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CONTRACTING ORGANIZATION: Baylor College of Medicine
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13. ABSTRACT *(Maximum 200*

The goal of this project was to understand the role of human ubiquitin-conjugating enzyme (UBC), hCdc34 in normal and malignant mammary cells. To elucidate the mode of hCdc34 action in human cells, we used a genetic assay called two-hybrid cloning to identify proteins that interact with hCdc34, screening 1.5 million human cDNAs. Thirty cDNA clones found to be active in this assay were isolated and analyzed. Four of these clones are previously known regulators of meiosis and spermatogenesis, including two that belong to the bZIP family of transcriptional repressors, and one has distinct role in sister chromatid cohesion and DNA double strand break repair. We have authenticated the two-hybrid interactions by clearly demonstrating the targeting of three repressors of cAMP-induced transcription, hICERIIγ, hATF5 and CREMβ by hCdc34 and a structurally similar UBC enzyme, hRad6B for ubiquitin-proteasome mediated proteolysis (Pati et. al., 1999, *Mol Cell Biol* **19**: 5001-13). This study provides novel information that stability of specific mammalian transcription factors is the result of complex targeting by multiple UBC enzymes, and may have far reaching physiologic implications in cAMP-inducible gene regulation which governs diverse cellular processes including cell cycle and oncogenesis. These results will also be critical to designing new therapeutic strategies that could modulate the activity of the Cdc34 enzyme to prevent the growth and/or genesis of breast tumor.

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N/C In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

7/29/99

PI - Signature Date

Table of Contents

Introduction

A feature common to breast cancer and other human malignancies is the alteration of the normal cell division cycle. Therefore, understanding the regulation of the mammalian cell cycle is essential to elucidate the mechanisms of carcinogenesis. A fundamental mechanism by which proteins control the cell cycle is ubiquitin-mediated destruction of regulatory proteins. *CDC34* is an essential gene for the early phases of the cell division cycle in yeast. It encodes a ubiquitin conjugating enzyme required for ubiquitination and proteolytic degradation of many cell cycle regulators including cyclins and cyclin dependent kinase inhibitors (for review see Deshaies, 1997, Krek, 1998). Budding yeast (*S. cerevisiae*) cells mutant in *cdc34* arrest in G1 and are unable to begin DNA replication (Goebl et al., 1988, Byers and Goetsch, 1974). Highly related functional *CDC34* genes in the human and the mouse have been cloned and characterized (Plon et al., 1993, Hemzawi et al., 1995, Pati, D. and Plon, S.E., unpublished), which share significant homology at the protein level with the *S. cerevisiae* Cdc34. These studies have shown that human *CDC34* fully complements *cdc34*^{ts} mutant yeast for growth at the restrictive temperature (Plon et al., 1993), and also rescues the lethality of the *cdc34* null strain (Plon, S.E., unpublished). These results suggest a conserved role for Cdc34 in the cell cycle control of the G1/S transition. Recent studies from a number of laboratories have also indicated a conserved Cdc34 ubiquitin conjugating enzyme (UBC)-Skip1/Cullin/F-Box (SCF) ubiquitin-protein ligase complex functions in both yeast and human cells to mediate ubiquitin-mediated proteolysis of a variety of targets. To elucidate the mode of human Cdc34 action in human cells it was necessary to identify the substrates for this enzyme. Until the present study, very little was known about the *in vivo* targets of Cdc34 in higher organisms, including humans. We have used a yeast-based *in vivo* genetic assay (two-hybrid cloning) to identify proteins that interact with hCdc34. cDNAs found to be active in this assay have been isolated and have been analyzed. In a recent set of studies published in the July issue of "Molecular and Cellular Biology", two of these two-hybrid clones, hICERIIγ and hATF5 have been verified as authentic targets of hCdc34-mediated ubiquitination and degradation (Pati, et. al., 1999). Following is the summary progress on both the first **(Identification of Cdc34 Target Proteins),** and second technical objectives **(Role of Cdc34 and its interacting proteins in carcinogenesis)**. As described in the original proposal, tasks #1-4 were performed over the first year of the grant period and Tasks #5-9 have been attempted in the 2nd and 3rd year. Part of the results have been presented in a number of national and international conferences and a peer-reviewed manuscript has recently been published (Pati et al., 1999).

Body of the Report

Technical Objective #1: Identification of Cdc34 Target Proteins

Isolation and Sequencing of Activation Domain Fusion Plasmids From True Positives of *hCDC34* two-hybrid screen: Success of a two-hybrid screen depends primarily on the production of a properly folded bait-fusion protein in the host cells. pC97-*hCDC34* bait was found to encode a functional hCdc34 protein, as evidenced by its ability to complement a temperature sensitive *cdc34^{ts}* mutant yeast strain, SJ1098-3d. In this assay, unlike the control plasmid (pPC97), the pPC97-*hCDC34* bait construct was able to completely suppress the *cdc34^{ts}* mutation, allowing growth at 37^{0}C. Suppression of the cdc34 ^{ts}-mutation suggests that the hCdc34 bait forms a functional complex with the recently described E3 (ubiquitin-protein ligase complex), Skp1/Cullin/F-box (SCF) in yeast. Production of *GAL4*-hCdc34 fusion protein in host two-hybrid yeast was also confirmed in Western blot using a monoclonal hCdc34 antiserum. In the screen of 1.5 million transformants 30 clones were found positive for all the reporters (His/3AT+, Ura+, FOA-, Xgal+) tested whereas 60 others were partially positive (3AT+, Ura+, FOA +/-, X-Gal +/-). Out of the 30 clones, 18 clones have been sequenced; seven of them implicated in control and proliferation of cell cycle and four are novel clones. However, none of these 18 sequences represent human cDNAs encoding products with significant homology to the members of the SCF complex, cyclins, or cyclin-dependent kinase inhibitors. Surprisingly, four of these 18 positive clones represent genes which have been previously identified in the regulation of meiosis and spermatogenesis, one also has a distinct role in DNA double strand break repair and sister chromatid cohesion. These genes are also known to be expressed in lymphocytes, presumably due to the requirement for recombination of immunoglobulin and T cell receptor genes in lymphocytes.

ICER IIγ and hATF5 are Targets of Cdc34: Three of the clones found to interact genetically with hCdc34 in the above 2-hybrid screen are members of bZIP family namely, ICERIIγ , ATF5 (Genbank Accession # AF101388) and Clone #30-17. ICERIIγ, is an ICER isoform generated by alternate splicing, It encodes a 108 aa protein and lacks the characteristics γ-exon and one of the DNA binding and dimerization domains. ICERIIγ has 97.2% homology with the mice isoform ICERIIγ and 76.7% with the human ICERI, the 120aa full length ICER protein. hATF5 is a novel clone, a partial sequence of its homolog in mouse has been reported as ATFX (Nishizawa and Nagata, 1992). hATF5 clone encoded a 122 aa protein, has extensive homology in the C-terminal domain with human (52.1%) and mouse ATF4 (50.9%). Mouse ATFX and human ATF5 are 96.6% homologous in their available sequence. In the leucine zipper domain, hATF5, like that of mouse has only three leucines instead of five that are present in ATF4 and ATF3, with the last two leucines replaced by valines.

Independent Biochemical Conformation of Authentic Two-Hybrid Interactions: Interaction between hCdc34 and two of the interactors identified in the 2-hybrid assay, belonging to the b-ZIP trascriptional repressor family, hICERIIγ and hATF5 have been verified through a variety of *in vivo* and *in vitro* biochemical assays (Pati et al., 1999, manuscript attached). A few specific highlights of these sets of studies include:

- In both human and yeast cells, Cdc34 (UBC2) and the structurally similar Rad6B (UBC3) enzyme target bZIP transcription factor for ubiquitination.
- In mammalian cells, repressors of cAMP-induced transcription are degraded by these UBC's. Consistent with these results, the endogenous repressor protein ICER is elevated in cells which are null for murine Rad6B (*mHR6B$^{-/-}$*) or transfected with antisense and dominant negative constructs of hCDC34.
- The level of Cdc34 and Rad6B is rate limiting for degradation of these substrates.
- Other processes regulated by ICER/ATF repressors that Cdc34 and/or Rad6B may modulate, include proliferation through regulation of Cyclin A transcription.

Thus, in diverse eukaryotes the stability of specific transcription factors is the result of complex targeting by multiple UBC's and this process impacts on cAMP-inducible gene regulation during both meiotic and mitotic cell cycles. Our data also suggests that for ubiquitination of some proteins, although specificity of targeting is mediated by the E3 complex the rate of targeting is dependent on the availability of the UBC enzyme.

hRad21, a Potential Interactant of hCdc34: In the two-hybrid screen Clone# 28C was identified as a strong interactor of hCdc34. Sequence analysis revealed that Clone#28C is a homolog of fission yeast (*S. pombe) rad21* gene, implicated in DNA repair and mitosis (Birkenbhil and Subramani, 1992, 1995, Tatebayashi et al., 1998), and is highly conserved among eukaryotic organisms (McKay et al., 1996). However, the specific role of Rad21 was not revealed until recently. Both genetic and biochemical studies in the budding yeast *S. cerevisiae* and frog, *Xenopus* respectively, have identified homologs of fission yeast Rad21 protein (called Scc1/Mcd1) as a key subunit in sister chromatid cohesion during DNA replication (Guacci et al., 1997, Losada et al., 1998). In *S. cerevisiae*, loss of cohesion at the metaphase-anaphase transition is accompanied by the proteolytic cleavage of Scc1/Mcd1 protein (Uhlmann et al., 1999) followed by the dissociation from chromatid (Michaelis et al., 1997). Recent evidence indicates that this process depends on Esp1 protein (called separin), which is complexed with its inhibitor Pds1 before anaphase (Ciosk et al., 1998). It has been proposed that at the metaphase-anaphase transition, the anaphase-promoting complex (APC), an ubiquitin-protein ligase (E3) triggers proteolysis of Pds1, which in turn liberates Esp1's function that lead to the dissociation of Scc1/Mcd1 from sister chromatids. Although the dissociated Scc1/Mcd1 is subsequently degraded, the exact mechanism and its functional significance is not fully understood. In summary, Scc1/Mcd1/Rad21 plays a critical role in the eukaryotic cell division cycle by regulating the sister chromatid cohesion and its separation at the metaphase to anaphase transition.

Presently very little is known about the physiologic role of Rad21 in higher vertebrates and its impact in human malignancies. In this regard, our finding represents unique and novel information on a link between hCdc34 and hRad21. We have made a number of reagents to study the interaction between hCdc34 and hRad21 as well as hRad21 function in the human cell cycle and its role, if any, in breast tumorigenesis. Several constructs of the hRAD21 in myc, flag and HA epitope vectors have been constructed. A polyclonal antisera using the C-terminal 14 amino acids of hRad21 has been raised. A stably transfected hRAD21 cell line is under development. Preliminary experiments using Rad21 transfected Jeg3 cells have following results:

1) Co-immunoprecipitation assays provides indication for the physical association of both hCdc34 and hRad21.
2) hRad21 protein level is significantly enhanced in the presence of proteasome inhibitor MG115 and MG132.

In addition, hRad21 is differentially expressed in a number of breast cancer derived cell lines in comparison to the normal breast epithelial cells (see below). Currently our focus has been to dissect the role of hCdc34 interaction with rad21in the sister chromatid cohesion and separation. One hypothesis is that Cdc34 may facilitate the degradation of the dissociated Rad21/cohesin complex after sisters are separated following its proteolytic cleavage at the onset of anaphase, and thereby striping the chromosome of any residual cohesin which then allows the cells to proceed for next round of cell division. This would help prevent misalignment of sisters during mitosis resulting in aneuploidy. We are presently testing this hypothesis in our cell culture model. Our ultimate aim is to identify the role of this interaction if any, in the development of human malignancies including breast cancer.

