed in the carboxyl-terminal domains of yeast and human Cdc34, both proteins exhibit highly acidic tails. Our past studies suggest that human Cdc34 is phosphorylated in vivo by CK2 at five possible residues (S203, S222, S231, T233, and S236).[17] When these five residues of Cdc34 are mutated to alanine and examined in vivo, the subcellular localization of Cdc34 is shifted from the nucleus to the cytoplasm.[17]

In order to determine the role of the acidic tail domain of human Cdc34 and the possible role of phosphorylation within this domain, we tested the ability of several human Cdc34 mutants to complement the budding yeast ts strain, *cdc34-2*. Wildtype human Cdc34 (1–236), C-terminal truncation mutant 1-200, and point mutants disrupted at putative CK2 phosphorylation sites within the carboxyl-terminal domain by alanine mutation (three point mutant or 3 PT MUT: S231A, T233A, S236A and five point mutant or 5 PT MUT: S203A, S222A, S231A, T233A, S236A) were cloned into the yeast expression vector, pSF315B (Fig. 1A). At the nonpermissive temperature of 37°C, wildtype human Cdc34 complemented the growth defect of the *cdc34-2* ts strain as expected, while the active-site point mutant, CL-S (C93S, L97S), did not (Fig. 1B). In contrast to wildtype Cdc34, the Cdc34 1-200 truncation mutant was completely defective for complementation of the *cdc34-2* ts strain, while the Cdc34 3 PT MUT and 5 PT MUT complemented the *cdc34-2* ts strain to similar extents as wildtype (Fig. 1B). Immunoblot analysis demonstrated that the human Cdc34 wildtype and 1–200 proteins were expressed in the *cdc34-2* ts strain to similar levels indicating that the inability of the human Cdc34 1–200 to complement the *cdc34-2* ts strain was not merely due to an instability of the human 1–200 protein in yeast (Fig. 1C). This result demonstrates that the acidic tail domain of human Cdc34 is critical for its complementation function in yeast, while alanine mutation of human Cdc34 at residues 203, 222, 231, 233 and 236 does not disrupt the complementation function of human Cdc34. Because sequences within the tail domain of budding yeast Cdc34p have been shown to be important for its association with the SCF, we postulate that the human Cdc34 1-200 mutant may be compromised in its ability to associate with the yeast SCF to mediate the ubiquitination of $p40^{Sic1}$.[28]

The human Cdc34 truncation mutant, 1-200, is charged by E1. One trivial explanation for the inability of the human Cdc34 1–200 to complement the budding yeast *cdc34-2* ts strain is that this mutant may not be efficiently charged with ubiquitin by E1 compared to the wildtype protein. Previous studies have indicated that the acidic tail domain of human Cdc34 is not required for charging by E1, but to directly examine this possibility, we measured the efficiency of E1 and ATP to charge recombinant wildtype and mutant human Cdc34 proteins in a thiolester assay.[15] The results demonstrated that wildtype Cdc34, the Cdc34 1-200 truncation mutant, and Cdc34 point mutants (5 PT MUT A and 5 PT MUT E) were all efficiently

charged in the presence of E1 (Fig. 2A). This indicates that the defect of the Cdc34 1-200 mutant did not lie in its ability to be charged by E1.

The C-terminal 36 amino acids of Cdc34 are required for the ubiquitination of p27^{Kip1}. The inability of the human Cdc34 1–200 mutant to complement a budding yeast ts strain indicated that the carboxyl-terminal 36 amino acids of Cdc34 were critical for its ubiquitination function. Instead of pursuing the defect of the human Cdc34 1–200 mutant in yeast, we wanted to determine whether this Cdc34 mutant could support the ubiquitination of a known human substrate. In mammalian cells, the CDK inhibitor protein, p27^{Kip1}, inhibits CDK2-cyclins during the G_1 to S phase transition.[29-31] Before the onset of S phase, p27^{Kip1} is targeted for ubiquitination and degradation by the ubiquitination machinery, Cdc34 and the SCF^{Skp2}.[11,14,32] The polyubiquitination of p27^{Kip1} is dependent upon p27^{Kip1} phosphorylation on threonine 187 by CDK2-cyclin E and subsequent binding to the F-box protein, Skp2.[12,32-35]

To further study the role of the Cdc34 acidic tail domain, we tested the ability of wildtype and mutant Cdc34 proteins to ubiquitinate p27^{Kip1} in vitro in the presence of SCF^{Skp2}. For these studies, we examined the function of the Cdc34 truncation mutant missing the C-terminal 36 amino acids (1–200), as well as two point mutants at CK2 consensus phosphorylation sites (5 PT MUT A: S203A, S222A, S231A, T233A, S236A bearing alanine mutations to prevent phosphorylation, and 5 PT MUT E: S203E, S222E, S231E, T233E, S236E bearing glutamic acid mutations to mimic constitutive phosphorylation).[17] Using a reconstituted in vitro ubiquitination assay, our studies indicated that while the WT, 5 PT MUT A, and 5 PT MUT E Cdc34 proteins all supported p27^{Kip1} ubiquitination, the Cdc34 1–200 truncation mutant was significantly reduced for the ability to ubiquitinate p27^{Kip1} (Fig. 2B). Under the conditions used, the phosphorylation status of WT Cdc34 is unclear because although no CK2 was added to the assay, CK2 activity able to phosphorylate recombinant Cdc34 is present in rabbit reticulocyte lysate which is used in the assay to in vitro transcribe and translate p27^{Kip1} (data not shown). Nevertheless, the Cdc34 5 PT MUT A will not be phosphorylated, while the Cdc34 5 PT MUT E will partially mimic the negative charge of phosphorylation at these residues. The result suggests that the carboxyl-terminal 36 amino acids of human Cdc34 are critical for its ubiquitination function, but possible phosphorylation of serine and threonine residues within the acidic tail domain of Cdc34 at five potential CK2 sites is not required for, and does not appear to enhance, the ubiquitination of p27^{Kip1} in vitro.

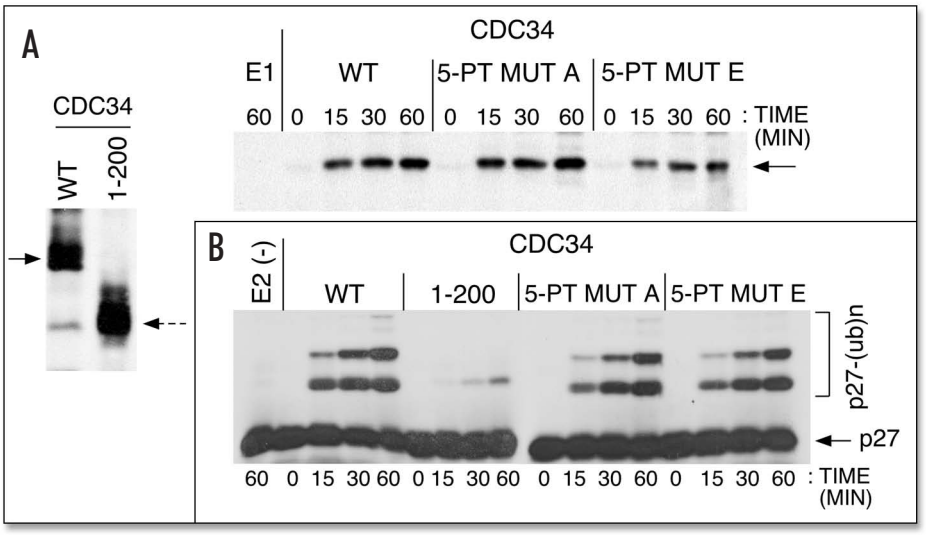

Figure 2. Truncation mutant Cdc34 1–200 is charged with ubiquitin by E1, but is significantly reduced for the ability to ubiquitinate p27^{Kip1}. (A) E2 charging reaction. Left panel: Recombinant wildtype (WT) or 1-200 mutant (1–200) Cdc34 proteins were incubated with E1, ATP, and biotin ubiquitin for 60 min and then analyzed by nonreducing SDS-PAGE and blotting with Streptavidin-HRP. The solid arrow indicates the charged WT Cdc34 and the broken arrow indicates the charged 1-200 Cdc34. Right panel: Recombinant wildtype Cdc34 (WT), Cdc34 quintuple alanine mutant (5-PT MUT A; S203A, S222A, S231A, T233A, S236A), and Cdc34 quintuple glutamic acid mutant (5-PT MUT E; S203E, S222E, S231E, T233E, S236E) were incubated with E1, ATP, and biotin ubiquitin for 0 to 60 min as indicated and then analyzed by nonreducing SDS-PAGE and blotting with Streptavidin-HRP. The solid arrow indicates the charged Cdc34 species. Lane 1 indicates the reaction containing E1 alone at 60 min (E1). (B) p27^{Kip1} in vitro ubiquitination reaction. ^{35}S-methionine labeled p27^{Kip1} was incubated in the ubiquitination reaction as described in the absence [E2 (-)] or presence of wildtype Cdc34 (WT), Cdc34 truncation mutant deleted of the C-terminal 36 amino acids (1-200), Cdc34 quintuple alanine mutant (5-PT MUT A; S203A, S222A, S231A, T233A, S236A), or Cdc34 quintuple glutamic acid mutant (5-PT MUT E; S203E, S222E, S231E, T233E, S236E). Samples were terminated at 0 to 60 min as indicated and analyzed by reducing SDS-PAGE. The solid arrow indicates the unconjugated p27^{Kip1} protein band and the bracket indicates the ubiquitinated p27^{Kip1} species [p27-(ub)n].

The C-terminal 36 amino acids of Cdc34 are dispensable for efficient binding to the SCF components, Cul1 and Roc1. In vitro binding studies of Cdc34 with Cul1 and Roc1 and Cdc34 auto-ubiquitination assays suggest that Cdc34 is recruited to the SCF through an interaction with a Cul1-Roc1 heterodimer.[15,36] More specifically, in vitro binding studies indicate that residues 195–236 of Cdc34 are required for maximal binding to GST-Roc1-Cul1$^{324-776}$.[15] This suggested to us that the Cdc34 1–200 defect in complementation and p27^{Kip1} ubiquitination might be attributable to an inability of the truncated Cdc34 to bind efficiently to the SCF through its association with Cul1-Roc1.

To study the binding efficiency of the Cdc34 1–200 mutant with Cul1 and Roc1, an in vitro binding assay was employed. Recombinant wildtype and mutant (1-200, 5 PT MUT A, and 5 PT MUT E) Cdc34 proteins (Fig. 3C, left panel) were incubated with cotranslated ^{35}S-labeled Cul1 and Roc1 (Fig. 3C, right panel). The samples were then immunoprecipitated with anti-Cdc34 antibody and the coprecipitated Cul1 and Roc1 were quantitated by Phosphorimager analysis. The binding results indicated that the Cdc34 1–200 mutant bound to Cul1 and Roc1 at a level similar to the WT Cdc34 protein, suggesting that the C-terminal 36 amino acids of Cdc34 containing acidic residues and the CK2 phosphorylation sites are not required for in vitro binding to Cul1 and Roc1 (Fig. 3A, B). This is contrary to published studies of Cdc34 binding to Cul1 and Roc1.[15] Paradoxically, the binding of the Cdc34 5 PT MUT A to Cul1 and Roc1 was reduced compared to WT Cdc34 and the Cdc34 1–200 mutant, while the binding of the Cdc34 5 PT MUT E to Cul1 and Roc1 was similar to WT Cdc34 (Fig. 3A, B). Based on our unpublished observations, it is likely that the recombinant WT Cdc34 protein is phosphorylated to some extent by the reticulocyte lysate used in the binding assay. However, it is unclear to what extent the Cdc34 protein may be phosphorylated. Supplementing the binding assay with recombinant CK2 did not appear to alter the binding of WT Cdc34 to Cul1 and Roc1 (data not shown). Therefore, we were not able to fully determine how phosphorylation of Cdc34 may affect its association to Cul1 and Roc1, but based on the result with the Cdc34 1–200 mutant, phosphorylation within the C-terminal tail domain of Cdc34 is not required for Cul1 or Roc1 binding. We postulate that alanine mutation of residues in the Cdc34 5 PT MUT A within the Cdc34 C-terminus may alter the inherent structure of Cdc34 and inhibit its binding to Cul1 and Roc1.