Technical Objective#2: Role of Cdc34 and its interacting Proteins in Carcinogenesis

Study of the Structure and Expression of hCDC34 and its partner proteins in Malignant Mammary Cells: In order to characterize the hCdc34-mediated regulation of its interactors and their physiologic role in the cell cycle progression and development of malignancy, initially we decided to study the expression pattern of hCDC34 and its interactors in a panel of breast cancer cell lines, including MCF10, MCF7, MDA-MB-157, MDA-MB-231, MDA-MB-136, BT-20, HBL100, and SKBR-3. These cell lines were chosen due to previous analysis of the expression and structure of other human cell cycle genes including BRCA1 and Cyclin A, B1, D1, and E (Band et al., 1986, Keyomarsi et al., 1993, Gudas et al., 1996). This panel includes both estrogen receptor positive and negative lines and vary in tumorigenecity in animal models. Choice of a normal control for expression studies is more difficult. We have used both the MCF-10F (immortal but not tumorigenic) cell line and normal human mammary epithelial cells, HMEC, obtained from Clonetics Corp. (San Diego, CA). These cell lines were maintained under similar conditions as recommended by ATCC in DFCI media. DNA, RNA and proteins from these cells were isolated as described above in the methods section. The expression pattern of hCDC34 and its 2-hybrid interactors were examined using Northern analysis. hCdc34 is found to be expressed in all the breast cancer lines tested. No apparent variation in the transcripts was observed compared to its expression in HMEC and MCF-10F cells. However, preliminary studies involving Western analysis of the proteins isolated from these cells using a monoclonal hCdc34 antibody indicated lower level of immunoreactive protein in MDA-MB-436 and HBL-100 in comparison to other breast cancer cells.

In a recent set of studies we have examined the expression pattern of fourteen of hCdc34 interactors including ICERIIγ, ATF5, Clone #28C (Fission yeast Rad21 homolog), and #42-2 (Leukemia cell differentiation factor) in these breast cancer cell lines. Using Northern analysis we were unable to detect the expression of ICERIIγ in these cell line. We plan to use more sensitive RNAse protection techniques to monitor their expression. However, clone #28C, #42-2 and ATF5 appear to have altered expression patterns. Compared to the normal H-MEC cells, clone#28C appeared to be overexpressed in MDA-MB-436 and SKBR-3, and clone#42-2 in SKBR-3 and MDA-MB-157. However, ATF5 on the other hand is found to be down regulated in the MDA-MB-436. It is interesting to note that, in MDA-MB-436 cells, hCdc34 protein is down regulated and clone #28C transcripts are up regulated. Further experiments will be performed to elucidate the altered differences in the expression of hCdc34 and its interactors in this cell line. Expression pattern of other interactors are being currently examined in this panel of breast cancer cell lines.

Discussion: We have demonstrated that mammalian Cdc34 ubiquitin conjugating enzyme, a protein essential for cell cycle progression in yeast, has a wide variety of potential targets in humans, including regulators of transcription, meiosis, spermatogenesis, cell proliferation, sister chromatid cohesion and DNA repair. This is analogous to the finding in yeast, where yeast Cdc34 has diverse targets ranging from cell cycle regulators (Krek, 1998) to the regulators of amino acid biosynthesis (Kornitzer et al., 1994). The present study is the first demonstration of the targeting of potent transcriptional repressors by human Cdc34, and may have physiologic implications in regulating transcription. Here we have clearly demonstrated that members of the CREM/ICER/ATF family, belonging to the bZIP transcriptional factors, can be targeted by the Cdc34/Rad6B ubiquitin conjugating enzyme for degradation. ICERIIγ, a known ICER isoform, and hATF5, a novel gene isolated in the present study are potent repressors of cAMP-induced transcription. Both ICERIIγ and ATF5, interact in a two-hybrid screen as well *as in vivo* with Cdc34 and Rad6B. In addition to the genetic evidence in yeast, several observations supported the notion that both these transcriptional factors are targeted by hCdc34 for ubiquitination and subsequent degradation via 26S proteasome. First, co-expression of the ICERIIγ or hATF5 and hCDC34 or hRAD6B in mammalian cells resulted in significant and sometimes complete loss of the target proteins, which could be reversed in the presence of proteasome inhibitors. Second, co-expression of a hcdc34

mutant, deficient in the active site of the enzyme was unable to destabilize the targets. Third, ubiquitin conjugates of target proteins are formed in the presence of ectopic ubiquitin and peptide-aldehyde that inhibits proteasome function. Fourth, the steady state loss of the target protein is enhanced considerably in the presence of hCdc34 and hRad6B and evidenced from the lower half-life of the targets. In addition, these findings have been strengthened by a recent report suggesting the involvement of ubiquitin-proteasome pathway in the degradation of ICER protein (Koren et al., 1997) in primary cardiocytes and myogenic cell lines. ICER turn over and its degradation via ubiquitin-proteasome pathway may act as a regulatory mechanism to relieve the repression and to control the negative effect of ICER on the cAMP-inducible transcriptional response. Ubiquitin-mediated degradation of transcriptional repressors, ICER and ATF5 proteins may therefore have far reaching implications in rapidly turning on transcriptions by destruction of repressors as opposed to the production of transcriptional activators. These results highlight the role of ubiquitination in regulating the steady state levels of potent transcriptional repressors of cAMP-induced transcriptional responses and signal transduction. This work has been presented at national and international meetings and a manuscript (attached) published. Acknowledgement of support from the US Army fellowship was included.

From our studies involving the expression of Cdc34 and its targets it is evident that Cdc34 and some of its targets (e.g. ATF5 and hRad21) are differentially expressed in the breast cancer cell lines compared to the normal breast epithelial cells. Further studies are in progress to elucidate such differences and their significance in the development of breast cancer. In this context it has becoming increasingly evident that the ubiquitin-proteasome pathway is often the target of cancer-related deregulation and can underlie processes, such as oncogenic transformation, tumor progression, escape from immune surveillance and drug resistance (reviewed in Spataro et al., 1998).

Conclusion: Knowledge gained about mammalian Cdc34 function and its interacting partners will be instrumental in understanding how selective destabilization of proteins through ubiquitination regulates both the meiotic and mitotic cell division cycles and the role of Cdc34 in the development of breast cancer and oncogenesis in general. Eventually this knowledge can be used to develop therapies which modulate the stability of tumor suppressor and oncogene proteins.

Key Research Accomplishments

- Identification of 18 potential targets of human Cdc34 ubiquitin-conjugating enzyme using yeast two-hybrid system. These interactants include a wide variety of proteins in human, including regulators of transcription, meiosis, spermatogenesis, cell proliferation, sister chromatid cohesion and DNA repair. These findings hilight that two-hybrid screens are capable of identifying the potentially transient interaction between a ubiquitin-conjugating enzyme (UBC) and its targets.
- Two of the targets that have been characterized are repressors of cyclic AMP-induced transcription: hICERIIγ, a product of the CREM gene, and hATF5, a novel ATF homolog. Both human Cdc34 and the tructurally similar UBC enzyme hRad6B can target these bZIP transcriptional repressors for degradation, which parallels the Cdc34/Rad6B-mediated degradation of Gcn4 in yeast. Thus, in diverse eukaryotes the stability of specific transcription factors is the result of complex targeting by multiple UBC's and this process impacts on cAMP-inducible gene regulation during both meiotic and mitotic cell cycles.
- hRad21, a protein critical for sister chromatid cohesion during DNA replication is a potential target of hCdc34, which is also found to be upregulated in a number of breast cancer cell lines. Our finding represents unique and novel information on a link between hCdc34 and hRad21
- Cdc34 and some of the targets (e.g. ATF5 and hRad21) are differentially expressed in the breast cancer cell lines compared to the normal breast epithelial cells.
- Polyclonal antisera against hRad21 have been raised and a monoclonal hRad21 antiserum is under development.

Reportable Outcomes

Manuscript:

Pati, D., Meistrich, M.L., Plon, S.E. (1999). Human Cdc34 and Rad6B ubiquitin-conjugating enzymes target repressors of cyclic AMP-induced transcription for proteolysis. *Molecular and Cellular Biology*, **19**: 5001-5013.

Pati, D., Vidal, M., Plon, S.E. (1997). Identification of novel targets of the human cell cycle regulatory protein Cdc34, an ubiquitin ligase using yeast reverse two-hybrid system. In the proceedings of the Department of Defense Breast cancer Research Program Meeting "Era of Hope" Vol.-III: 463-64.

Abstract:

Pati, D., Meistrich, M. L., and Plon, S.E. (1999). Human Cdc34 and Rad6B ubiquitin-conjugating enzymes target repressors of cAMP-induced Transcription for proteolysis. Abstract book of the Yeast Genetics and Human Disease II (American Society of Microbiology), Vancouver, British Columbia, June 24-27, 1999, p108.

Pati, D., Meistrich, M. L., and Plon, S.E. (1999). Human Cdc34 and Rad6B ubiquitin-conjugating enzymes targetbZIP transcriptional repressors for proteolysis: implications for spermatogenesis. Abstract book of the Biology of Proteolysis, Cols Spring Harbor Laboratory, NY, May 5-9, 1999, p83.

Pati, D., and Plon, S.E. (1998). Regulators of meiosis and spermatogenesis as potential targets of human Cdc34 ubiquitin ligase. Abstract# 327, Abstract book of the Cell Cycle meeting, Keystone Symposia, Keystone, CO, March 27-April 2, 1998, p70.

Plon, S.E., and **Pati, D**. (1997). Identification of meiotic cell cycle regulators as potential targets of the human Cdc34 ubiquitin ligase. *Am. J. Human Genetics*: **61**: A160.

Presentations:

Pediatric Research Conference, Department of Pediatrics, Baylor College of Medicine
Topic: Ubiquitin-Mediated Proteolysis: Regulator of Diverse Physiological Processes. April 29, 1999

Reproduction and Development Workshop, Department of Cell Biology, Baylor College of Medicine
Topic: Cdc34 ubiquitin ligase-mediated targeting of bZIP transcriptional repressors: implication for meiosis and spermatogenesis. October 15, 1998.

Funding:

Based on the work supported by this award, I have been succeeded in getting a special fellowship from the Leukemia Society of America for a period of July 1999-2002 to further investigate the physiologic role of human Cdc34 in leukemia and other human malignancies. Mouse knockout models with mutant Cdc34 is expected to be developed in the course of this project. In addition, a few selected Cdc34 interactants obtained in the above two-hybrid screen will be studied for their role in tumorigenicity.

Employment:

Based on the progress supported by this award, I have been promoted to the rank of assistant Professor as of July 1, 1999 in the Department of Pediatric, Hematology/Oncology at the Baylor College of Medicine, Houston, TX.

Appendices

Appendix-I

Tasks Completed:

Technical objective #1: Identification of Cdc34 target proteins.

Task 1: Month 1-3: Cloning of *CDC34* cDNA (the bait) into DNA-binding domain vector pAS2 and determination of stable expression in yeast.

Task 2: Month 2-3: Excision of the library (the prey) encoding candidate interacting proteins fused to the activation domain from λACT.

Task 3: Month 4-6: Transformation of library plasmids into yeast host strains containing bait with sequential selection.

Task 4: Month 6-12: Determination and elimination of false positives.

Task 5: Month 10-16: Isolation and sequencing of the activation-domain fusion plasmids from true positives.

Task 6: Month 14-24: Independent biochemical confirmation of authentic two-hybrid interactions between Cdc34 and two of its interactors, ICERIIγ and ATFX..

Technical objective #2: Role of Cdc34 and its interacting proteins in carcinogenesis.

Task 7 & 8: Month 18-28: Study of the structure and expression of human *CDC34* and its novel partner proteins in malignant mammary cells.

Task 9: Month 28-36: Creation of epitope-tagged partner proteins and expression in a breast carcinoma cell line.

Appendix-II

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Appendix-III

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Human Cdc34 and Rad6B Ubiquitin-Conjugating Enzymes Target Repressors of Cyclic AMP-Induced Transcription for Proteolysis

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Ubiquitin-mediated proteolysis controls diverse physiological processes in eukaryotes. However, few in vivo targets of the mammalian Cdc34 and Rad6 ubiquitin-conjugating enzymes are known. A yeast-based genetic assay to identify proteins that interact with human Cdc34 resulted in three cDNAs encoding bZIP DNA binding motifs. Two of these interactants are repressors of cyclic AMP (cAMP)-induced transcription: hICERIIγ, a product of the *CREM* gene, and hATF5, a novel ATF homolog. Transfection assays with mammalian cells demonstrate both hCdc34- and hRad6B-dependent ubiquitin-mediated proteolysis of hICERIIγ and hATF5. This degradation requires an active ubiquitin-conjugating enzyme and results in abrogation of ICERIIγ- and ATF5-mediated repression of cAMP-induced transcription. Consistent with these results, the endogenous ICER protein is elevated in cells which are null for murine Rad6B (*mHR6B*$^{-/-}$) or transfected with dominant negative and antisense constructs of human CDC34. Based on the requirement for CREM/ICER and Rad6B proteins in spermatogenesis, we determined expression of Cdc34, Rad6B, CREM/ICER isoforms, and the Skp1–Cullin–F-box ubiquitin protein ligase subunits Cul-1 and Cul-2, which are associated with Cdc34 activity during murine testicular development. Cdc34, Rad6B, and the Cullin proteins are expressed in a developmentally regulated manner, with distinctly different patterns for Cdc34 and the Cullin proteins in germ cells. The Cdc34 and Rad6B proteins are significantly elevated in meiotic and postmeiotic haploid germ cells when chromatin modifications occur. Thus, the stability of specific mammalian transcription factors is the result of complex targeting by multiple ubiquitin-conjugating enzymes and may have an impact on cAMP-inducible gene regulation during both meiotic and mitotic cell cycles.