The discrepancy between our studies and previous studies may be due to several different factors. In previous studies, Wu et al. studied direct binding of recombinant Cdc34 to a heterodimer containing full-length recombinant Roc1 and a truncated form of Cul1 (amino acids 324–776).[15] In our studies, we use full-length Roc1 and Cul1 provided as an in vitro translation and

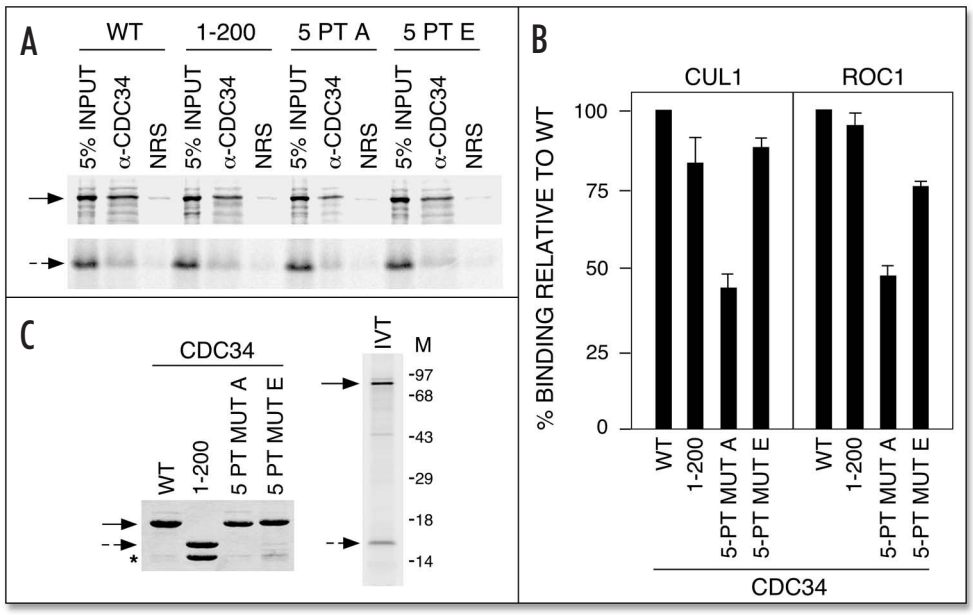

Figure 3. The C-terminal 36 amino acids of human Cdc34 are dispensable for in vitro binding to Cul1-Roc1. (A) In vitro coimmunoprecipitation assay. Recombinant wildtype Cdc34 (WT), Cdc34 truncation mutant deleted of the C-terminal 36 amino acids (1–200), Cdc34 quintuple alanine mutant (5 PT A; S203A, S222A, S231A, T233A, S236A), or Cdc34 quintuple glutamic acid mutant (5 PT E; S203E, S222E, S231E, T233E, S236E) were incubated with full-length in vitro cotranslated Cul1-Roc1 followed by immunoprecipitation with anti-Cdc34 antibody (α-CDC34) or normal rabbit serum (NRS). Five percent of the Cul1-Roc1 input is shown (5% INPUT). The solid arrow indicates the Cul1 protein band and the broken arrow indicates the Roc1 protein band. (B) Quantitation of in vitro coimmunoprecipitation assay. The percentage of Cul1 (left panel) or Roc1 (right panel) binding was determined relative to the wildtype Cdc34 which was normalized to 100% binding. The Standard Error of the Mean (SEM) was calculated and is represented by the error bars. For more details on quantitation, see Materials and Methods. (C) Left panel: Coomassie blue staining of gel showing the purified recombinant Cdc34 proteins used in the in vitro binding assay. The solid arrow indicates the full-length Cdc34 proteins, the broken arrow indicates the Cdc34 1-200 mutant protein, and the asterisk indicates a degradation product from the Cdc34 1-200 protein preparation. All proteins were normalized to the concentration of the full-length species. Right panel: In vitro cotranslated ^{35}S-methionine-labeled Cul1-Roc1 (IVT) used in the in vitro binding assay. The solid arrow indicates the Cul1 protein band and the broken arrow indicates the Roc1 protein band. Molecular weight markers are as indicated in kilodaltons (M).

therefore we cannot rule out the presence of other proteins from the reticulocyte lysate which may influence binding. To understand the Cdc34 requirements for Cul1 and Roc1 binding under more physiological conditions, we studied the binding of the WT and 1–200 Cdc34 proteins to Cul1 and Roc1 in mammalian cells. We cotransfected human U2OS cells with HA-tagged WT Cdc34 or mutant Cdc34 1–200 along with HA-tagged Cul1 and Myc-tagged Roc1. We then immunoprecipitated the transfected Roc1 using anti-Myc agarose beads and analyzed the beads for coprecipitated Cul1 and Cdc34 proteins by western blotting with anti-HA antibody (Fig. 4, right panel, lanes 5 and 6). The results indicated that equivalent amounts of WT and 1–200 Cdc34 proteins were coprecipitated with Cul1 and Roc1, suggesting that the C-terminal 36 amino acids of Cdc34 are not required for efficient in vivo binding to Cul1 and Roc1. These studies further indicate that the defect in the function of Cdc34 1–200 in complementing the budding yeast ts strain or in the ubiquitination of $p27^{Kip1}$ does not lie in its association with the SCF, but instead appears to lie in a step following the association of the SCF with Cdc34.

DISCUSSION

Our studies address the role of the *cdc34* ts strain, to ubiquitinate $p27^{Kip1}$, and to associate with the SCF components, Cul1 and Roc1. Our studies demonstrated that the C-terminal 36 amino acids were critical for complementation of a *cdc34* budding yeast ts strain and for the in vitro ubiquitination of $p27^{Kip1}$ by Cdc34- SCF^{Skp2}. In contrast, phosphorylation within the C-terminal 36 amino acids of Cdc34 at 5 potential CK2 sites was dispensable for complementation and $p27^{Kip1}$ ubiquitination. Contrary to past studies, our results indicated that the C-terminal 36 amino acids of Cdc34 were not required for efficient in vitro or in vivo binding of Cdc34 to the SCF components, Cul1 and Roc1, suggesting that the defect of the Cdc34 C-terminal truncation mutant called 1–200 lay in steps following the association of Cdc34 with the SCF.[15]

In budding yeast, studies have demonstrated that ubiquitin charging of Cdc34p is critical for its dissociation from the SCF^{Cdc4} which in turn is essential for efficient ubiquitination of $p40^{Sic1}$.[16] Although no studies have been performed to study a requirement for dissociation from the SCF for the ubiquitination function of human Cdc34, it is predicted that human Cdc34 may function in a manner similar to budding yeast Cdc34p. We have shown that the C-terminal 36 amino acids of human Cdc34 are not required for ubiquitin charging by E1, a result that is consistent with previous findings.[15] Our results also indicate that the C-terminal 36 amino acids of Cdc34 are not required for the association of Cdc34 with Cul1 and Roc1, both in vivo and in vitro. Therefore, given the significant reduction in the ability of the Cdc34 mutant, 1–200, to ubiquitinate $p27^{Kip1}$ compared to WT Cdc34, we postulate that the C-terminal 36 amino acids of Cdc34 may be important for the efficient dissociation of human Cdc34 from the SCF. Current studies are being conducted to address this possibility. If the tail domain of human Cdc34 is not found to be required for its efficient dissociation from the SCF, then this may indicate that the C-terminal 36 amino acids of Cdc34 may instead play an important direct role in ubiquitin transfer to the substrate. It is possible that the acidic C-terminal residues of Cdc34 contact the basic lysine residues of the substrate and form a transient association that is important for proper ubiquitin transfer to the substrate. Because we have not studied the ubiquitination of other Cdc34 substrates, we cannot exclude the possibility that the defect of the Cdc34 1–200 mutant may be specific to only $p27^{Kip1}$, but we predict that the defect of Cdc34 1–200 will be generally observed with other Cdc34 substrates.

Studies have indicated that residues 171 to 244 within the tail domain of budding yeast Cdc34p are important for the cell cycle function of Cdc34p, while residues 171 to 209 are necessary and sufficient for Cdc34p binding to the yeast SCF.[28,37-39] Our studies demonstrate that residues 201 to 236 within the tail domain of human Cdc34 are required to complement the cell cycle function of the budding yeast *cdc34* ts mutant strain. Although the tail domain of human Cdc34 is significantly shorter than the yeast Cdc34p tail domain and no amino acid sequence homology exists between the Cdc34 tail domains in yeast and humans, structural elements must

exist which can be satisfied by the acidic sequences of the human Cdc34 tail domain when expressed in budding yeast. The exact defect of the human Cdc34 truncation mutant, 1–200, in budding yeast is unclear. However, given the role of the yeast Cdc34 tail domain in SCF binding, it is possible that the human Cdc34 1–200 mutant does not efficiently bind to the yeast SCF to mediate the ubiquitination of Cdc34 substrates. The inability of the 1–200 mutant to complement the cell cycle defect of the *cdc34* ts strain suggests a failure of the human Cdc34 mutant to mediate the ubiquitination and degradation of the critical substrate of Cdc34 in yeast, the CDK inhibitor $p40^{Sic1}$.[40-44] It is also possible that the human Cdc34 1–200 mutant may efficiently bind to the yeast SCF^{Cdc4}, but may instead be defective in another step such as release from the yeast SCF.

Studies by Wu et al. have indicated that residues 170–194 of human Cdc34 are important for catalyzing the auto-ubiquitination of Cdc34 in the presence of E1 and that residues 209 to 236 are necessary for the formation of Roc1-Cul1-dependent ubiquitin polymers.[15] A Cdc34 mutant (1–208) could mediate the assembly of multi-ubiquitin chains, but at an efficiency 100 fold lower than wildtype Cdc34, while residues 195–208 of Cdc34 were essential for the generation of extensive Roc1-Cul1 polyubiquitin chains.[15] Our studies measured the ubiquitination of $p27^{Kip1}$, a physiological substrate of human Cdc34, in the presence of E1, Cdc34, and the SCF^{Skp2}, rather than examining the formation of polyubiquitin chains mediated by E1, Cdc34, and only the Roc1-Cul1 components of the SCF. Binding studies by Wu et al. suggested that residues 195 to 208 of human Cdc34 play a critical role in the association of Cdc34 with Roc1-Cul1 and that residues 209 to 236 of Cdc34 were required for optimal binding efficiency to the SCF.[15] Our studies indicated that removal of the C-terminal 36 amino acids of Cdc34 in the 1–200 mutant did not significantly influence the ability of Cdc34 to associate with Roc1-Cul1 in vitro, nor did it affect the binding of Cdc34 to the SCF in vivo. The differences observed in Cdc34 enzymatic function between our studies and the studies of Wu et al. are most likely attributable to our study of substrate ubiquitination versus the study of ubiquitin polymer formation by Wu et al. The differences in Cdc34 binding to the SCF are most likely due to differences in the binding assay conditions used. While Wu et al. used a truncated Cul1 (aa 324–776) we used full-length Cul1.[15] Additionally, Wu et al. used exclusively bacterially expressed and purified proteins while our assay utilized in vitro translated Roc1-Cul1. We also conducted in vivo binding studies between ectopically expressed Cdc34 and Roc1-Cul1 in the presence of endogenous SCF components. In general, our studies attempted to examine the interaction of the Cdc34 1-200 truncation mutant with the SCF under more physiological conditions compared to the binding studies of Wu et al.

Our studies indicate that phosphorylation of human Cdc34 at residues S203, S222, S231, T233, and S236 in budding yeast is not required for complementation of the *cdc34* ts strain. Although our in vitro binding study and $p27^{Kip1}$ ubiquitination assay were not performed in the presence of CK2, our unpublished results indicate that CK2 activity appears to be present in rabbit reticulocyte lysate which was included in both assays. Because of this, we do not know the exact status of Cdc34 phosphorylation within these experiments. However, the ability of the Cdc34 5 PT MUT A to mediate the ubiquitination of $p27^{Kip1}$ at a level comparable to WT Cdc34 suggests that Cdc34 phosphorylation is not essential for $p27^{Kip1}$ ubiquitination in vitro. Whether CK2 phosphorylation of Cdc34 may promote the binding of Cdc34 to the SCF and contribute to the nuclear localization of Cdc34 in vivo is still unclear. Our studies were conducted using in vitro assays which could not address the possible effects of phosphorylation on the subcellular localization of Cdc34. We have attempted to study the possible biological function of Cdc34 phosphorylation on the mammalian cell cycle by overexpressing the Cdc34 5 PT MUT A in mammalian cells. While we observed that this mutant significantly inhibited the entry of cells into S phase, we also observed a similar effect on cell cycle progression upon the overexpression of wildtype Cdc34 and other non-phosphorylation mutants of Cdc34. Future studies will be required to understand the potential biological significance of phosphorylation on Cdc34 function.

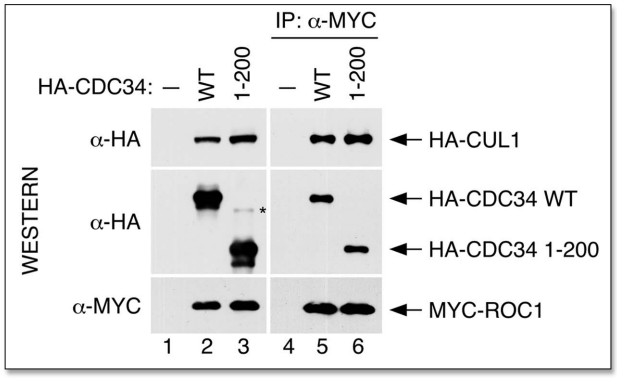

Figure 4. The C-terminal 36 amino acids of human Cdc34 are dispensable for in vivo binding to Cul1-Roc1. In vivo binding study of Cdc34 with Cul1 and Roc1 in transfected U2OS cells. Human osteosarcoma U2OS cells were either not transfected (-) (lanes 1 and 4) or were transiently transfected with Myc-tagged Roc1 (MYC-ROC1), HA-tagged Cul1 (HA-CUL1), and either HA-tagged human Cdc34 wild type (WT) (lanes 2 and 5) or HA-tagged Cdc34 truncation mutant deleted of the C-terminal 36 amino acids (1–200) (lanes 3 and 6). Left panel (WESTERN; lanes 1 to 3): Immunoblot analysis of Cdc34-transfected or not transfected (-) cell lysates using anti-HA antibody (α-HA; top and middle panels) or anti-Myc antibody (α-MYC; bottom panel). Right panel (IP-Western; lanes 4 to 6): Cdc34-transfected or not transfected (-) cell lysates were immunoprecipitated with anti-Myc antibody (IP: α-MYC) followed by immunoblotting with anti-HA antibody (α-HA; top and middle panels) or anti-Myc antibody (α-MYC; bottom panel). The solid arrows indicate the protein bands for HA-Cul1 (top panel), HA-Cdc34 wildtype (WT) or 1–200 mutant (1–200) (middle panel), and MYC-Roc1. The asterisk indicates a nonspecific band in the Cdc34 1–200 sample analyzed by immunoblotting.

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BIOCHEMICAL CHARACTERIZATION OF A HUMAN CDC34 COMPLEX THAT RESCUES DNA REPLICATION IN CDC34-DEFICIENT *XENOPUS* EGG EXTRACTS

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Running Title: Characterization of Human Cdc34 DNA Replication Activity

SUMMARY

Previous studies have shown that Cdc34 in a large molecular size complex is required for the initiation of DNA replication in eggs from *Xenopus laevis*. Our studies characterize a human Cdc34 complex from HeLa cells which is able to complement DNA replication in Cdc34-immunodepleted *Xenopus* egg extracts. We find that while immunoaffinity purified HeLa nuclear extract cannot restore DNA replication to Cdc34-depleted *Xenopus* extract, immunoaffinity purified HeLa nuclear extract passaged over phosphocellulose can restore DNA replication to Cdc34-depleted extracts. We further fractionated HeLa nuclear extract passaged over phosphocellulose using DEAE anion exchange, gel filtration, and immunoaffinity chromatography and found that only Cdc34 present in a large molecular size complex of ~400 kDa was able to rescue DNA replication in Cdc34-depleted *Xenopus* extract. Immunoaffinity purification of the Cdc34 complex from partially purified HeLa nuclear extract indicated that SCF components, Cul1 and $p19^{Skp1}$, were not tightly associated with human Cdc34, despite the ability of this Cdc34 complex to restore DNA replication in Cdc34-depleted *Xenopus* extracts. Further, the addition of recombinant human Cdc34 and $SCF^{p45Skp2}$ was not sufficient to restore DNA replication to Cdc34-depleted *Xenopus* extract. These studies suggest that human Cdc34 in association with as yet unidentified Cdc34-interacting proteins can complement DNA replication in Cdc34-deficient *Xenopus* egg extract.

INTRODUCTION

Cell cycle progression and homeostasis require ubiquitin–dependent proteolytic degradation. Regulated protein degradation involves a pathway in which ubiquitin is activated, in an ATP dependent manner, for transfer to a substrate. Subsequently the poly-ubiquitinated substrate is recognized by the 26S proteosome and destroyed (Jentsch, 1992,Ciechanover, 1994). These cascades of events begin when ubiquitin-activating enzyme (E1) forms a thioester bond to the carboxy-terminus of ubiquitin. Ubiquitin is transferred, through thioesterification, to a member of a family of ubiquitin-conjugating enzymes (E2). Finally, the activated ubiquitin will be transferred to a lysine residue on the substrate directly by the E2 or it will be passed to the ubiquitin ligating enzyme (E3) and then to the substrate. E3s bind the substrate directly, suggesting they provide specificity in ubiquitin–dependent proteolytic degradation. A requirement for ubiquitin–dependent protein degradation at the G1/S transition was first observed in *S. cerevisae* where a temperature sensitive (ts) mutant arrested at the initiation of DNA replication because p40Sic1, an inhibitor of S phase promoting Clb/Cdc28 complexes, was unable to be degraded (Schwob, 1994).

In Xenopus egg extracts, depletion of a ubiquitin conjugating enzyme, Cdc34, abrogated DNA replication initiation suggesting a role for Cdc34 at the onset of DNA replication, presumably through its function in protein degradation (Yew and Kirschner 1997). Purified

[3] The abbreviations used are: AP Cdc34, affinity purified Cdc34; BSA, bovine serum albumin; CDC, cell division cycle; Ctrl, control; CK2, Casein Kinase 2; CK1, CSA, chicken serum albumin; DTT, dithiothreitol E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzymes; E3, ubiquitin ligating enzyme; hCK2β, human Casein Kinase 2β; hCdc34, human Cdc34; Ig, immunoglobulin; IP, immunoprecipitation; SCF, Skp1, Cullin, F-box; ScCdc34p, Cdc34p in *S. cerevisiae*; MUT, mutant; NEXT, nuclear extract; No Depl, not depleted; NRS, normal rabbit serum; PCFT, phosphocellulose flow through; RIgG, rabbit immunoglobulin; LSS, low speed supernatant; PCR, Polymerase Chain Reaction; RER, energy-regenerating system; RU, rescuing units; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; UBC, ubiquitin conjugating enzyme; WCE, whole cell extract; WT, wildtype.

Cdc34 alone is not sufficient to rescue DNA replication activity in the Cdc34 immunodepleted egg extracts, suggesting that Cdc34 in association with other proteins are required. In mammals, Cdc34 interacts and functions with different ubiquitin ligating enzymes targeting substrates that play a role in cell cycle progression, signal transduction, and development (Tyers M 2000, Koepp 1999). The most characterized ubiquitin ligating enzyme that functions with Cdc34 is p19Skp1/Cul1/p45Skp2 (F-box)/Roc1 (ring) or SCF/ring complex.

We have also identified the regulatory (β) subunit of human Casein Kinase 2 (CK2) as a novel Cdc34 interacting protein through a yeast two hybrid screen. We have shown that Cdc34 is a phosphoprotein that immunoprecipitates with kinase activity. This associated kinase activity is biochemically characteristic of CK2 (Block et al., 2001). CK2 activity has also been implemented in cell cycle progression, signal transduction, and more recently, protein stability (Uhle et al., 2003, Semplici, oncogene 2002, Allende and Allende 1998, Guerra and Issinger 1999, Pinna and Meggio, 1997). Although protein degradation has been linked to cell cycle progression for some time now, ongoing investigations are identifying novel interacting proteins and post-translational modifications of the ubiquitin conjugating enzymes as well as the ubiquitin ligating enzymes which are continuing to provide insights as to the levels of complexity of protein degradation and their regulation in higher eukaryotes. The Xenopus system has proven to provide mechanistic insights to cell cycle events. It is currently unclear what degradation event/events are required for DNA replication in a vertebrate. Here we describe the partial purification and biochemical characterization of the Cdc34-associated protein complex in HeLa extracts required for DNA replication activity in Cdc34-immunodepleted *Xenopus* egg extracts.

EXPERIMENTAL PROCEDURES

Sucrose gradient centrifugation. Isokinetic 10-40% sucrose gradients (HKME buffer: 50mM Hepes pH 7.2, 100mM KCL, 5mM $MgCl_2$, 0.2 mM EDTA, 2mM DTT, 1x protease inhibitors) were poured in a two chamber gradient connected to a peristaltic pump. Gradients were poured by displacement method with a glass capillary tube at the bottom of the centrifugation tube. The glass tube was carefully removed vertically afterwards without disturbing the gradient. A mixture of standards including Thyroglobulin, Ferritin, Catalase, Aldolase, BSA and Lysozyme, each 375 ug, were layered onto the gradient. HeLa extracts were dialyzed in buffer D- (20mM Hepes pH 7.9, 100mM KCl, 5mM B-mercaptoethanol, 0.5 mM PMSF, 0.1mM EDTA). 5 mgs of HeLa whole cell extract (HeLa WCE), 5 mgs of HeLa nuclear extract (HeLa NEXT), or 4.2 mgs of HeLa phosphocellulose flow through (HeLa PCFT) were layered onto the gradient. Samples were subjected to centrifugation at 37,500 rpm for 17.5 hours. Fractions were collected by cutting 0.5 mL aliquots from the top of the gradient. 52.5 uL of each fraction was added to sample buffer and resolved on a SDS-Page gel and immunoblots were performed. In parallel, 0.5 mL fractions of standard markers were resolved on SDS-PAGE and subsequently coomassie blue stained and calibration curves plotted.

Immunodepletion/Rescue studies. Xenopus laevis interphase low speed supernatant (LSS) extracts were generated as previously described (Yew Kirscner1997). Demembranated sperm nuclei were prepared as described (J.J. Blow and R.A. Laskey, Cell 47 577 1986). DNA replication was measured by tricloroacetic acid precipitation of radiolabeled fragments (Yew and Kirchner 1997) and presented as percent replication of the input template (5ng/ul). Antibodies were covalently cross-linked to protein A (pA)-sepharose(Seph) or pA-affiprep as described

(Yew and Kirschner, 1997). Cyclohexamide (0.1mg/ml) and energy-regenerating system (RER) was added to LSS before replication was assayed. Immunodepletetion was performed by mixing 1 volume of LSS and 1.25 volume antibody coupled pA-seph beads for 2-3 hours at 0^0C with resuspension every 10 minutes. Rescue assays were performed by incubating 8uL coupled pA-affiprep beads with indicated *X. laevis* LSS or indicated fractionated mammalian extracts overnight at 4^0C in the presence of 1mM ATP. The beads were washed 3x with XB-(10mM Hepes pH 7.2, 100mM KCL, 0.1mM $CaCl_2$, 1mM $MgCl_2$). Immunodepleted LSS was added to washed rescue beads and assayed for replication as described. For SCF/ring, CK2 rescue studies, 200nM of bacterial 6xhis Cdc34 with 200nM of each baclovirus SCF/ring component indicated or purified enzyme CK2 was added to immunodepleted LSS and assayed for replication. Rescuing units are calculated as %replication of rescue sample/% replication of control rescue (Ctrl).

Immunoprecipitation-western (IP-western) and Direct Westerns. 2mgs of mammalian HeLa Phosphocellulose flow through (HeLa PCFT) was incubated overnight with antibody coupled Cdc34 pA-affiprep beads or control serum (NRS) coupled pA-affiprep beads. Beads were washed 3x with XB- and boiled in Laemmli sample buffer and resolved by SDS-Page. Immunoblots were performed on the immunoprecipitated material or on 50 ug of indicated cell lysate per lane. Immunoblots were incubated with affinity purified Cdc34 anti-body, Cullin 1 antibody (neomarkers) or p19Skp1 (Santa Cruz). The immunoblots were washed and incubated with pA-coupled horse radish peroxidase (Biorad) followed by chemiluminescence using ECL reagent (Amersham-Parmacia). Other immunoblots were incubated with Casein Kinase 2 (calbiochem) or p45Skp2 (Santa-Cruz). Immunoprecipitation from HeLa PCFT/DEAE 150mM

KCl fraction was performed by taking 10mgs of the purified 150mMKCl DEAE bump/IP and incubating the fraction with affinity purified Cdc34 antibody covelantly coupled to pA-affiprep or RigG covelantly coupled to pA-affiprep. The immunoprecipitate was washed (XB-/250mMKC1) and eluted by acid base. The eluent was concentrated and 1/5th was resolved on SDS-PAGE for western blot analysis and the remaining 80% was resolved on SDS-PAGE and subjected to silver stain.