A fundamental mechanism for control of cellular processes is ubiquitin-mediated destruction of regulatory proteins. *CDC34*, a gene essential for the transition into S phase of the cell division cycle in *Saccharomyces cerevisiae* (6, 20), encodes a ubiquitin-conjugating (UBC) enzyme (named UBC3). Cdc34 is required for ubiquitination and proteolytic degradation of cyclins and cyclin-dependent kinase inhibitors (for reviews, see references 14 and 36). The Cdc34 protein consists of a highly conserved catalytic domain common to all UBC enzymes, including the DNA repair protein Rad6 (23, 30), and a unique carboxy-terminal extension or tail (20), which is essential for cell cycle function (34, 64). A chimera consisting of the Rad6 catalytic domain and the Cdc34 carboxy terminus can fulfill some of the in vivo function of both proteins (34, 64). Yeast *RAD6* (UBC2) has been implicated in DNA repair, induced mutagenesis, retrotransposition, sporulation, and the degradation of proteins with destabilizing N-terminal amino acid residues (15, 25, 37, 67).

Highly related *CDC34* genes in humans and mice have been cloned and characterized (26, 53, 55). Human *CDC34* (*hCDC34*) can fully complement a *cdc34-1* mutant strain for growth at the restrictive temperature (55). Similarly, both human *RAD6* homologs (*hRAD6A* and *hRAD6B*) can complement the DNA repair and mutagenesis deficiencies of *rad6*Δ strains (33). Disruption of the mouse homolog of the *hRAD6B* gene results in partial arrest of gametes at the postmeiotic spermatid stage and alteration of postmeiotic chromatin remodeling (57). The role of *hRAD6B* in spermatogenesis appears to be indirect, since its deletion does not cause a complete and uniform block at a given point of spermatogenic differentiation.

Ubiquitination is a process in which ubiquitin, a small polypeptide, is covalently attached to a cellular protein and normally results in proteasome-dependent proteolysis (for reviews, see references 24 and 29). The UBC enzyme transfers the ubiquitin from a ubiquitin-activating enzyme (E1) to specific target proteins, in some cases requiring a third factor, the ubiquitin-protein ligase (E3), to mediate transfer (for reviews, see references 27 and 54). The yeast Cdc34 UBC enzyme is recruited to a large E3 complex called Skp1–Cullin–F-box protein (SCF) by interaction with the Cullin protein Cdc53 (32, 36, 54).

In *S. cerevisiae*, Cdc34 in conjunction with the SCF complex ubiquitinates cell cycle regulators (14) and other diverse substrates (for a review, see reference 61), including transcription factor Gcn4 (35). Phosphorylation of at least some Cdc34 targets (e.g., Sic1 and Cln2) is a prerequisite for its recognition and subsequent degradation by Cdc34-mediated ubiquitination (39, 63). The yeast Cdc34 and Rad6 enzymes appear to share certain targets, including Gcn4 (35). However, it has been difficult to identify specific targets of the mammalian enzymes because of the large number of UBC enzymes and the lack of cells containing conditional or null UBC alleles. We have used a yeast-based in vivo assay (two-hybrid cloning) to identify proteins that interact with mammalian Cdc34. As described here, two of the hCdc34 interactants obtained in this assay contain bZIP motifs and are repressors of cyclic AMP (cAMP)-induced transcription: inducible cAMP early repressor (ICER) and activating transcription factor 5 (ATF5).

In eukaryotes, cAMP-mediated transcription regulates multiple physiological processes, including gametogenesis, circadian rhythm, and neuroendocrine functions (for a review, see

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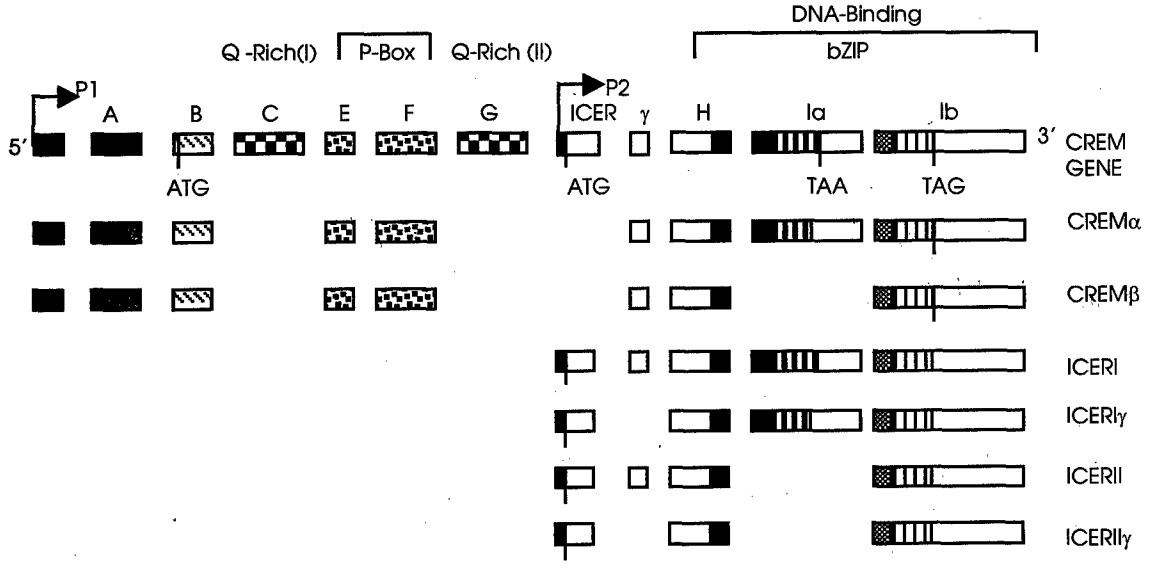

FIG. 1. Schematic representation of the *CREM/ICER* gene structure. The *CREM* repressor isoforms, *CREM* α and *CREM* β, and *ICER* isoforms are indicated. The P1 promoter is GC rich and directs constitutive expression; the P2 promoter is strongly inducible by activation of the PKA-cAMP-dependent signaling pathway (modified from reference 38, printed with permission of the Royal Society).

reference 60). Stimulation of this pathway is mediated via phosphorylation by protein kinase A (PKA) of a single serine in the structurally similar transcription factors called cAMP-responsive element (CRE) binding proteins (CREB), CRE modulators (CREM), and ATFs. Transcription factors which regulate the response to cAMP belong to the bZIP family and bind as dimers to an 8-bp pallindromic DNA consensus sequence called the CRE (59). The *CREM* gene is controlled by two promoters and results in a large number of alternately spliced transcripts encoding activators and repressors of cAMP-dependent transcription that are expressed in a tissue- and developmentally regulated manner (Fig. 1). The upstream promoter (P1) directs the CREM τ, τ1, and τ2 activators and the CREM α, β, γ, and S repressors. The downstream intronic promoter (P2) directs the potent early repressors of cAMP-induced transcription (ICER) (18, 19, 38, 48). Four types of ICER transcripts (*ICERI*, *-Iγ*, *-IIγ*, and *-II*) are generated by alternate splicing of the DNA binding domain and γ domain exons (48). ICER proteins are small (estimated molecular mass, ~13 kDa) and, unlike CREM, lack both the phosphorylation box (P box) and activation domain and escape from PKA-dependent phosphorylation. The principal determinant of ICER activity is not its degree of phosphorylation but its intracellular concentration (48), which depends on the transcription rate of the P2 promoter and the degradation rate of the ICER polypeptide (48, 49).

CREM/ICER isoforms play a regulatory role in gene expression in haploid germ cells in mammals (for reviews, see references 11 and 12) and have recently been implicated in spermatogenesis in humans (40). CREM activates a number of testis-specific promoters of haploid cell-expressed genes. *CREM* gene products are highly abundant in adult testis, and their expression follows a developmental and quantitative switch (19); the activator forms are the dominant forms in postmeiotic germ cells, while the repressor forms are observed at only low levels before meiosis (12). Targeted disruption of the *CREM* gene in mice (5, 50) results in abrogation of spermatogenesis at the spermatid stage.

Here, we report that an hICER isoform (hICERIIγ) and a new ATF homolog (hATF5) are targeted by both hCdc34 and hRad6B UBC enzymes for degradation in vivo. Both Cdc34 and Rad6B proteins are expressed at high levels in pre- and postmeiotic germ cells, and targeting of CREM/ICER and ATF proteins by Cdc34 and Rad6B UBC enzymes may have a role in spermatogenesis. Thus, complex targeting of these repressors by multiple UBC enzymes may have a major impact on cAMP-dependent gene regulation during both meiotic and mitotic cell cycles.

MATERIALS AND METHODS

Two-hybrid reagents. Reagents used in the two-hybrid screening include the Gal4 activation domain library, the Gal4 DNA binding domain vector (pPC97), the yeast host strain MV103 (*MAT***a** *leu2 trp1 his3 gal1::HIS3 gal1::lacZ SPAL::URA3*), and five constructs in MV103 for use as reference controls during screening (69, 70) and were kindly provided by M. Vidal (Massachusetts General Hospital Cancer Center, Charlestown).

Construction of the DNA binding domain-Cdc34 fusion (bait). Human *CDC34* cDNA (pKS-6110) (55) was digested with *Not*I followed by *Sma*I at the codon encoding the first methionine and inserted in frame into pPC97 (*LEU2*). The junction between *GAL4* and *hCDC34* has been verified by sequence analysis. The hCdc34C93S active-site mutant was generated from pKS-6110 by standard PCR mutagenesis methods (28), and the mutation was confirmed by sequencing. The mutant was then subcloned into pPC97 in a manner analogous to that for the *hCDC34* bait construct described above. The *cdc34-1* mutant yeast strain SJ1098-3d (*MAT***a** *cdc34-2 leu2-3 ura3 trp1*) was obtained from B. Byers, University of Washington, Seattle.

Activation domain cDNA library. A human T-lymphocyte cDNA fusion library in the activation domain vector pPC86 (Trp^+) was kindly provided by J. La Baer (Massachusetts General Hospital Cancer Center). The cDNAs were cloned into the *Eco*RI (5′) and *Spe*I (3′) sites. This library has approximately 2×10^6 clones, and the average insert size is 1 kb. The library was amplified once by electroporation with electrocompetent *Escherichia coli* JS4 cells (Bio-Rad, Hercules, Calif.) followed by replica plating onto Luria-Bertani agar–ampicillin plates. The DNA was prepared by using a Plasmid Maxi kit from Qiagen (Valencia, Calif.).

Selection of *hCDC34*-interacting genes. The bait (*LEU2*) and the library plasmid (*TRP1*) were sequentially transformed into the yeast host strain MV103 by using the modified Li-acetate transformation protocol of Schiestl and Giets (62) with yeast total RNA and denatured salmon sperm DNA as the carrier to achieve a transformation efficiency of 300,000 colonies/μg of plasmid DNA. Three independent pools of library DNA were used to transform the MV103(pPC97-*hCDC34*) cells, and 500,000 transformants from each pool were obtained. The two-hybrid screen was performed by first selecting for growth of hCdc34 bait-library cotransformants on SC-His-Leu-Trp plus 25 mM 3-amino-1,2,4-triazole (3AT) (Sigma). Subsequently, activities of the additional reporter genes *URA3* and *lacZ* in the 3AT-positive clones were determined. *URA3* gene positive selection was on uracil-deficient medium and negative selection medium with 0.1% 5-fluoro-orotic acid. Induction of the *lacZ* gene was assayed qualitatively in

the presence of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for blue colonies (58). The cDNA inserts of positive clones were PCR amplified and cloned into a pPCRII vector by using a TA cloning kit from Invitrogen (Torrey Pines, Calif.), and both strands were sequenced by using an automated sequencer (LI-COR, Inc., Lincoln, Nebr.).