Gel Filtration Fractionation/silver stain: DEAE 250mMKCL/SDEX200 purification: HeLa PCFT was dialyzed in DEAE binding buffer (50mM Tris pH 8.0, 50 mM KCL, 5mM $MgCl_2$, 0.2 mM EDTA, 10% Glycerol, 1mM DTT). 133.6 mgs of HeLa PCFT in binding buffer was loaded onto DEAE and washed in binding buffer. The flow through was collected and subsequent washes of eluting buffer (50mM Tris pH 7.7, 250 mM KCl or 600 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 10% Glycerol and 1mM DTT were passed over the column and collected. Pooled peaks were combined and each fraction was assayed for protein by western analysis. In addition, each fraction was assayed for DNA replication/rescue (data not shown) activity. Subsequently, the 250 mM KCl fraction was equilibrated in 150 mM KCL, concentrated and 22 mgs was passed over a HiLoad 16/60 SDex 200 column (Pharmacia). Fractions were collected and concentrated. 1/60th of each fraction or 50ug of indicated cell lysate was used for immunoblot analysis. 1/4th of each fraction was used in a rescuing assay as described. 3uL of each purified fraction was analyzed by silver staining. **DEAE 150mM purification:** HeLa PCFT was dialyzed in DEAE binding buffer (50mM Tris pH 8.0, 50 mM KCL, 5mM $MgCl_2$, 0.2 mM EDTA, 1mM DTT) and 158mgs HeLa PCFT loaded onto the DEAE column. The column was washed with binding buffer, flow through fraction (FT) and subsequently eluted with 50mM Tris

pH 7.2, 150 mM KCl, 5mM $MgCl_2$, 0.2 mM EDTA or 50mM Tris pH 7.2, 300 mM KCl, 5mM $MgCl_2$, 0.2 mM EDTA or 50mM Tris pH 7.2, 600 mM KCl, 5mM $MgCl_2$, 0.2 mM EDTA. The peak of each fraction was pooled, dialyzed in HKME (50mM Hepes pH 7.2, 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA), and concentrated. 25uL of each fraction was used in a western analysis and $1/41^{th}$ of each fraction was used in a rescue assay. 3uL of each purified fraction was analyzed by silver staining.

RESULTS

Immunoaffinity purified HeLa nuclear extract passaged over phosphocellulose can restore DNA replication in Cdc34-immunodepleted *Xenopus* egg extracts. It has been characterized in *Xenopus laevis* that a low speed supernatant (LSS) egg extract immunodepleted of Cdc34 cannot support DNA replication onset. Incubating LSS with pA-affiprep covelantly coupled anti-Cdc34 beads rescues the Cdc34 depleted egg extract but not purified bacterial Cdc34 alone (Yew and Kirschner 1997). To determine the ability of a mammalian cell extract to rescue Cdc34 immunodepleted Xenopus egg extract, we incubated 1100 ug of HeLa nuclear extract (HeLa NEXT) or HeLa nuclear extract passaged over a phosphocellulose column (HeLa PCFT) with pA-affiprep covelantly coupled anti-Cdc34 beads. The results show that LSS incubated with normal rabbit serum covelantly coupled to pA-sepharose beads (Ctrl Depl) support DNA replication (Fig 1 left panel). However, depletion of Cdc34 with antibody covelantly coupled to pA-sepharose incubated in LSS (Cdc34 Depl) does not support DNA replication (Fig 1 left panel). This Cdc34 immunodepleted extract was incubated with pA-affiprep beads covelantly coupled to Cdc34 or normal rabbit serum that had been incubated in LSS, HeLa NEXT, or HeLa PCFT. Normal rabbit serum incubated with LSS could not rescue DNA replication in the Cdc34

depleted egg extract although anti-Cdc34 beads incubated in the LSS could (Fig 1 right panel, Ctrl and XlLSS respectively). pA-affiprep coupled anti-Cdc34 beads incubated in a HeLa PCFT can rescue DNA replication in a Cdc34 depleted egg extract whereas anti-Cdc34 beads incubated in a nuclear extract could not (HeLa NEXT) (Fig 1 right panel). These results suggest that mammalian HeLa PCFT extract incubated with pA-affiprep beads covelantly coupled to anti-Cdc34 can rescue DNA replication in a Cdc34 depleted Xenopus egg extract. Importantly these findings also suggest that Cdc34 in a HeLa nuclear extract may be associated with an inhibitor of DNA replication onset in Xenopus LSS egg extract and that passage of a HeLa nuclear lysate over a phosphocellulose column restores DNA replication rescuing activity.

The Cdc34 associated DNA replication activity in HeLa extracts is found in a higher molecular weight complex. We wanted to purify and characterize the Cdc34 associated DNA replication rescuing activity in mammalian cells. We further fractionated the HeLa Phosphocellulose Flow Through (PCFT) by passing it onto a DEAE anion exchange column and took three fractions, the flow through, a 250mM KCl bump and a 450 mM KCl bump. We examined each fraction by western analysis for Cdc34p and known Cdc34 interacting proteins (Fig 2A). SCF components, Cullin1, p45Skp2, and p19Skp1 all co-fractionated with Cdc34 in the 250mM KCl bump. However, another known Cdc34 interacting protein Casein Kinase 2 (CK2) did not (Fig 2A). Each fraction was tested for Cdc34 rescuing activity as described in Fig 1. The HeLa PCFT/DEAE 250 mM KCl fraction showed DNA replication rescuing activity in the Xenopus Cdc34 depleted egg extract (data not shown). We further fractionated the 250 mM KCl bump over a SDEX 200 sizing column. Each fraction of the SDEX 200 column was collected, concentrated resolved on a SDS-Page gel and transferred to nitrocellulose and

analyzed by western analysis for Cdc34p and other known Cdc34 interacting proteins (Fig 2B). The molecular weight sizes listed above represent the elution of standards used to calibrate the column (Fig 2B above top panel). These western results suggest that endogenous Cullin 1 in fractionated HeLa extracts is only found in a higher molecular weight complexes of 232-440 kDa. Cullin1 encodes for a protein of ~85kDa. p45Skp2 is also found in several fractions of higher molecular weight from ~67-660 kDa. p19Skp1 is spread over many fractions from 20-660 kDa. (Fig 2B). This is consistent with other studies that show that fractionation of p19Skp1 and p45Skp2 in HeLa cells over a gel filtration column exist in many different fractions (Raymond et al., 1998). Endogenous hCdc34 in fractionated HeLa cells is found predominately in a free form, however 1-3 percent is found in higher molecular weight complexes of 232-660kDa. This is consistent with fractionated S100 extracts from Xenopus laevis following Cdc34 protein (Yew and Kirschner, 1997). There is a modified form of Cdc34 that migrates slower in SDS-Page in fractions 9 and 10. We do not think this modified Cdc34 species is a Cdc34 phosphorylation form as we do not observe a shift of Cdc34 protein in our phosphorylation studies (Block et al., 2001). This shift may represent a ubiquitinated species of Cdc34 as Cdc34 is autoubiquitintated in vitro and in vivo (Banerjee et al., 1993, Goebl et al., 1995, Ohta et al., 1999, Seol et al., 1999, Chen et al., 2000). We analyzed each fraction by silver staining to see the complexity of the purified SDEX fractions (Fig 2C). In addition, each fraction was tested for Cdc34 rescuing activity in Xenopus Cdc34 depleted egg extracts as previously described. The results show that LSS not depleted and LSS incubated with normal rabbit serum covelantly coupled to pA-sepharose beads support DNA replication (Fig 2D left panel). However, depletion of Cdc34 with antibody covelantly coupled to pA-sepharose incubated in LSS does not (Fig 2D left panel). This Cdc34 immunodepleted extract was incubated with pA-affiprep beads

covelantly coupled to Cdc34 or normal rabbit serum that had been incubated in LSS or each eluted SDEX 200 fraction. Normal rabbit serum covelantly coupled pA-affiprep beads incubated with LSS could not rescue DNA replication in the Cdc34 depleted egg extract although anti-Cdc34 coupled pA-affiprep beads incubated in the LSS could (Fig 2D right panel). pA-affiprep coupled anti-Cdc34 beads incubated in HeLa PCFT/DEAE 250mM KCl/SDEX200 fractions indicate that the Cdc34 associated rescuing activity is found in fractions 10,11 of the SDEX200 column (Fig 2D right panel noted by an astrick (*)). Importantly, these results suggest that the Cdc34 associated rescuing activity is found with the smaller percent of Cdc34 in a higher molecular weight complex of ~ 400 kDa rather than the higher percent of Cdc34 that is in a monomer form.

Fractionation of HeLa WCE, HeLa NEXT, HeLa PCFT extracts by sedimentation. To characterize endogenous Cdc34 in non fractionated HeLa cell extracts, we prepared a HeLa whole cell extract (HeLa WCE) and resolved it on a sucrose gradient. A linear sucrose gradient was prepared so that the 40% was on the bottom of the gradient and 10% on the top. Standards were run in parallel and 0.5 mL fractions were cut from the top of the gradient and subjected to a SDS-Page gel and subsequently coomassie blue stained. The molecular weight sizes listed above (Fig 3A.) represent the fractionation of standards used to calibrate the sucrose gradient. Western analysis was performed on each fraction of the HeLa WCE using antibodies against Cdc34 (Fig 3A middle panel) and known Cdc34 interacting proteins, Casein Kinase 2 (CK2) and p19Skp1 (Fig 3A top panel and bottom panel respectively). These results suggest that the majority of Cdc34 (~90%) is free and only a small percent of Cdc34 is present in a large molecular size complex of 275-300 kDa. CK2 and p19Skp1 proteins can also be detected in the Cdc34 higher

molecular weight fraction. Because the results in Fig 1 suggest that Cdc34 in a HeLa nuclear extract (HeLa NEXT) may be associated with an inhibitor of DNA replication activity, we passed a HeLa PCFT and a HeLa NEXT over a linear sucrose gradient as just described to determine if there was a shift in the Cdc34 protein or Cdc34 high molecular weight protein complex. Western analysis on each of these extracts did not show a difference in the free Cdc34 (Fig 3B). However, we observed that Cdc34 in the higher molecular weight fractions in a HeLa NEXT appeared to shift to fractions of lower molecular weight when passaged over a phosphocellulose column. These results suggest, a protein that associates with Cdc34 in the HeLa nuclear extract is fractionated away or dissociated when passed over a phosphocellulose column. This, however, needs to be further investigated. We cannot rule out that the higher molecular weight complex of Cdc34 breaks apart when further fractionated over the phosphocellulose column. This fractionated HeLa PCFT extract does have Cdc34 rescuing activity in a Cdc34 depleted Xenopus egg extract as shown in Fig 1. Cdc34 fractionated in HeLa PCFT by gel filtration indicated Cdc34 in higher molecular weight complexes of 232-660kDa. The difference of molecular weight of Cdc34 in a HeLa WCE by sedimentation versus Cdc34 HeLa PCFT over gel filtration suggest that the Cdc34 protein complex is not spherical in nature rather it is globular.

HeLa PCFT passed over a DEAE anion exchange column and eluting at 150mM KCl separates the small percent of Cdc34 associated DNA replication activity from the majority of Cdc34 free form. We passed HeLa PCFT over a DEAE anion exchange column and eluted with more KC1 bumps to see if we could further fractionate the Cdc34 associated rescuing activity. We examined, by western analysis, the flow through, a 150mM KCl bump, a 300 mM

KCl bump and a 600 mM KCl bump for Cdc34 and other Cdc34 interacting proteins. We found that a very small percent of Cdc34 eluted and could be detected in the 150 mM KCl fraction, whereas the majority of other known Cdc34 interacting proteins, Cul1/Skp2 and Skp1 did elute in the 150 mM KCl bump (Fig 4A). The majority of Cdc34 eluted at the 300 mM KCl bump (Fig 4A). We ran each fraction on a SDS-Page gel and analyzed the complexity of these fractions by silver stain (Fig 4B). We then tested each fraction for Cdc34 associated DNA replication activity. The results show that LSS not depleted and LSS incubated with normal rabbit serum covelantly coupled to pA-sepharose beads support DNA replication. However, depletion of Cdc34 with antibody covelantly coupled to pA-sepharose incubated in LSS does not (Fig 4C left panel). This Cdc34 immunodepleted extract was incubated with pA-affiprep beads covelantly coupled to Cdc34 or normal rabbit serum that had been incubated in LSS, or each eluted DEAE fractions. Normal rabbit serum coupled pA-affiprep beads incubated with LSS could not rescue DNA replication in the Cdc34 depleted egg extract although anti-Cdc34 coupled pA-affiprep beads incubated in the LSS could (Fig 4C right panel). pA-affiprep coupled anti-Cdc34 beads incubated in the HeLa PCFT/DEAE fractions indicate that the Cdc34 associated rescuing activity is found in the 150 mM KCl fraction (Fig 4C right panel noted by an astrck (*)). This purification step is very efficient, as it appears to separate the small percent of Cdc34 that is associated with DNA replication rescue activity from the bulk of Cdc34 that is not complexed and does not exhibit any associated DNA replication activity.

Known Cdc34 associated proteins are not sufficient for rescuing DNA replication in a Cdc34 depleted Xenopus egg extract. A requirement for proteolysis and DNA replication has well been established. It has been shown through this study and in studies using Xenopus egg

extracts that Cdc34 in a higher molecular weight complex and not free Cdc34 exhibits associated DNA replication activity. It is also known that Cdc34 associates and functions with its E3, the SCF/ring complex in regulated protein degradation via the ubiquitin pathway. In addition, components of the SCF complex, Cullin1, p45Skp2 and p19Skp1 co-fractionates with Cdc34 associated rescuing activity in the DEAE 150mM KCl fraction. It was therefore important to determine if the SCF complex is necessary and or sufficient for rescuing a Cdc34 depleted Xenopus egg extract. We took two approaches to address this question. First, we know that pA-affiprep coupled to Cdc34 antibodies and incubated in a HeLa PCFT immunoprecipitates proteins that associate with Cdc34 that are both necessary and sufficient for DNA replication rescuing activity in a Cdc34 depleted Xenopus egg extract. Therefore, we immunoprecipitated 2 mgs of HeLa PCFT with pA-affiprep coupled to Cdc34 antibodies or pA-affiprep coupled to normal rabbit serum and examined the immunoprecipitate by western analysis (IP-western). The results show that endogenous CUL 1 and Skp1 are not detectable by western analysis in the co-precipitate using anti-Cdc34 coupled pA-affiprep beads although Cdc34 is easily detected (Fig 5A lanes 3,4). HeLa PCFT or HeLa PCFT/DEAE 250 mM KCl lysate were loaded as western controls (Fig 5A lanes 1,2 respectively). Importantly, we next took the HeLa PCFT/DEAE 150 mM KCl fraction that exhibited high Cdc34 rescuing activity and immunoprecipitated (IP) Cdc34. This fraction was enriched for Cul1 and p19Skp1 and only the small percent of Cdc34 in a complex required for DNA replication rescuing activity. We immunoaffinity purified Cdc34 antibodies (AP-Cdc34) and covelantly coupled AP-Cdc34 or RIgG to pA-affiprep beads as described in the methods. We incubated the HeLa PCFT/DEAE 150mM KCl fraction to AP-Cdc34 or RIgG coupled beads, washed the beads and eluted the Cdc34 immunoprecipitate. The eluted immunoprecipitate was concentrated and and $1/5^{th}$ was resolved by SDS-Page analyzed by

western analysis. The RIgG precipitate did not immunoprecipitate Cul1/Cdc34 nor p19Skp1. The Cdc34 immunoprecipitate easily detected Cdc34 however, Cul1 and p19Skp1 were not detected by western analysis (Fig 5B). This suggests that known SCF proteins that function with Cdc34 do not immunoprecipitate with Cdc34 nor with Cdc34 rescuing activity. Finally, we tested the ability of purified bacterially expressed 6xhisCdc34 with purified baclovirus SCF/Roc1 or another known Cdc34 interacting protein, CK2, to rescue a Cdc34 depleted Xenopus egg extract. The results show that LSS not depleted and LSS incubated with normal rabbit serum covelantly coupled to pA-sepharose beads support DNA replication (Fig 5C left panel). However, depletion of Cdc34 with antibody covelantly coupled to pA-sepharose incubated in LSS does not (Fig 5C left panel). This Cdc34 immunodepleted extract was incubated with pA-affiprep beads covelantly coupled to Cdc34 or normal rabbit serum that had been incubated in LSS. Normal rabbit serum pA-affiprep coupled beads incubated with LSS could not rescue DNA replication in the Cdc34 depleted egg extract although anti-Cdc34 pA-affiprep coupled beads incubated in the LSS could (Fig 5C right panel). Further, 200nM of 6xHisCdc34 with 200nM baclovirus purified Cul1/ROC1 (Fig 5C lane 6) or 200nM of 6xHis Cdc34 with 200nM baclovirus purified Cul1/ROC1/Skp1/Skp2 (Fig 5C lane 7) or 6xHis Cdc34 with 200nM purified enzyme of Casein Kinase 2 (CK2) (Fig 5C lane 8) was added to the Cdc34 depleted egg extract. We did not detect any rescuing activity over background in these studies. This suggests that the SCF/ring complex alone is not tightly associated with Cdc34 and that these known interacting proteins are not sufficient when combined with Cdc34 to rescue a Cdc34 depleted egg extract although anti-Cdc34 coupled pA-affiprep beads incubated in a HeLa PCFT is. Taken together, this suggests that there are yet unidentified associated proteins of Cdc34 that are required for the onset of DNA replication in Xenopus.

Potential Cdc34 interacting proteins. We have fractionated Cdc34 rescuing activity and our fractionation protocol is shown schematically in Figure 6A. We have found that HeLa PCFT over a DEAE column and eluting at 150mM KCl followed by immunoaffinity with Cdc34 antibody exhibits the highest DNA replication Cdc34 rescuing activity as assayed in a Cdc34 Xenopus Cdc34 depleted egg extracts. Because it appears that known Cdc34 interacting proteins are not sufficient for our Cdc34 rescuing activity, we wanted to next examine the HeLa PCFT/DEAE 150mM fraction for candidate Cdc34 interacting proteins. We took the concentrated and eluted fraction from the Cdc34 immunoprecipitation PCFT/DEAE 150mM fraction and resolved it on a SDS-PAGE gel and performed silver stain. The results indicate candidate Cdc34 interacting proteins that are not found in the RIgG immunoprecipitation (Figure 6B). We have identified seven candidate Cdc34 interacting proteins with approximate molecular weights of; 56 kDa, 60 kDa, 90 kDa, 110 kDa, 120 kDa, 150 kDa, and 220 kDa. A Western Blot for Cdc34 was performed on the immunoprecipitates (Fig 6B α-Cdc34 immunoblot). Consistent with our earlier data, there appear to be novel Cdc34 interacting proteins as compared by the proteins of higher molecular weight in the Cdc34 immunoprecipitate.

DISCUSSION

Here we describe the partial purification and biochemical characterization of the Cdc34-associated protein complex in HeLa extracts required for DNA replication activity in Cdc34-immunodepleted *Xenopus* egg extracts. We show that anti-Cdc34 coupled beads incubated in a HeLa cell extract can rescue a Cdc34 immunodepleted *Xenopus* extract, which we call the Cdc34 associated DNA replication activity. Importantly, we have found that only a HeLa nuclear

extract that has been passaged over a phosphocellulose column exhibits the Cdc34 associated DNA replication activity (Fig 1). Further fractionation of the HeLa nuclear extract phosphocellulose flow-through by DEAE anion exchange, gel filtration and immunoaffinity chromatography found that only Cdc34 present in a large molecular size complex of ~400 kDa was able to rescue DNA replication in Cdc34-depleted *Xenopus* extract (Fig 2D). Immunoaffinity purification of the Cdc34 complex from partially purified HeLa nuclear extract indicated that SCF components, Cul1 and $p19^{Skp1}$, were not visibly associated with Cdc34, despite the ability of the Cdc34 complex to restore DNA replication to Cdc34-depleted *Xenopus* extract (Fig 5A,B). Further, the addition of recombinant human Cdc34 and $SCF^{p45Skp2}$ or another known Cdc34 interacting protein, CK2, was not sufficient to restore DNA replication to Cdc34-depleted *Xenopus* extract (Fig 5C). We cannot say by these studies, however, that the SCF/ring and or CK2 proteins are not necessary for complementation. These studies do suggest that the human Cdc34 complex does not consist merely of Cdc34 and SCF/ring components, but may instead consist of previously unidentified Cdc34-interacting proteins. Earlier purification studies in Xenopus indicate that only Cdc34 in a multi-protein complex of ~400 kDa was able to rescue a Xenopus Cdc34 immunodepleted egg extract [67]. Our studies in mammalian cells are consistent with these results and suggest the human and Xenopus protein complex may be conserved. It is unclear from the Xenopus studies if Cdc34 is associated with an inhibitor during any part of the cell cycle. The inhibition we observe in a nuclear extract could be another level of regulation for Cdc34 by direct or indirect means and are currently being investigated. We see a shift of Cdc34 in higher molecular weight fractions to that of lower molecular weight fractions in our sucrose gradients. In the HeLa NEXT the Cdc34 is found in higher molecular weight fractionates up to ~ 350 kDA by western analysis. This higher molecular weight form of Cdc34

shifts to ~ 180-225 kDa when the NEXT has been passaged over a phosphocellulose column (Fig 3). This shift suggests there may be a protein associated with the higher molecular weight Cdc34 complex that binds to the phosphocellulose column. We tried, unsuccessfully, to test these sedimentation fractions for DNA replication activity (data not shown). Importantly, we have found a purification step, HeLa PCFT/DEAE 150mM KCl bump, that appears to separate the bulk of Cdc34 that is not in a complex from a small percent of Cdc34 that is in a complex that rescues DNA replication activity in a Xenopus Cdc34 depleted egg extracts (Fig 4 A, C). When we immunopurify the HeLa PCFT/DEAE 150mM KCl bump on affinity purified Cdc34 beads we find candidate Cdc34 interacting proteins that do not appear to be known Cdc34 interacting proteins by molecular weight (Fig 6B). We have identified seven candidate Cdc34 interacting proteins with approximate molecular weights of; 56 kDa, 60 kDa, 90 kDa, 110 kDa, 120 kDa, 150 kDa, and 220 kDa. These molecular weights combined total a ~800 kDa which is greater than the ~400 kDa complex required for our Cdc34 rescuing activity. Identification of these proteins will help determine which ones are specific Cdc34 interacting proteins. In Xenopus, the functional Cdc34 that was able to rescue DNA replication was also found in a 440 KD complex. In addition, Xenopus extract (S100) immunoprecipitated with anti-Cdc34 coupled beads identified potential interacting proteins of Cdc34 corresponding to 60, 85, 180 and 220 kDa. The human immunopurified Cdc34 complex is sufficient for rescuing a Xenopus Cdc34 immunodepleted egg extract, suggesting this complex may be conserved between Xenopus and humans. The unidentified potential Cdc34 interacting proteins in mammalian cells appear to be similar (by molecular weight) to the candidate Cdc34 interacting proteins in Xenopus. Importantly, in both mammalian cells and in Xenopus, the associated Cdc34 proteins to not appear to be known SCF/ring proteins based on western analysis. A requirement for Cdc34 at

the initiation of DNA replication has been established. The substrates that must be degraded prior to the onset of DNA replication are still unknown. Identification of the Cdc34 interacting proteins will help elucidate the molecular mechanisms required for DNA replication initiation.

REFERENCES

FIGURE LEGENDS

Figure 1. *HeLa nuclear extract passaged over phosphocellulose can complement DNA replication in Cdc34-immunodepleted Xenopus egg extract*. Left panel (Depletion): Xenopus LSS was control depleted with pA-Seph coupled to normal rabbit serum Ig (Ctrl Depl), or pA-Seph coupled rabbit 1 anti-hCdc34 Ig (Cdc34 Depl) and sperm nuclei was added and assayed for DNA replication. Replication (%) is normalized to 100% of that in the control-depleted sample (Ctrl Depl). Right panel (Cdc34 Depletion/Rescue): 10uL of Cdc34 depleted Xenopus LSS left panel (Cdc34 Depl), was added to 8 uL pA-affiprep coupled to normal rabbit serum Ig (Ctrl) or pA-affiprep coupled anti-hCdc34 Ig incubated in LSS (XL LSS). Cdc34-depleted LSS (Cdc34 Depl) was also incubated with 8uL pA-affiprep coupled to anti-Cdc34 Ig and incubated with Hela nuclear extract (HeLa NEXT) or Hela nuclear extract passaged over a phosphocellulose column (HeLa PCFT). Rescuing Units were calculated by % replication of the rescue sample/% replication of control rescue.

Figure 2. *Cdc34 associated rescuing activity in mammalian cells is found in a high molecular weight complex.* **(A)** HeLa extract passaged over phosphocellu**se** (HeLa PCFT) was applied to a DEAE anion exchange column and eluted at increasing KCl bumps. The flow through (FT), 250 mM KCl bump (250), and 450mM KCl bump (450) fractions were subjected to western blot analysis with Cullin 1 (CUL 1), p45Skp2 (Skp2), affinity purified Cdc34 (Cdc34), p19Skp1 (Skp1) and Casein Kinase 2 (CK2). **(B)** The HeLa PCFT/DEAE 250mM KCl fraction was applied to SDEX 200 gel filtration chromatography. The molecular sizes listed above represent the elution of standards used to calibrate the column. Fractions were collected and subjected to western blot analysis with Cullin 1 (Cul1), p45Skp2 (Skp2), affinity purified Cdc34 (Cdc34) and

p19Skp1 (Skp1) or the starting material (HeLa PCFT/DEAE 250 mM KCl). **(C)** 3 μL of each SDEX200 fraction above were ran on a SDS-PAGE and silver stained. **(D)** Xenopus LSS was not depleted (No depl) or depleted with pA-Seph coupled to normal rabbit serum Ig (Ctrl), or pA-seph coupled rabbit 1 anti-hCdc34 Ig (Cdc34) and sperm nuclei was added and assayed for DNA replication. Replication (%) is normalized to 100% of that in the control-depleted sample (Ctrl). Right panel: 10uL of Cdc34 depleted LSS used in the left panel (Cdc34 Depl) was added to 8uL pA-affiprep coupled to normal rabbit serum Ig (Ctrl Rescue) or 8uL pA-affiprep coupled anti-hCdc34 Ig incubated in LSS. 10uL of Cdc34-depleted LSS (Cdc34 depl) was also incubated with 8 uL pA-affiprep coupled to anti-Cdc34 Ig incubated with HeLa PCFT/DEAE 250mM KCl fraction or SDEX 200 column fractions. Rescuing Units (RU) were calculated by % replication of the rescue sample/% replication of control rescue. The (+) represents the rescuing activity (Fig 4D fractions 10,11).