Cell cultures and transfection. NIH 3T3 and human choriocarcinoma JEG3 cells (both obtained from the American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium (DMEM) and MEM, respectively, supplemented with 10% fetal bovine serum and were maintained at 37°C, 95% humidity, and 5% CO_2. Cells used in the experiments were between 130 and 140 passages. Cells were transfected with appropriate plasmids in 100-mm-diameter dishes by the calcium phosphate method as previously described (58). A fixed amount of plasmid DNA was used in any given experiment. The total amount of expression vector DNA was equalized by adding blank vectors to control for the promoter competition effect. When necessary, transfection efficiency was monitored by use of 1 μg of cytomegalovirus β-galactosidase plasmid per transfection, and calorimetric β-galactosidase assays were performed with *o*-nitrophenyl β-D-galactopyranoside as a substrate (58). Fibroblast cell lines from wild-type *mHR6B*$^{+/+}$ and knockout *mHR6B*$^{-/-}$ mice at passage 3 (kindly provided by H. P. Roest and J. Hoeijmaker) were grown in F10-DMEM supplemented with 10% fetal calf serum, and protein lysates were prepared as described below.

Plasmids. The following plasmids were used for transfection. pCS2MT-*hICERIIγ* was constructed by ligation of the 500-bp *Eco*RV/*Pmac*I fragment bearing the *hICERIIγ* cDNA, in frame at the end of the sixth Myc epitope in pCS2MT (B. Kelley, Fred Hutchinson Cancer Center, Seattle, Wash.), which had been digested with *Stu*I. pFLAGCMV2-*hCDC34* was generated by cloning the region of the *hCDC34* gene corresponding to the N terminus of the product contained on a 1,298-bp *Nru*I/*Kpn*I fragment from pKS6110 (55) into pFLAGCMV2 (Kodak) that had been digested with *Eco*RV and *Kpn*I. pFLAGCMV2-*hCDC34 C93S*, which encodes the active-site C93S mutation, was also constructed analogously. pFLAGCMV2-*hCDC34ΔCT* was generated by cloning the *hCDC34* cDNA corresponding to the amino-terminal end contained on a 629-bp *Nru*I/*Sca*I fragment from pKS6110, encoding the first 189 amino acids (aa) from the putative first methionine of hCdc34 (55). Plasmid pCB6-*hCDC34DN* (kindly provided by M. Goebl, Indiana University, Indianapolis), containing the dominant negative human *CDC34* mutant cDNA (66), was sequenced to verify the mutations C93S and L97S and subcloned into the pSG5 (Promega) (22) mammalian expression vector (pSG5-*hCDC34DN*). The *hCDC34* antisense plasmid pSG5-*hCDC34AS* was constructed by subcloning the *CDC34* fragment from pFLAGCMV2-*hCDC34* (described above) in the reverse orientation relative to the promoter. pFLAGCMV2-*hRAD6B* was constructed by cloning a 766-bp *Hin*cII/*Ssp*I fragment from pRR518 (L. Prakash, University of Texas Medical Branch, Galveston) corresponding to the N terminus in frame into pFLGCMV2 digested with *Sma*I and *Eco*RV. Other plasmids used were pSomCAT and pSV-mouse CREM β (P. Sassone-Corsi, Institut de Genetique et de Biologie Moleculaire et Cellulaire, Strasbourg, France), pSG5 (Promega, Madison, Wis.), pCαEV (G. McKnight, University of Washington School of Medicine, Seattle), and the pMT133 and pMT107 vectors (hemagglutinin [HA] and His-tagged ubiquitin, respectively) (D. Bohman, EMBL, Heidelberg, Germany).

CAT assay. *hICERIIγ*, *hATF5*, and *hCDC34* sequences were cloned into the expression plasmid pSG5 for expression in mammalian cells. Forty-eight hours following transfection, protein extracts were made from freeze-thaw-lysed cells. β-D-Galactosidase activity was used for normalization of the amount of lysates to be used in the subsequent chloramphenicol acetyltransferase (CAT) assay. The CAT reaction was performed with cellular extracts, acetyl coenzyme A (Boehringer Mannheim, Indianapolis, Ind.), and [^{14}C]chloramphenicol (NEN, Boston, Mass.), with incubation at 37°C for 3 to 4 h. Reaction products were extracted in ethyl acetate and separated on thin-layer chromatography plates (Whatman, Maidstone, England) in 95:5 chloroform-methanol. The CAT plates were visualized with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.), and the activity was quantified by measuring the percentage of chloramphenicol acetylated by using ImageQuant software (Molecular Dynamics).

Antisera. Monoclonal antisera for human Cdc34 and Cul-2 (Transduction Lab, Lexington, Ky.), FLAG epitope and mouse β-actin (Sigma), c-Myc epitope and bacterial TrpE (Oncogene Research Products, Cambridge, Mass.), and HA epitope (BabCO, Richmond, Calif.) and polyclonal antisera for hRad6B (H. P. Roest and J. Hoeijmaker, Erasmus University, Rotterdam, The Netherlands), ICER (C. A. Molina, University of Medicine and Dentistry of New Jersey, Newark), CREM (P. Sassone-Corsi), pan-CREM (Upstate Biotechnology, Lake Placid, N.Y.), and Cul-1 (W. Krek, Friedrich Miescher Institut, Basel, Switzerland) were used.

Protein analysis and immunoprecipitation. Cells were lysed directly on 100-mm-diameter tissue culture dishes in radioimmunoprecipitation assay buffer (1× phosphate-buffered saline [PBS], 1% Nonidet P-40, 0.1% sodium dodecyl sulfate [SDS], 0.5% sodium deoxycholate) or PBSTDS buffer (1× PBS, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate) containing protease and phosphatase inhibitors (1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg of pepstatin per ml, 30 μl of aprotinin per ml, 0.5 μg of leupeptin per ml, and 100 mM sodium orthovandate) (all from Sigma), followed by scraping and passing through a 21-gauge needle. After protein quantification (with Bio-Rad protein dye and bovine serum albumin as standards) and normalization, 10 to 40 μg of protein extract was electrophoresed on SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, Mass.) with a Bio-Rad Mini Protein Blot apparatus according to the manufacturer's protocol. The filters were initially blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 for 1 to 2 h at room temperature and then probed with Myc (1.5 μg/ml), FLAG (2.5 μg/ml), β-actin (1:100,000), Cdc34 (1:2,000), Rad6B (1:250), Cul-1 (1:1,000), Cul-2 (1:500), ICER (1:1,000), and CREM (1:500) antibodies. The bound antibodies were visualized with the enhanced chemiluminescence detection system (Amersham, Buckinghamshire, England) in combination with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies as appropriate, and the intensities of the specific bands in the exposed films were quantified. Immunoprecipitation was performed as follows. Cell lysates (1.0 ml) were precleared by incubation with 10 μl of normal mouse immunoglobulin G and 20 μl of protein G plus agarose (Oncogene Research Products) at 4°C for 1 h on a rotator. The precleared lysate was collected after centrifugation at 750 × *g* for 15 min. Precleared lysates normalized for protein concentration (0.5 to 1.0 ml) were incubated at 4°C for 1 h with appropriate dilutions of the primary antibodies, and then 20 μl of protein G plus agarose was added. The mixture was then incubated at 4°C for another 12 to 16 h on a rotator. Precipitates were then washed four times with 1 ml of ice-cold PBS before electrophoresis and Western blot analysis.

Pulse-chase assay. JEG3 cells at passages 138 and 140 transiently transfected with various plasmids were incubated for 2 h in methionine- and cysteine-free DMEM. Cells were incubated for 1 h with 125 μCi of [^{35}S]methionine (NEN) per ml in the same medium. Cells were harvested (time zero) or washed four times in PBS and incubated for 3, 6, or 9 h in complete medium (MEM) supplemented with 4 mM methionine. At the end of each time period, cells were washed three times in ice-cold PBS and lysed in 2.5 ml of PBSTDS buffer. Following centrifugation at 1,250 × *g* for 15 min, the supernatant was collected and analyzed for protein content with a Bio-Rad protein assay kit and for incorporation of the [^{35}S]methionine by trichloroacetic acid precipitation. The average incorporation among samples was found to be 40% ± 2.0%. An amount corresponding to 25 × 10^6 trichloroacetic acid-precipitable counts was immunoprecipitated with Myc antibody in accordance with the instructions of the manufacturer (Oncogene Research Products), resolved by SDS-polyacrylamide gel electrophoresis, fixed in acetic acid (10%)-methanol (40%), and analyzed in a Storm imager.

Proteasome inhibitors and detection of ubiquitin-ICERIIγ conjugates. Peptide aldehydes MG115 and MG132 were obtained from Peptide Institute Inc. (Lexington, Ky.) and dissolved at 10 mM in dimethyl sulfoxide. Approximately 36 h after transfection and 5 h before harvest, cells were treated with 0.025 mM proteasome inhibitors. Cells were lysed as described above, with the addition of 5 mM *N*-ethylmaleimide (Sigma) to the lysis buffer as previously described (10). For the detection of HA-tagged ubiquitin-ICERIIγ conjugates, cells growing in 100-mm-diameter dishes were cotransfected with ICERIIγ and either HA (pMT133)- or His$_6$ (pMT107)-tagged ubiquitin expression plasmid, followed by treatment with proteasome inhibitor, lysis, immunoprecipitation, and Western analysis as described above.

Testicular protein lysates and isolation of germ cells. Testes were collected from a colony of wild-type C57 mice at various ages of development from birth until 40 days old at intervals of 5 days (kindly provided by S. Sharan and A. Bradley, Baylor College of Medicine, Houston, Tex.). Testes were dissected, washed several times with PBS, snap frozen in liquid nitrogen, and kept at −80°C until protein lysates were made. Germ cells were prepared from adult C57BL/6 × 129 (hybrid) mouse and Sprague-Dawley rat testes by trypsin digestion, followed by centrifugal elutriation as described previously (21, 45). Enriched populations of pachytene spermatocytes, round spermatids, and late spermatids were prepared by centrifugal elutriation, with a purity of ~80% as shown by microscopic analysis. Protein lysates were made from these fractions by using radioimmunoprecipitation assay buffer. Testicular lysates from 12-week-old C3H × C57BL/6 *jsd* heterozygote (*jsd*/+) and mutant (*jsd*/*jsd*) mice (3) (kindly provided by G. Shetty) were made as described above. C57BL/6J mice (6 to 7 weeks old) (kindly provided by G. Wilson) treated with 16 Gy of radiation were sacrificed 4 weeks postirradiation, testes were collected, and lysates were made as described above (44). Testicular lysates from 7- to 8-week-old unirradiated littermates from the same colony were used as controls.

RNA extraction and Northern analysis. Total RNA was extracted from transfected JEG3 cells by using a total RNA isolation kit from Qiagen. DNase-I treated RNAs were Northern blotted from 1% formaldehyde gels with Hybond N$^+$ sheets (Amersham). The blots were hybridized overnight at 65°C with the nick-translated [γ-^{32}P]dCTP-labeled *hICERIIγ* probe in 10% dextran sulfate–2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% SDS–250 μg of salmon sperm DNA per ml. The final wash was in 0.1× SSC–0.1% SDS at 65°C.

Nucleotide sequence accession number. The *hATF5* sequence reported in this paper has been deposited in the GenBank database under accession no. AF101388.

RESULTS

***hCDC34* two-hybrid screen.** The two-hybrid cloning system used in this study has been described previously (69, 70). Reagents used in this screen include a human activated T-lym-

phocyte cDNA library fused to the Gal4 activation domain, the full-length *hCDC34* cDNA (55) fused to the Gal4 DNA binding domain as bait (pPC97), and the yeast host strain MV103 with appropriate reporter constructs. The success of a two-hybrid screen depends on the production of a properly folded bait fusion protein in the host cells. The pPC97-*hCDC34* bait construct encoded a functional hCdc34 protein as demonstrated by its ability to complement a *cdc34-1* mutant yeast strain, SJ1098-3d, for growth at 37°C (data not shown). Complementation of *cdc34-1* also suggests that the *hCDC34* bait can form a functional complex with the SCF in yeast. Production of Gal4-hCdc34 bait and mutant Gal4-hCdc34C93S fusion proteins was also confirmed in Western blots with a monoclonal hCdc34 antiserum (result not shown). In a screen of 1.5 million transformants, 30 clones were found to be positive for all reporters tested (His/3AT$^+$, Ura$^+$, 5-fluoro-orotic acid negative, and X-Gal$^+$). Of the 30 positive clones, 18 clones have been sequenced and 4 of them are novel genes. None of these 18 sequences represent human cDNAs encoding products with significant homology to members of the SCF complex, cyclins, or cyclin-dependent kinase inhibitors.