Figure 3. *Endogenous Cdc34 in mammalian cell extracts fractionated via sedimentation.* **(A)** HeLa whole cell extract (HeLa WCE) was layered on an isokinetic 10-40% sucrose gradient in parallel with protein standards. Fractions were collected from the top of the gradient (10%) followed by western blot analysis with Casein Kinase 2 antibody (ANTI-CK2), affinity purified Cdc34 antibody (ANTI-Cdc34) or p19Skp1 antibody (ANTI-p19Skp1). Molecular weights are shown above the panel. **(B)** HeLa Phosphocellulose flow through (HeLa PCFT) or HeLa nuclear extract (HeLa NEXT) were layered on an isokinetic 10-40% sucrose gradient as just described. Fractions were collected from the top of the gradient (10%) followed by western blot analysis with affinity purified Cdc34 antibody (ANTI-Cdc34). Molecular weights are shown above the panel.

Figure 4. *Fractionation of HeLa Phosphocellulose Flow Through over a DEAE column and bumping at 150 mM KCl seperates the small percent of Cdc34 in an active complex.* **(A)** HeLa extract passaged over phosphocellu**se** (HeLa PCFT) was applied to a DEAE column and eluted at increasing KCL bumps. The flow through (FT), 150 mM KCl, 300mM KCl, and 600mM KCl bump fractions were subjected to western blot analysis with Cullin 1 (CUL 1), p45Skp2 (Skp2), affinity purified Cdc34 (Cdc34), p19Skp1 (Skp1). **(B)** 3 μL of each DEAE fraction above were ran on a SDS-PAGE and silver stained. **(C)** Left panel: Xenopus LSS was not depleted (No depl) or depleted with pA-Seph coupled to normal rabbit Ig (Ctrl), or pA-seph coupled rabbit 1 anti-hCdc34 Ig (Cdc34) and sperm nuclei was added and assayed for DNA replication activity. Replication (%) is normalized to 100% of that in the control-depleted sample (Ctrl). Right panel: 10uL Cdc34 depleted LSS used in the left panel (Cdc34 Depl) was added to pA-affiprep coupled to normal rabbit Ig (Ctrl) or pA-affiprep coupled anti-hCdc34 Ig incubated in LSS. Cdc34-depleted LSS (Cdc34 depl) was also incubated with pA-afffiprep coupled to anti-Cdc34 Ig and incubated with Hela PCFT/DEAE Flow Through (FT) fraction, 150mM KCl bump, 300mM KCl bump, and 600mM KCl bump. Rescuing Units were calculated by % replication of the rescue sample/% replication of control rescue. The astric (*) represents rescuing activity (Fig 4C).

Figure 5. *Known Cdc34 interacting proteins do not immunoprecipitate with Cdc34 nor rescue a Cdc34 depleted Xenopus egg extract.* **(A)** 2mgs of HeLa phosphocellulose flow through (HeLa PCFT) were incubated with pA-affiprep coupled to anti-Cdc34 Ig (anti-Cdc34) or to pA-affiprep coupled to normal rabbit serum Ig (Ctrl) and washed. Sample buffer was added to the beads and boiled. The eluent or 50ug of HeLa PCFT or HeLa PCFT/DEAE 250mM KCl fraction.was

subjected to western analysis with Cullin 1 (Cul1), affinity purified Cdc34 (Cdc34) or p19Skp1 (Skp1) **(B)** Left panel: Xenopus LSS was not depleted (No depl) or depleted with pA-Seph coupled to preimmune Ig (Ctrl), or rabbit 1 anti-hCdc34 Ig (Cdc34) and sperm nuclei was added. Replication (%) is normalized to 100% of that in the control-depleted sample (Ctrl). Right panel: Cdc34 depleted LSS used in the left panel (Cdc34 Depl) was added to pA-affiprep coupled to preimmune Ig (Ctrl Rescue) or anti-hCdc34 Ig incubated in LSS. 200nM Cdc34p with 200nM Cul1/ROC1, 200nM Cdc34p with Cul1/ROC1/Skp1/Skp2 or 200nM Cdc34p with 200nM Casein Kinase 2 (CK2) was added to the Cdc34 depleted LSS and examined for Cdc34 rescuing activity. Rescuing Units were calculated by % replication of the rescue sample/% replication of control rescue.

Figure 6. *Fractionation schematic of Cdc34 rescuing activity in mammalian cell extracts.* **(A)** Fractionation scheme. HeLa cell extracts were fractionated and subjected to Cdc34 rescuing assays in Xenopus Cdc34 depleted extracts. For details see Results. **(B)** Silver Stain SDS-PAGE. Top Panel: (Silver Stain): M indicate molecular weight markers in kDa. .1% of the immunoprecipitation input of the HeLa PCFT/DEAE 150mM KCl fraction (.1% Input). HeLa PCFT/DEAE 150mM KCl fraction was immunoprecipitated with affinity purified anti-Cdc34 (Cdc34 IP) or RIgG (RigG IP) and eluted by acid base. The eluent was concentrated and resolved by SDS-PAGE and silver stained as described in the methods. Bottom Panel (Western). $1/5^{th}$ of the concentrated eluent from each IP (Top panel) was resolved on a SDS-Page gel, transferred to nitrocellulose and examined by western blot for Cdc34 (α-Cdc34 immunoblot.

Figure 1

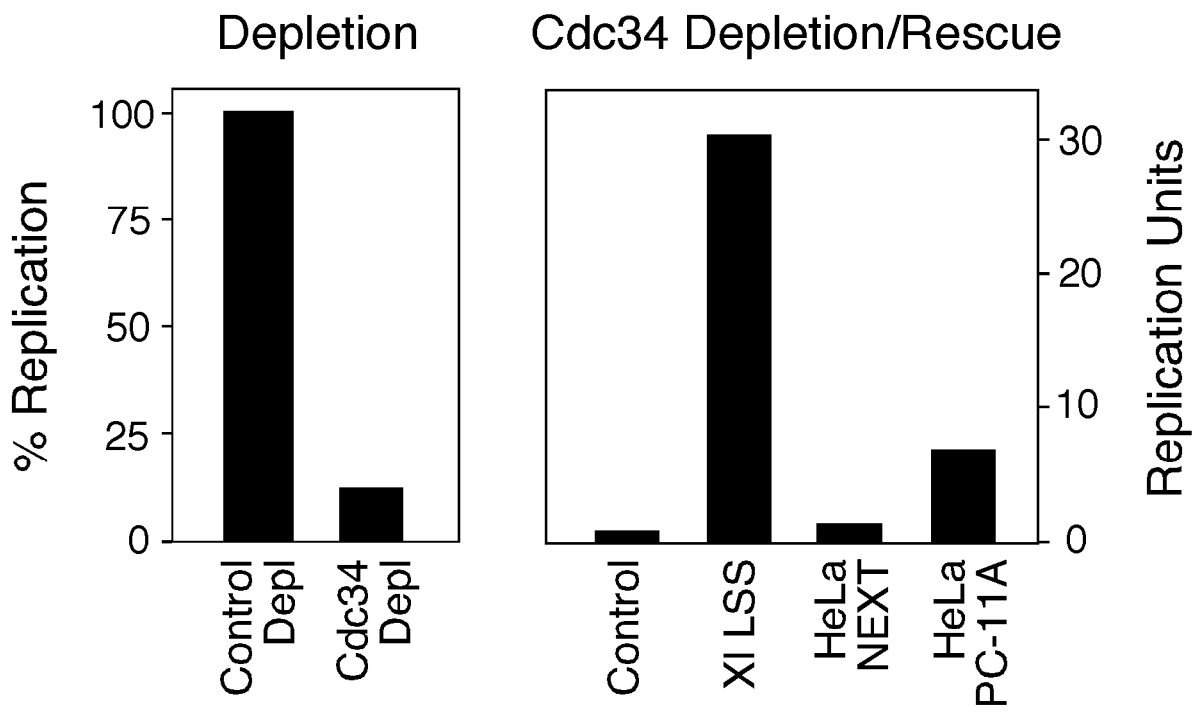
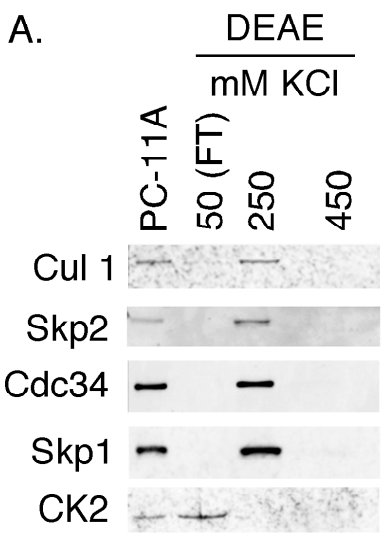
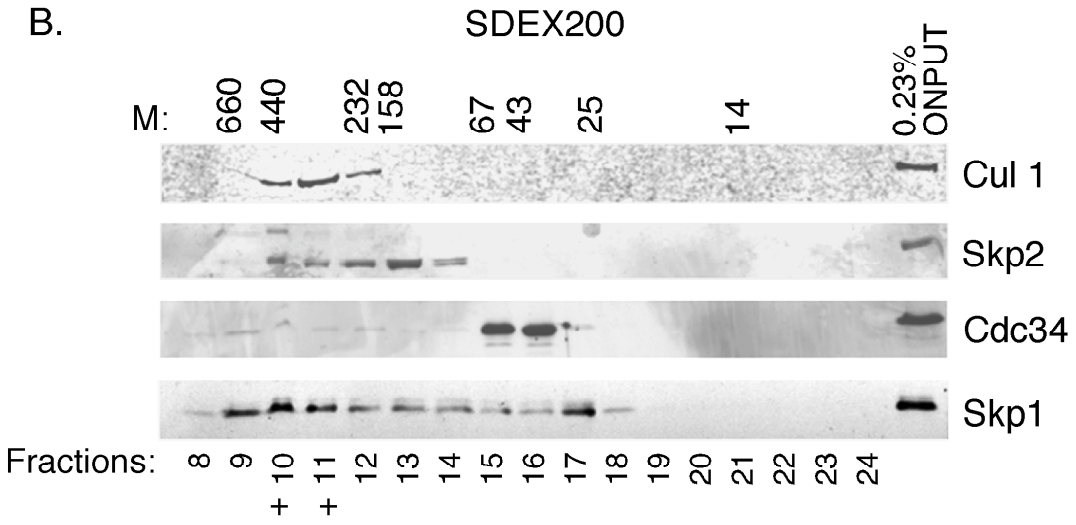
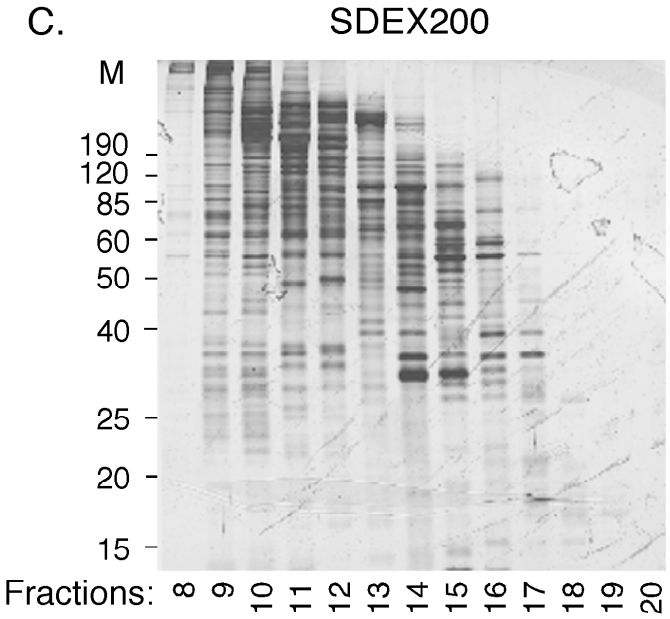