Three of the 18 clones, *hICERIIγ*, *hATF5*, and clone 30-17, encode proteins with bZIP motifs. *hICERIIγ*, an *ICER* isoform generated by alternate splicing, encodes a 108-aa protein and lacks the γ exon and one of the DNA binding and dimerization domains (Fig. 1). hICERIIγ has 97.2% homology with the mouse isoform mICERIIγ and 76.7% homology with hICERI, a 120-aa hICER protein. *hATF5* is a novel clone; a partial sequence of a mouse homolog called *ATFX* has been reported (51). *hATF5* encodes a 122-aa protein with extensive homology in the C-terminal domain with human (52.1%) and mouse (50.9%) ATF4. Mouse ATFX and human ATF5 are 96.6% homologous in their available sequences. In the leucine zipper domain, hATF5, like mouse ATFX, has only three leucines instead of the five that are present in hATF4 and hATF3, with the distal two leucines replaced by valines.

Expression of Cdc34/Rad6B and its targets in spermatogenesis. Isolation of a particular interaction in a two-hybrid screen does not necessarily imply that these two proteins are co-expressed or normally interact in mammalian cells. Currently little is known about the expression pattern of the Cdc34 protein in normal mammalian tissues. Considering the requirement for ICER/CREM and Rad6B proteins during spermatogenesis, we determined whether human Cdc34 is also expressed during spermatogenesis.

During spermatogenesis, the temporal appearance of the most advanced wave of spermatogenic cells has been well characterized (4). The ontogeny of germ cell development in mice is as follows: spermatogonia (types A and B), primary spermatocytes (preleptotene, leptotene, zygotene, and pachytene), secondary spermatocytes, spermatids, and sperm. By day 3 most of the germ cells are spermatogonia, by day 13 the most advanced stage of spermatogenesis is the pachytene spermatocyte stage, by day 22 the first wave of meiosis is completed and early spermatids first appear, and by day 35 mature spermatozoa are produced. To establish the time of appearance of Cdc34 and Rad6B proteins during development, testis extracts from mice at different ages were studied by Western analysis (Fig. 2A). Cdc34 protein is found at very low levels at birth. However, the level increases considerably at day 20, correlating with the time of an increased number of spermatocytes. The Cdc34 profile shows an initial peak at day 20, followed by a significant rise again by day 30, when spermatids become the predominant cell type. A similar pattern of expression is also observed for Rad6B. Rad6B protein appears at day 15, and the level increases considerably at day 20 and reaches a peak by day 35 (Fig. 2B). The levels of Cdc34 and Rad6B proteins remain high during postmeiotic development of round and then late spermatids. Given the requirement for the SCF complex in Cdc34-mediated degradation of proteins in budding yeast (1, 16, 65) and conservation of the SCF complex in higher eukaryotes (41), we studied the expression of the Cdc53 homologs Cul-1 and Cul-2 during testicular development. As shown in Fig. 2, both Cul-1 and Cul-2 follow a developmental profile which is distinct from that of Cdc34. Their levels, especially that of Cul-1, are extremely low in prepubertal testis (until day 6) and then increase sharply and reach a peak at day 20, coinciding with the initial increase in levels of Cdc34 and Rad6B proteins (Fig. 2B). However, the levels of both Cul-1 and Cul-2 proteins then decline gradually and are low in adult testis. The maximum expression of Cul-1 and Cul-2 along with elevated levels of Cdc34 at day 20, when pachytene spermatocytes are the predominant germ cell, indirectly suggests the formation of an active SCF complex during meiosis.

Due to the presence of both germ cells and stromal cells in the analysis of whole testis, we also compared expression of Cdc34 and Rad6B proteins in testes from mice lacking differentiated germ cells, including the recessive mutant juvenile spermatogonial depletion (*jsd*) and irradiated testes. The adult testes from a *jsd* homozygous mouse are one-third of the normal size and lack cells undergoing spermatogenesis, compared to the *jsd* heterozygotes (*jsd*/+) (3). The majority of the tissue is composed of Sertoli's, Leydig's, and other interstitial cells, with rare type A spermatogonia. Figure 3A shows the result of Western blot analysis with antibodies to hCdc34 and hRad6B of lysates from whole testes of homozygous and heterozygous animals. These blots demonstrate a loss of Rad6B and a decrease in Cdc34 staining in testes from the homozygous *jsd*/*jsd* mice compared with heterozygous *jsd*/+ littermates. In addition, the higher-molecular-weight band recognized by the Cdc34 antibody which is seen in normal adult testis is absent. A similar change in the patterns of expression for Rad6B and Cdc34 is seen in the testes of irradiated mice (Fig. 3A), which also are deficient in germ cells secondary to radiation damage (44).

To further characterize the roles of Cdc34, Cul-1, Cul-2, and Rad6B in the germ cell component of the testis, we examined the expression of these proteins in separated germ cells from mouse and rat testes. Pachytene spermatocyte-, round spermatid-, and late spermatid-enriched fractions were obtained by centrifugal elutriation as described previously (21, 45). Western blot analysis of the protein lysates of these fractions demonstrated lower levels of Cdc34 and Rad6B proteins in mouse pachytene spermatocytes than in the round and late spermatids (Fig. 3B). Consistent with the developmental profile, the levels of these proteins are significantly higher in late spermatids, with a three- to fivefold increase over the protein levels in pachytene spermatocytes and round spermatids. Similar results were also obtained for Cdc34 protein in elutriated rat cells (results not shown). In both the developmental profile and the separated germ cell fractions there is the appearance of a higher-molecular-weight form of Cdc34 protein in the later stages of spermatogenesis. The patterns of expression of Cul-1 and Cul-2 are different and are distinct from that of Cdc34. There is maximal expression of Cul-2 in the spermatocyte fraction and of Cul-1 in the round spermatids, with both expressed at low levels in the late spermatid fractions.

With regard to potential targets of the Cdc34 and Rad6B UBC enzymes isolated in our screen, we investigated the developmental profile of CREM/ICER protein expression in the mouse testis. The polyclonal anti-CREM/ICER antiserum recognizes all CREM/ICER isoforms with the same efficiency,

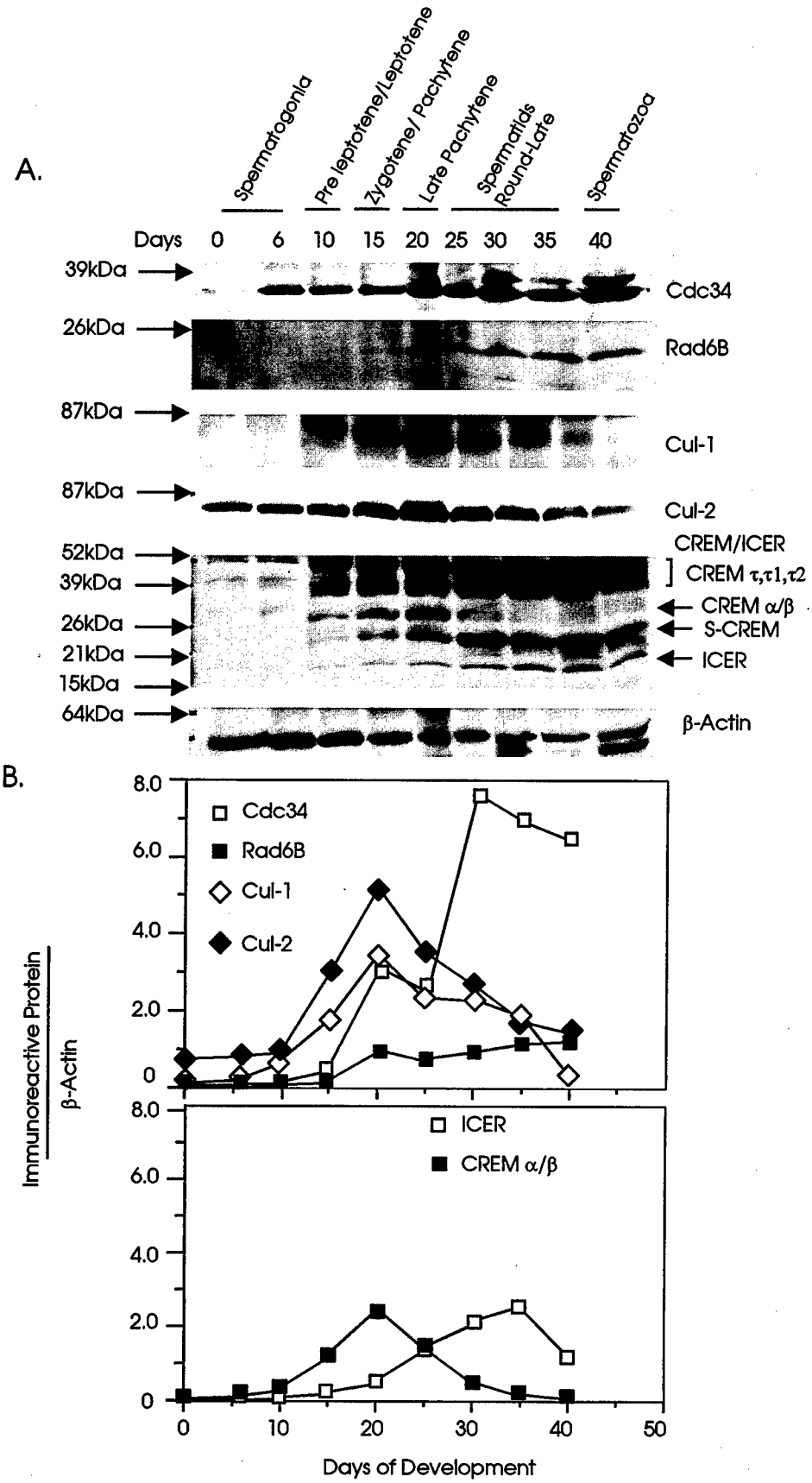

FIG. 2. (A) Western blot analysis of the expression profiles of Cdc34, Rad6B, Cul-1, Cul-2, and CREM/ICER proteins during mouse testicular development. Testis extracts (40 μg/lane) from C57 mice at various ages of development as indicated were probed with monoclonal anti-hCdc34, anti-Cul-2, and anti-β-actin and polyclonal anti-Rad6B, anti-CREM/ICER, and anti-Cul-1 antisera as described in Materials and Methods. Molecular mass markers are on the left. The most advanced germ cell types during testicular development, as previously described (4), are noted on top. The profiles of the immunoreactive proteins shown are from four different Western blots, and each blot was probed with β-actin; one β-actin blot is shown as a representative to compare loading. (B) Densitometric analysis of the bands of the immunoreactive proteins.

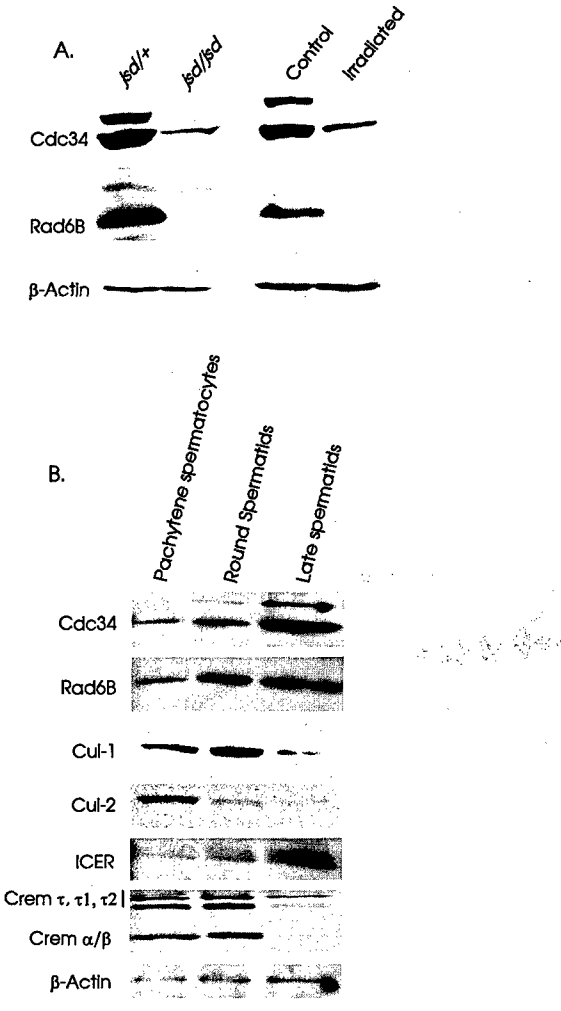

FIG. 3. (A) Expression profiles of Cdc34 and Rad6B proteins in germ cell-deficient testis. Left panel, analysis of the testes of adult spermatocyte-deficient juvenile spermatogonial depletion (*jsd/jsd*) mutant mice and unaffected *jsd* heterozygote (*jsd/+*) mice. Right panel, pattern of expression in the testes from irradiated mice in comparison to nonirradiated testes. Testicular extracts were probed with monoclonal anti-hCdc34 and polyclonal anti-Rad6B antisera as described in Fig. 2. β-Actin is shown to compare loading. (B) Western blot analysis of the expression profiles of Cdc34, Rad6B, CREM α/β, and ICER proteins in elutriated germ cell fractions of C57BL/6 mice. Germ cell extracts were probed with monoclonal anti-hCdc34 and anti-β-actin and polyclonal anti-Rad6B and anti-CREM-ICER antisera as described in Materials and Methods. The profiles of the immunoreactive proteins shown are from three different Western blots, and each blot was probed with β-actin; one β-actin blot is shown as a representative to compare loading.

indicating that a common domain constitutes the antigenic epitope (12, 48). Five distinct immunoreactive bands were detected (Fig. 2A), with apparent molecular masses of ~18 (ICER), 26 (S-CREM), ~30 (CREM α/β), 39 to 40 (CREM τ1 and τ2), and ~50 (CREM τ) kDa, respectively. ICER protein is found to be expressed at moderate levels in mouse testis after birth and reaches a maximum at around 35 days of age. The level of ICER is lower in prepubertal testis than in the postmeiotic testis in mice and remains higher through the stages of spermatids and spermatozoa. In contrast, the repressor CREM α/β protein (represented by the 30-kDa immunoreactive band) was found to be present at a very low level at birth, to increase significantly at day 10, and to reach a peak at day 20; thereafter its level declines sharply, and it remains undetectable after day 35 (Fig. 2B). The sharp decline and subsequent disappearance of CREM α/β coincide with the first wave of meiosis and the first appearance of spermatids.

The expression patterns of CREM/ICER isoforms were further examined in the separated germ cells of mouse (Fig. 3B) and rat (data not shown) testes as described above. ICER isoforms were expressed at low levels in pachytene spermatocytes but at increased levels in round and late spermatids of both mouse (Fig. 3B) and rat (data not shown). The 30-kDa CREM α/β, however, is found to be expressed at higher levels in pachytene spermatocytes and round spermatids, while it is not detected in late spermatids (Fig. 3B). Disappearance of the 30-kDa CREM α/β repressor isoform during the late puberty stage of testicular development and absence of the immunoreactive CREM α/β protein in the late spermatid fractions are correlated with high levels of Cdc34 and Rad6B proteins in late puberty and late spermatids (Fig. 2 and 3).

Ectopically expressed hICERIIγ and hATF5 proteins are degraded by the ubiquitin-proteasome machinery in a Cdc34- and Rad6B-dependent pathway. The results of the previous experiments demonstrate that mammalian Cdc34 and the ICERIIγ proteins are both expressed in a complex pattern in germ cells during spermatogenesis. We therefore next determined if Cdc34 and the ICERIIγ proteins can directly interact in mammalian cells as suggested by the two-hybrid screen. We expressed epitope-tagged versions of hCdc34 and its targets by transient transfection in mammalian cells. The human choriocarcinoma cell line JEG3 has been used extensively to characterize the expression and function of ICER and other *CREM* gene products (18, 48) and therefore was chosen for use in the present study. Expression of Myc-tagged hICERIIγ (pCS2MT-*hICERIIγ*) resulted in production of a 24-kDa protein recognized by the Myc epitope antibody (Fig. 4A). Cotransfection of Myc-tagged hICERIIγ with FLAG-tagged hCDC34 (pFLAGCMV2-*hCDC34*) into JEG3 cells (Fig. 4A) and NIH 3T3 cells (data not shown) resulted in considerable and in some cases complete loss of the hICERIIγ fusion protein in multiple experiments. In contrast to the loss of protein, Northern analysis of the mRNA isolated from pCS2MT-*hICERIIγ*-transfected cells demonstrated no significant difference in the steady-state *hICERIIγ* transcripts compared to the cells cotransfected with pFLAGCMV2-*hCDC34* (Fig. 4A).

To explore whether loss of hICERIIγ protein was secondary to ubiquitin-mediated proteolysis, we repeated the experiments in the presence of the potent 26S proteasome inhibitors MG115 and MG132 (42). Incubating the transfected cells for 5 to 6 h with 0.025 mM MG115 (Fig. 4B) or MG132 (data not shown) prevented the loss of hICERIIγ protein in cells cotransfected with *hCDC34*. The requirement for an active hCdc34 UBC enzyme in the loss of the hICERIIγ protein was determined by cotransfecting cDNAs encoding two different mutant hCdc34 proteins. The first, hCdc34C93S, has the highly conserved active-site cysteine of the enzyme replaced with a serine. Expression of this mutant protein, unlike the Cdc34 bait, was unable to complement the growth of *cdc34-1* yeast at the restrictive temperature (data not shown). The second mutant, hCdc34ΔCT, is a truncated protein which has the carboxy terminus distal to amino acid 189 deleted. In *S. cerevisiae*, the comparable truncation has been shown to remove the binding site required for association with Cdc53 in yeast (43) and results in a protein unable to complement a *cdc34* mutation (34, 64). Comparable expression of the wild-type, hCdc34C93S, and hCdc34ΔCT proteins in transfected JEG3 cells was verified by Western blot analysis (result not shown). In cotransfection assays with pCS2MT-*hICERIIγ*, the C93S and ΔCT mutant proteins had no effect on the steady-state level of hICERIIγ protein, compared to the loss of protein seen upon

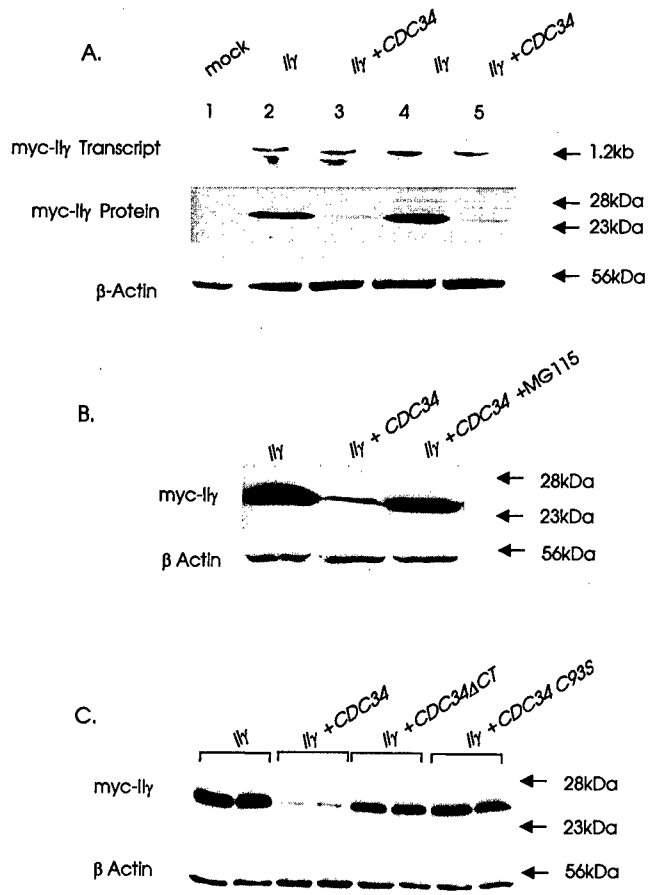

FIG. 4. Cotransfection assay for assessing the expression of ectopic *hICERIIγ* (IIγ) transcripts and protein in JEG3 cells. (A) Upper panel, Northern blot analysis of the mRNA isolated from the cells cotransfected with pCS2MT-*hICERIIγ* and the blank vector (pFLAGCMV2) or pFLAGCMV2-*hCDC34* as described in Materials and Methods. The RNA was probed with the full-length *hICERIIγ* cDNA. Lane 1, untransfected control; lanes 2 to 5, results of duplicate experiments. Middle panel, Western blot analysis of the protein collected from the identical sets of experiments described above. Protein lysates were prepared as described in Materials and Methods, electrophoresed on an SDS–5 to 15% polyacrylamide gel, transferred onto a polyvinylidene difluoride membrane, and probed with the Myc epitope antibody. Blots were reprobed with the mouse β-actin antibody to compare loading (bottom panel). The numbers on the right represent molecular mass markers. The data shown are representative of those from 12 independent experiments. (B) Effect of a proteasome inhibitor, MG115, on the hCdc34-induced destabilization of hICERIIγ protein. Transfected JEG3 cells were incubated for 5 to 6 h in the presence or absence of 0.025 mM peptide aldehyde MG115 before harvest. Protein was analyzed as described above. The data shown are representative of those from three independent experiments. (C) Effect of mutant hCdc34 enzymes (C93S and ΔCT) on the stability of hICERIIγ protein in cotransfection assays. Vectors expressing the wild-type or mutant enzymes were cotransfected with the *hICERIIγ* vector into JEG3 cells, and protein was analyzed as before. The data shown are representative of those from three independent experiments.

cotransfection of the wild-type hCdc34 (Fig. 4C), indicating that the loss of hICERIIγ protein by coexpression of hCdc34 required a fully functional UBC enzyme.

Given the homology between hCdc34 and hRad6B and the requirement for both murine Rad6B and CREM/ICER in spermatogenesis, we performed cotransfection assays with *hRAD6B* and the targets obtained in the *hCDC34* screen. Coexpression of FLAG-tagged hRad6B (pFLAGCMV2-*hRAD6B*) with Myc-tagged hICERIIγ can mimic the loss of hICERIIγ protein to a similar but lesser degree as hCdc34 (Fig. 5A). This loss of hICERIIγ can also be reversed in the presence of proteasome inhibitors. Similar to the case for *hICERIIγ*, cotransfection of a Myc-tagged *hATF5* fusion with either *hCDC34* (Fig. 5B) or *hRAD6B* cDNA resulted in a significant loss of hATF5 protein, which can be reversed by incubation with MG115. However, once again, in comparison to hCdc34, the loss of hATF5 protein in the presence of hRad6B is lower (data not shown).

To determine whether the change in steady-state levels of hICERIIγ protein upon cotransfection with *hCDC34* and *hRAD6B* is due to a change in protein half-life, we performed pulse-chase experiments with JEG3 cells (Fig. 6). The cells were transfected with pCS2MT-*hICERIIγ* in combination with pFLAGCMV2 vector, pFLAGCMV2-*hCDC34*, or pFLAG CMV2-*hRAD6B*. Forty hours following transfection, cells were metabolically labeled with [^{35}S]methionine in a pulse-chase protocol. The Myc-ICERIIγ protein was then immunoprecipitated from the samples collected at various time points during the chase by using a monoclonal antibody against the Myc epitope. Immunoprecipitates were resolved on an SDS–15% polyacrylamide gel, and labeled protein was quantitated. The half-life of the transfected ICERIIγ protein during the chase was calculated to be 3.82 h. In the presence of hCdc34 or hRad6B, hICERIIγ protein degraded more rapidly, with estimated half-lives of 2.3 and 2.9 h, respectively (Fig. 6B). In duplicate experiments the kinetics of hICERIIγ protein degradation in the presence of hRAD6B suggests a biphasic pattern of instability with a decreased half-life later in the pulse (Fig. 6B).

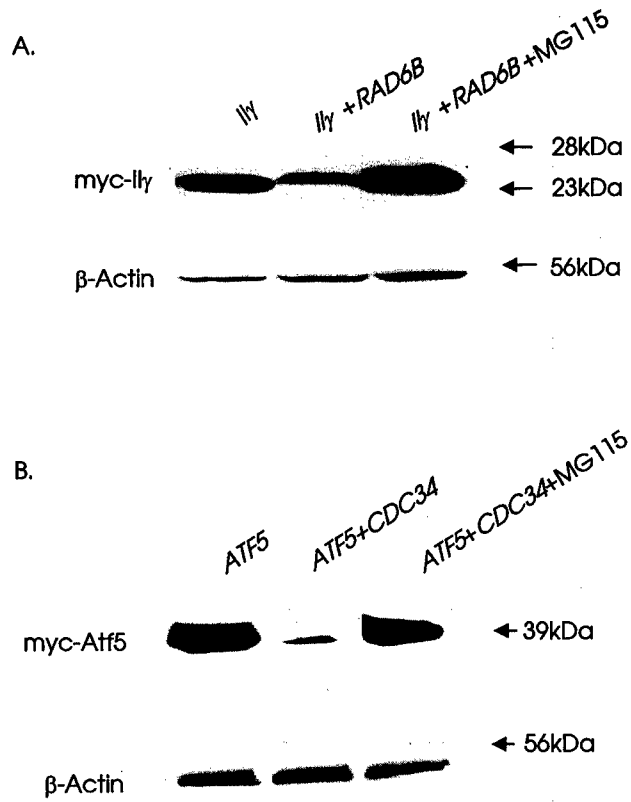

FIG. 5. (A) Effect of pFLAGCMV2-*hRAD6B* on the stability of pCS2MT-*hCERIIγ* in JEG3 cells in the presence and absence of a proteasome inhibitor, MG115. (B) Effect of pFLAGCMV2-*hCDC34* on the expression of ectopic pCS2MT-*hATF5* in cotransfected JEG3 cells in the presence and absence of a proteasome inhibitor, MG115. β-Actin is shown to compare loading. The treatment and Western analysis of the protein were the same as for Fig. 4. The data shown are representative results from three independent experiments.