Depletion
Cdc34 Depletion/Rescue
% Replication
100
75
50
25
0
Control Depl
Cdc34 Depl
30
20
10
0
Replication Units
Control
XI LSS
HeLa NEXT
HeLa PC-11A

Figure 2


A.
DEAE
mM KCl
PC-11A
50 (FT)
250
450
Cul 1
Skp2
Cdc34
Skp1
CK2


B.
SDEX200
M:
660
440
232
158
67
43
25
14
0.23% ONPUT
Cul 1
Skp2
Cdc34
Skp1
Fractions:
8
9
+ 10
+ 11
12
13
14
15
16
17
18
19
20
21
22
23
24


C.
SDEX200
M
190
120
85
60
50
40
25
20
15
Fractions:
8
9
10
11
12
13
14
15
16
17
18
19
20

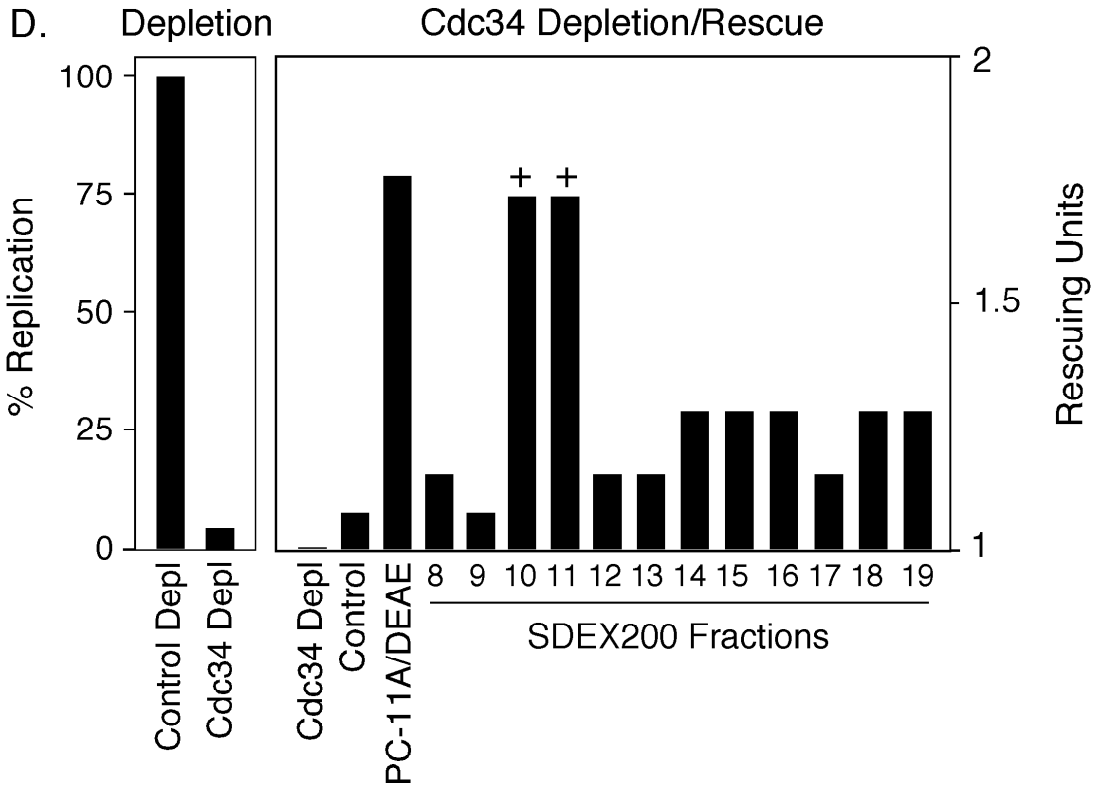

D.
Depletion
Cdc34 Depletion/Rescue
% Replication
100
75
50
25
0
Rescuing Units
2
1.5
1
+
+
Control Depl
Cdc34 Depl
Cdc34 Depl
Control
PC-11A/DEAE
8
9
10
11
12
13
14
15
16
17
18
19
SDEX200 Fractions

Figure 3

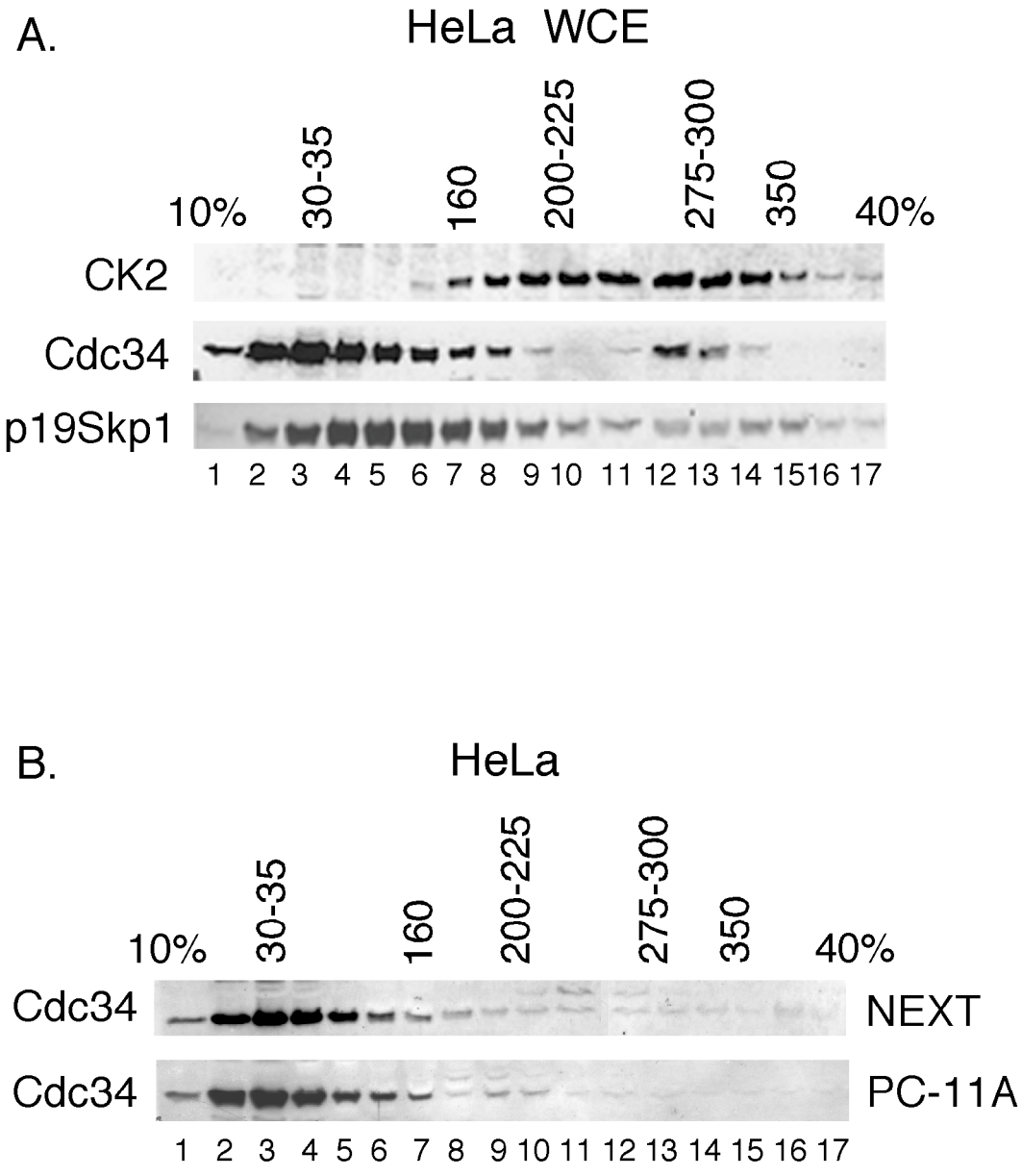

A.
HeLa WCE
10%
30-35
160
200-225
275-300
350
40%
CK2
Cdc34
p19Skp1
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
B.
HeLa
10%
30-35
160
200-225
275-300
350
40%
Cdc34
NEXT
Cdc34
PC-11A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Figure 4

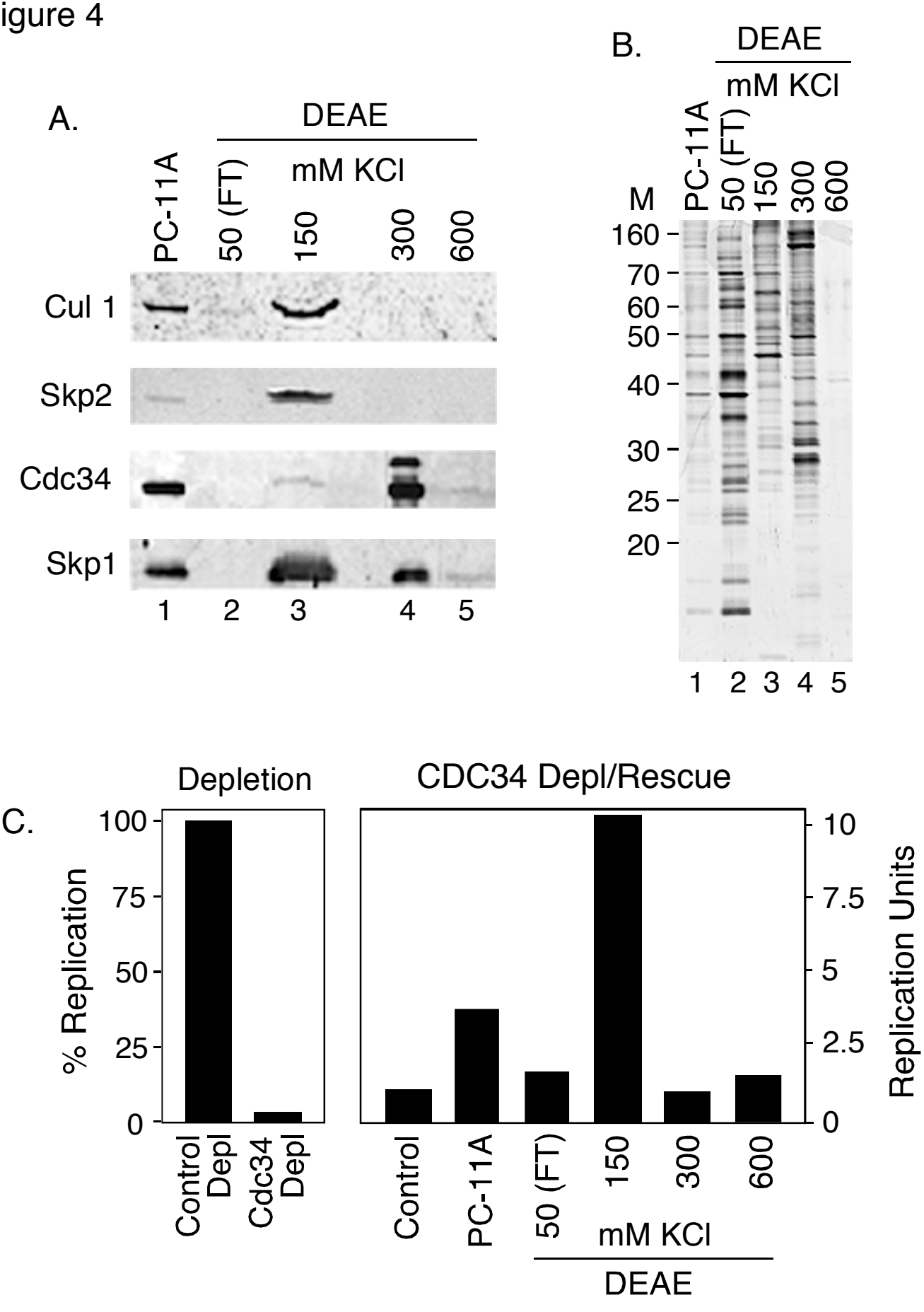
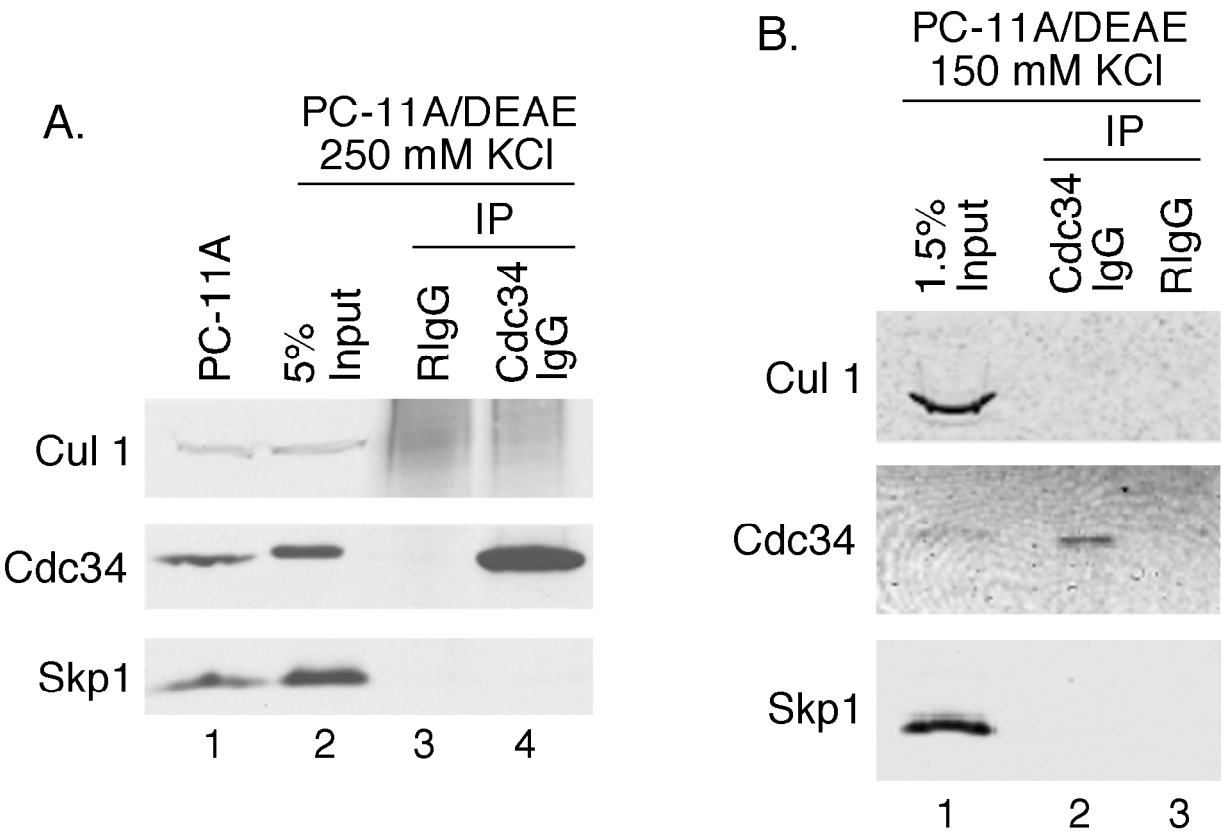