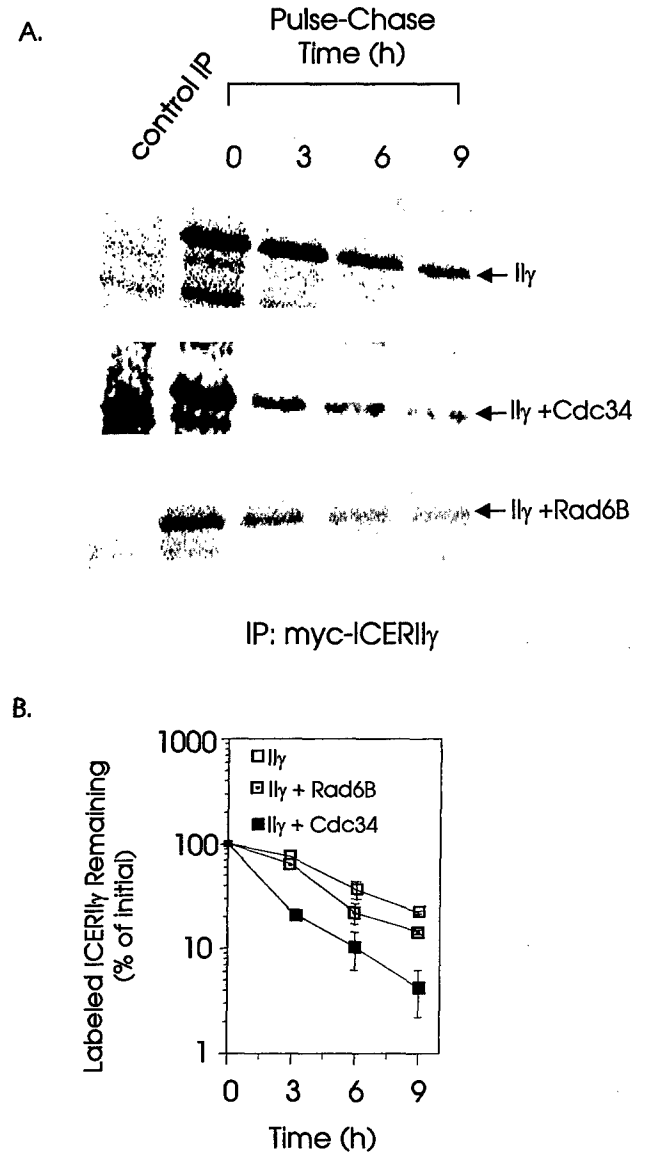

FIG. 6. Pulse-chase analysis of the stability of hICERIIγ protein in JEG3 cells alone or cotransfected with either pFLAGCMV2-*hCDC34* or pCS2MT-*hRAD6B*. ^{35}S pulse-chase labeling for the indicated periods of time was performed as described in Materials and Methods. Cell lysates were immunoprecipitated (IP) with monoclonal anti-Myc-tagged antibody, and the immunoprecipitates were analyzed on an SDS–15% polyacrylamide gel and quantitated. The control immunoprecipitation was performed with a mouse monoclonal antibody raised against the bacterial TrpE protein. (B) Quantitation of the bands corresponding to labeled hICERIIγ protein from panel A. Data are the averages and standard errors of the means from two experiments. In some cases the standard error of the mean is smaller than the symbol.

Coexpression of hCdc34 relieves the repression of cAMP-induced transcription mediated by hICERIIγ and hATF5 in JEG3 cells. An important question to address is whether Cdc34-mediated degradation affects the biologically active nuclear repressor protein and not just excess (potentially misfolded) hICERIIγ and hATF5 protein. As previously reported by Molina et al. (48), we have found that hICERIIγ is a powerful repressor of PKA (cAMP)-induced transcription (Fig. 7). We used a pSomCAT reporter plasmid that contains a canonical rat somatostatin CRE sequence inserted upstream from the herpesvirus thymidine kinase promoter and the bacterial CAT gene (19). Activation of pSomCAT transcription was obtained by cotransfection of the mouse PKA subunit expression vector (pCαEV) (46) or treatment with 10 mM forskolin for 2 to 3 h before harvest (data not shown). JEG3 cells were transiently transfected with pSomCAT, pCαEV, pSG5-*hICERIIγ*, or pSG5-*hATF5* in combination with pSG5-*hCDC34*. Both hICERIIγ and hATF5 show strong repression of PKA-induced CAT activity (Fig. 7). However, coexpression of *hICERIIγ* or *hATF5* with *hCDC34* completely abrogated the repression of cAMP-induced CAT activity. These results demonstrate that Cdc34 targets for degradation the biologically active transcriptional repressor proteins in mammalian cells.

The ICER-specific domain is not sufficient for degradation. All four isoforms of human ICER proteins possess the same 9-amino-acid N-terminal domain. The isoforms differ based on the presence or absence of two exons, exon γ and exon Ia (Fig. 1). To analyze the potential role of the ICER-specific domain in the stability of hICERIIγ in vivo, we made a Myc epitope-tagged construct (pCS2MT-*hICERIIγ* 1-33) bearing the N-terminal 33 amino acids. Cotransfection of the mini-ICER construct with pFLAGCMV2-*hCDC34* into either JEG3 cells (Fig. 8A) or NIH 3T3 cells (data not shown) did not result in significant loss of the truncated protein compared to the full-length hICERIIγ, indicating that the ICER-specific domain and exon γ are not sufficient for hCdc34 targeting and subsequent degradation. In addition, this result also demonstrates

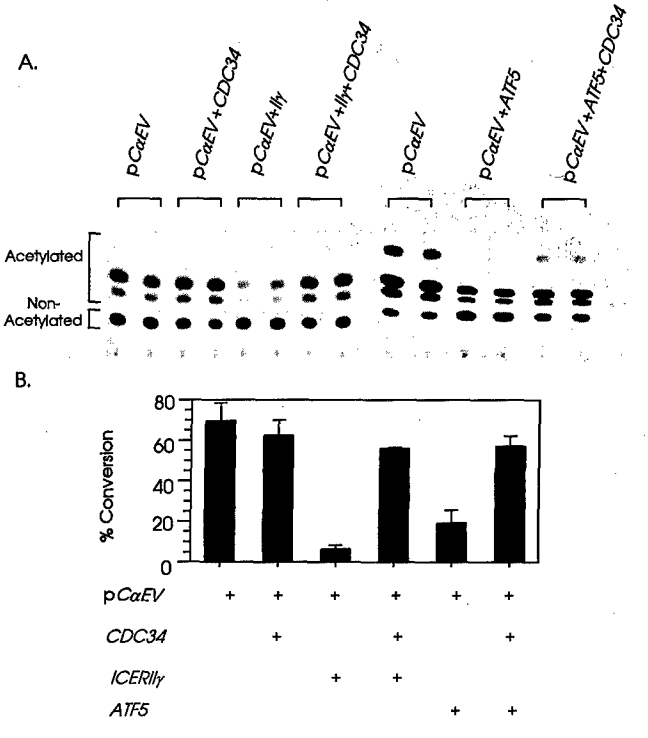

FIG. 7. (A) Assay for the effect of hCdc34 on the cAMP-induced transcriptional repression activities of hICERIIγ and hATF5. pSG5-*ICERIIγ* or pSG5-*hATF5* with or without pSG5-*hCDC34* was cotransfected into JEG3 cells along with β-D-galactosidase plasmid, pSomCAT, and pCαEV. Reporter pSomCAT was used to measure PKA-mediated activation of the somatostatin CRE element upstream of the CAT gene. pCαEV encodes murine PKA and activates the CRE element. β-D-Galactosidase plasmid was used to assess transfection efficiency. All experiments had equal amounts of plasmid DNA transfected into the JEG-3 cells by using the appropriate pSG5 vector control. (B) Average CAT activity (percent conversion of nonacetylated to acetylated chloramphenicol) from three different experiments as shown in panel A performed in duplicate. Each value represents the mean ± standard error of the mean for six observations.

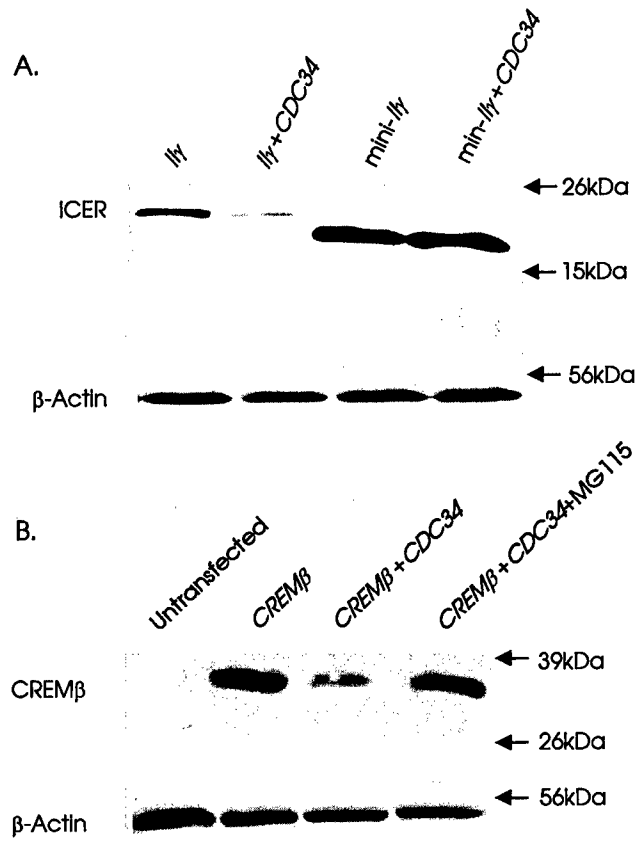

FIG. 8. (A) Role of the ICER-specific domain in the *hCdc34*-mediated destabilization of the ICERIIγ protein. A Myc epitope-tagged construct (pCS2MT-*ICERIIγ* 1-33) bearing the N-terminal 33 amino acids of the ICER-specific domain and exon γ of the hICERIIγ protein (mini-IIγ) was made as described in Materials and Methods. Cotransfection of full-length *hICERIIγ* (*IIγ*) or pCS2MT-*ICERIIγ* 1-33 (mini-*IIγ*) with pFLAGCMV2-*hCDC34* into JEG3 cells and Western analysis were performed as described for Fig. 4. Lanes 1 and 2, expression of the full-length Myc epitope-tagged hICERIIγ protein; lanes 3 and 4, expression of the mini-ICERIIγ protein; lanes 2 and 4, presence of FLAG-*hCDC34*; lanes 1 and 3, absence of FLAG-*hCDC34*. The data shown are representative results from three independent experiments. (B) Effect of pSG5-*hCDC34* on the expression of ectopic pSG5-*mCREMβ* in cotransfected JEG3 cells in the presence and absence of a proteasome inhibitor, MG115. The treatment and Western analysis of the protein were the same as for Fig. 4. The data shown are representative results from three independent experiments.

that specific sequences in the full-length hICERIIγ protein are required for targeting by hCdc34.

Coexpression of *hCDC34* destabilizes CREM β in JEG3 cells. Given these results and an inverse correlation between Cdc34 and CREM α/β during spermatogenesis, we investigated the targeting of the CREM α/β repressor isoforms by hCdc34. Mammalian expression vectors encoding both mouse CREM β (mCREM β), which does not contain the ICER-specific exon, and hCdc34 were cotransfected into JEG3 cells (Fig. 8B) and NIH 3T3 cells (result not shown). The protein lysates from the transfected cells were analyzed in a Western blot with CREM antiserum as a probe. Coexpression of *mCREMβ* and *hCDC34* resulted in a significant loss of the CREM β protein, which was reversed by addition of the proteasome inhibitor MG115.