A.
DEAE
mM KCl
PC-11A
50 (FT)
150
300
600
Cul 1
Skp2
Cdc34
Skp1
1 2 3 4 5
B.
DEAE
mM KCl
M
PC-11A
50 (FT)
150
300
600
160
70
60
50
40
30
25
20
1 2 3 4 5
C.
Depletion
CDC34 Depl/Rescue
% Replication
100
75
50
25
0
Control Depl
Cdc34 Depl
Replication Units
10
7.5
5
2.5
0
Control
PC-11A
50 (FT)
150
300
600
mM KCl
DEAE

Figure 5


A.
PC-11A/DEAE
250 mM KCl
IP
PC-11A
5% Input
RIgG
Cdc34 IgG
Cul 1
Cdc34
Skp1
1
2
3
4
B.
PC-11A/DEAE
150 mM KCl
IP
1.5% Input
Cdc34 IgG
RIgG
Cul 1
Cdc34
Skp1
1
2
3

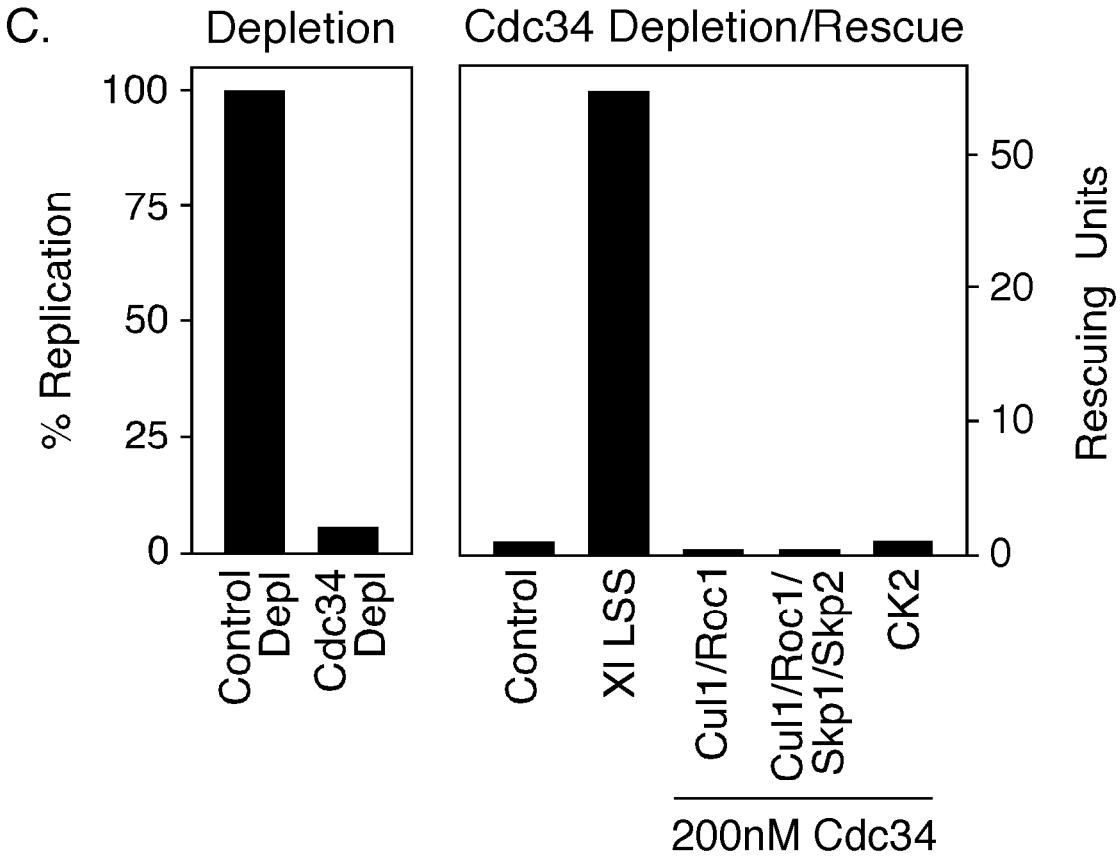
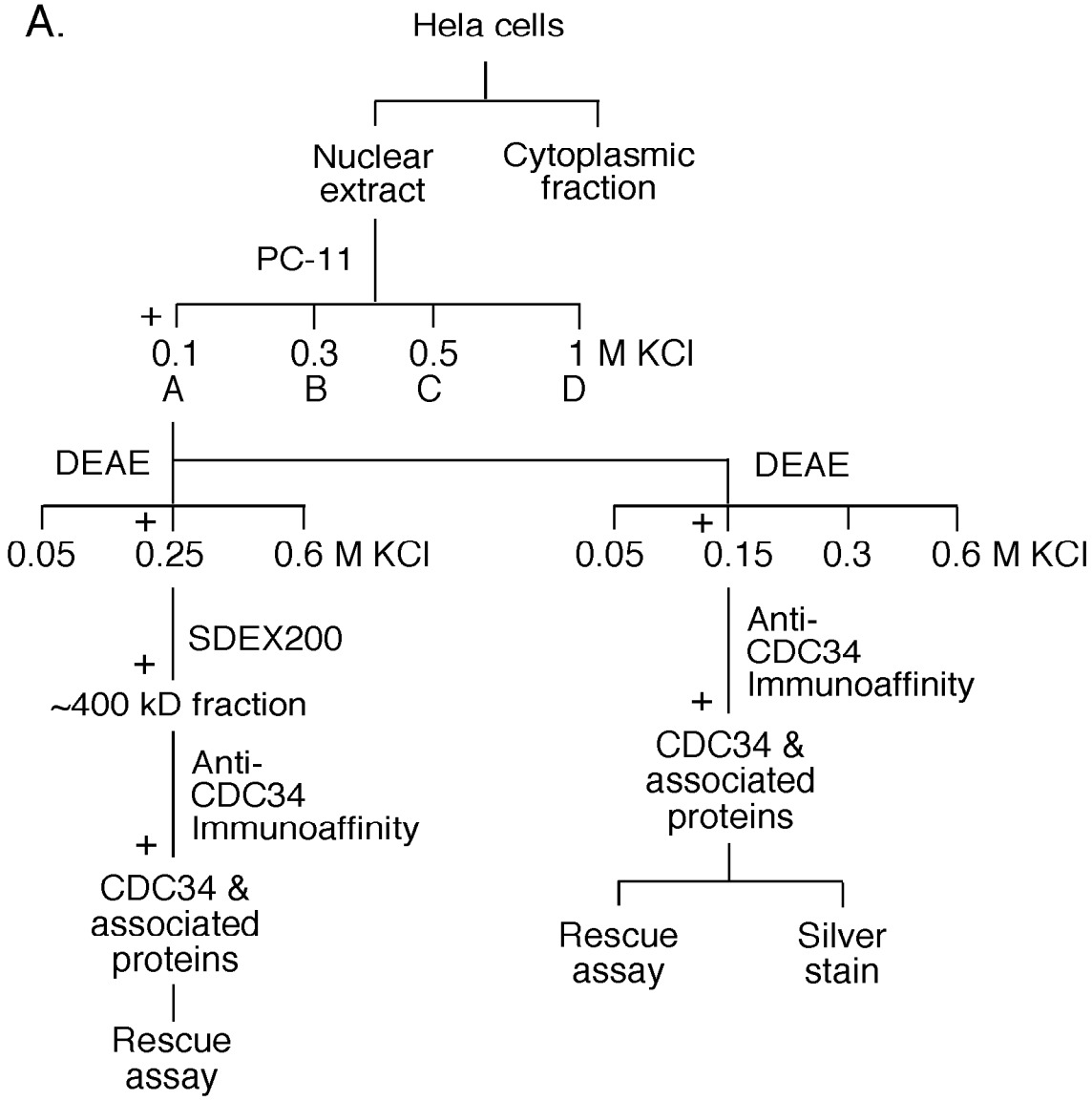
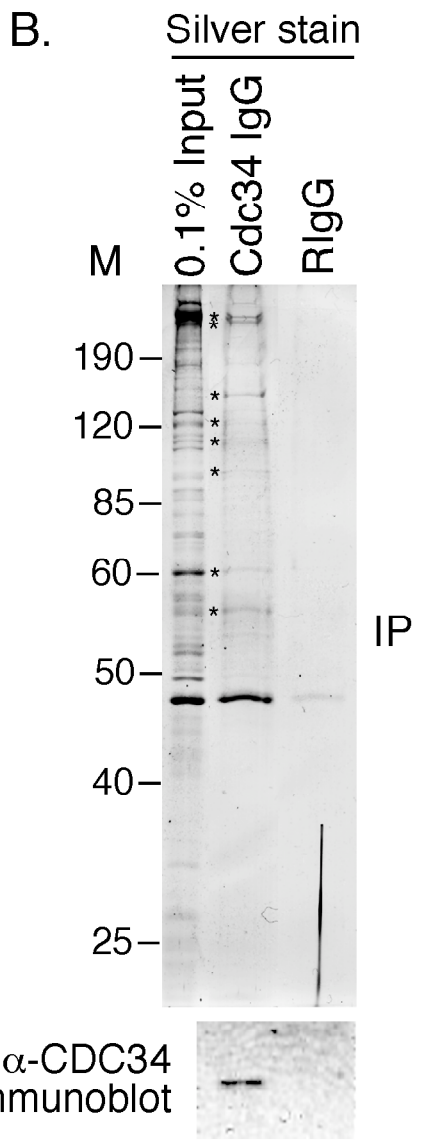

C.
Depletion
Cdc34 Depletion/Rescue
% Replication
100
75
50
25
0
Rescuing Units
50
20
10
0
Control Depl
Cdc34 Depl
Control
XI LSS
Cul1/Roc1
Cul1/Roc1/ Skp1/Skp2
CK2
200nM Cdc34

Figure 6


A.
Hela cells
Nuclear extract
Cytoplasmic fraction
PC-11
+
0.1
A
0.3
B
0.5
C
1 M KCl
D
DEAE
DEAE
+
0.05
0.25
0.6 M KCl
0.05
0.15
0.3
0.6 M KCl
SDEX200
+
~400 kD fraction
Anti-CDC34 Immunoaffinity
+
CDC34 & associated proteins
Rescue assay
Anti-CDC34 Immunoaffinity
+
CDC34 & associated proteins
Rescue assay
Silver stain


B.
Silver stain
M
0.1% Input
Cdc34 IgG
RIgG
190
120
85
60
50
40
25
IP
α-CDC34 immunoblot