The Myc-ICERIIγ protein is ubiquitinated by endogenous hCdc34. As ubiquitination is not a prerequisite for the degradation of proteins via 26S proteasomes (8), we examined the polyubiquitination of hICERIIγ protein. Polyubiquitination has been detected for a number of substrates, including c-Jun (68), cyclin E (10), and p27 (52). To demonstrate the formation of the polyubiquitin-hICERIIγ conjugates, an immunoprecipitation-Western assay in which an HA-tagged ubiquitin construct was cotransfected along with the Myc-tagged hICERIIγ in the presence and absence of pFLAGCMV2-*hCDC34* into JEG3 cells was used. Five hours before harvest, cells were treated with MG115. Extracts were made in the presence of *N*-ethylmaleimide and immunoprecipitated with either the anti-Myc or anti-HA monoclonal antiserum, followed by Western blotting with either anti-Myc or anti-HA antiserum. High-molecular-weight species of hICERIIγ protein were detected with either antibody and in cells transfected with both HA-ubiquitin and Myc-*hICERIIγ* or in cells transfected with FLAG-*hCDC34*, Myc-*ICERIIγ*, and HA-ubiquitin (Fig. 9A). However, no ubiquitin conjugates were observed in the presence of His_6-tagged ubiquitin as opposed to HA-ubiquitin. Thus, the slower-migrating species contain both Myc-hICERIIγ protein and HA-ubiquitin and represent multiubiquitinated forms of hICERIIγ protein. A ladder of bands was found in the presence and absence of transfected *hCDC34*, which indicates that ICERIIγ can be targeted by endogenous UBC enzymes, confirming an earlier report (17).

To determine whether endogenous hCdc34 is one of the endogenous UBC enzymes responsible for the ubiquitination of ICERIIγ, we transfected cells with the Myc-tagged *hICER IIγ* construct and two Cdc34-inhibitory constructs. The first is a mammalian expression vector containing the corresponding

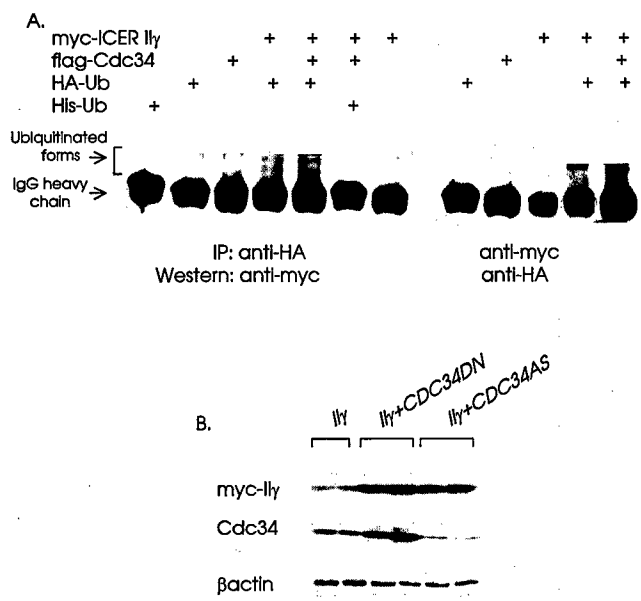

FIG. 9. (A) Identification of hICERIIγ-ubiquitin conjugates by an immunoprecipitation (IP)-Western blot analysis. JEG3 cells were transfected with the indicated plasmids and then treated with 0.025 mM MG115 5 h before harvest. Left panel, cell lysates were immunoprecipitated with HA antibody, followed by Western blotting with the Myc antibody (which recognizes the Myc epitope on hICERIIγ). Right panel, cell lysates were immunoprecipitated with the Myc antibody, and the Western blot was probed with anti-HA. Ubiquitin-hICERIIγ conjugates are detected only in cells transfected with both Myc- and HA epitope-tagged constructs and in the presence or absence of exogenous hCDC34. A His_6-tagged ubiquitin construct (His-Ub) was used as a negative control. Immunoglobulin G (IgG) heavy chain is visualized in this analysis and is indicated. (B) Effect of the expression of Cdc34-inhibitory constructs *hCDC34DN* and *hCDC34AS* on the stability of ectopically expressed myc-ICERIIγ protein. JEG3 cells were transiently transfected with pCS2MT-*ICERIIγ* and the pSG5 empty vector, pSG5-*hCDC34DN*, or pSG5-*hCDC34AS*. Cell lysates were prepared and subjected to Western analysis as described for Fig. 4, using anti-Myc and anti-Cdc34 antibodies. β-Actin is shown to compare loading. The data shown are representative of those from two independent experiments.

double mutation in the human sequence (*hCDC34DN*-C93S and -L97S) of a dominant negative allele of yeast *CDC34* (2). The *hCDC34DN* mutation has been previously reported to inhibit the ubiquitination of other Cdc34 substrates, including MyoD in mammalian cells (66). Transfection of the cDNA containing the dominant negative mutation subcloned into the pSG5 vector (pSG5-*hCDC34DN*) resulted in high-level expression of the mutant hCdc34 protein (Fig. 9B). A second inhibitory construct, containing the wild-type human cDNA subcloned into pSG5 in an antisense orientation (pSG5-*hCDC34AS*), was also constructed. Transfection of this construct resulted in a decrease in the steady-state level of endogenous Cdc34 protein (Fig. 9B). Cotransfection of JEG-3 cells with the Myc-tagged hICERIIγ and either pSG5 empty vector or dominant negative or antisense *hCDC34* constructs was carried out in parallel. Expression of both the dominant negative and antisense *hCDC34* constructs results in increased levels of Myc-tagged hICERIIγ protein compared to that with the empty vector control. These results suggest that the ubiquitination of the Myc-tagged hICERIIγ demonstrated in Fig. 9A is at least partially due to ubiquitination by the endogenous hCdc34 UBC enzyme.

Endogenous ICER protein expression is increased by mutation of Rad6B or inhibition of Cdc34 activity. The data presented here are consistent with both the human Cdc34 and Rad6B conjugating enzymes targeting transfected hICERIIγ and ATF5 proteins for ubiquitination. In order to determine the impact of these enzymes on the endogenous ICER target proteins, we obtained mouse embryo fibroblasts (MEF) mutant for the orthologous gene *mHR6B*, which have been previously described (57). Whole protein lysates of MEF from *mHR6B* homozygous null mice (*mHR6B*$^{-/-}$) or wild-type littermate controls (*mHR6B*$^{+/+}$) were hybridized with an antibody to ICER proteins as described above. A distinct increase in expression of the ~18-kDa band which comigrates with ICERIIγ in the null cells is seen compared with the wild-type cells (Fig. 10A). A number of other proteins recognized by this antibody are increased in the *mHR6B* null cells and represent other CREM isoforms.

Although *cdc34* null cells are not available, our results suggest that we can inhibit the endogenous Cdc34 enzyme by expression of either the dominant negative or antisense constructs. We therefore transfected JEG-3 cells with either the empty vector or dominant negative or antisense Cdc34 constructs and isolated whole-cell lysates 36 to 40 h after transfection. The transient-transfection technique used here results in a transfection efficiency of approximately 30% (data not shown). We therefore expect that the subpopulation of cells that are transfected with the antisense or dominant negative construct will have inhibition of Cdc34 activity, while the remainder of the cells will have normal Cdc34 activity. Despite this limitation, Western blot analysis of these lysates (Fig. 10B) with an ICER antibody demonstrates that the cells transfected with either the antisense or dominant negative construct have a significantly increased steady-state level of the endogenous ICER protein compared with the empty vector control.

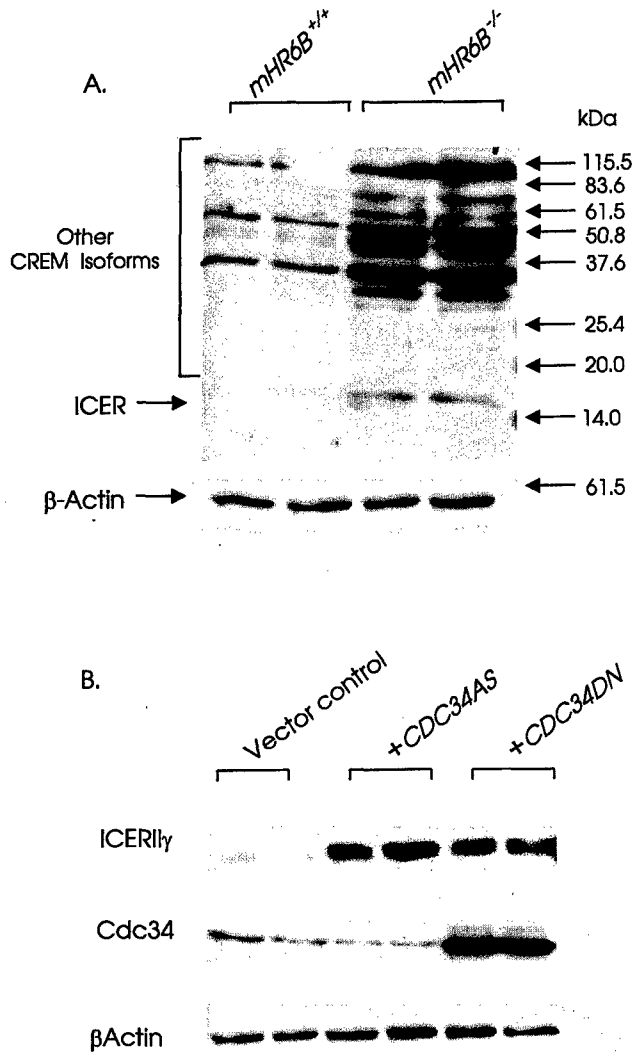

FIG. 10. (A) Western blot analysis of the expression profiles of endogenous ICER and other CREM isoforms in *mHR6B* wild-type (*mHR6B*$^{+/+}$) and mutant (*mHR6B*$^{-/-}$) MEF. Total cell lysates from exponentially growing cells were prepared and subjected to Western analysis as described for Fig. 4, using anti-ICER antibody. Molecular mass markers are indicated on the right. β-Actin is shown to compare loading. The data shown are representative of those from two independent experiments. (B) Effect of the expression of an *hCDC34AS* or *hCDC34DN* mutant construct on the stability of endogenous ICER protein. JEG3 cells were transiently transfected with vector alone (pSG5), pSG5-*CDC34DN*, or pSG5-*CDC34AS*. Cell lysates were prepared and subjected to Western analysis as described for Fig. 4, using anti-ICER and anti-Cdc34 antibodies. β-Actin is shown to compare loading. The data shown are representative of those from two independent experiments.

DISCUSSION

In this study, we have found that the human Cdc34 and Rad6B UBC enzymes can target repressors of cAMP-inducible transcription for ubiquitination, including hICERIIγ (a previously described ICER isoform), CREM β, and hATF5 (a novel protein isolated in the present study). Most substrates of the yeast UBC enzymes have been identified by changes in stability of candidate target proteins in UBC mutant strains. The target proteins obtained in our study were initially identified as the result of a two-hybrid screen in yeast with *hCDC34* cDNA as the bait, which demonstrates that this method is capable of detecting the potentially transient interaction between Cdc34 and its substrates. Our ability to detect this interaction was likely dependent on the finding that the *hCDC34* bait encoded a functional protein in yeast.

In addition to the interactions detected in the yeast two-hybrid assay, several other biochemical experiments directly support the model in which both hICERIIγ and hATF5 are targeted by hCdc34 for ubiquitination and subsequent degradation via the 26S proteasome. First, transfection of *hICERIIγ* or *hATF5* with *hCDC34* or *hRAD6B* expression constructs in

mammalian cells results in significant and sometimes complete loss of the target proteins; this loss is reversed in the presence of proteasome inhibitors. Second, coexpression of hCdc34 mutant proteins fails to destabilize the targets. Third, the half-life of the target protein is decreased in the presence of hCdc34 and hRad6B. Fourth, coexpression with *hCDC34* in mammalian cells completely abrogates the repression of cAMP-induced transcription by hICERIIγ and hATF5. These findings taken together demonstrate that Cdc34 coexpression results in the loss of the biologically relevant repressor protein.

The involvement of the ubiquitin-proteasome pathway in the degradation of endogenous ICER protein in primary cardiocytes and myogenic cell lines has been previously reported (17). We also detect ubiquitin conjugates of hICERIIγ-Myc fusion protein in the presence of ectopic ubiquitin and peptide-aldehyde without addition of exogenous *hCDC34* or *hRAD6B*. Further evidence for specific targeting of the endogenous ICER protein by endogenous Cdc34 and Rad6B enzymes was obtained by analysis of the *mHR6B*$^{-/-}$ MEF and JEG-3 cells transfected with Cdc34 inhibitory constructs. These experiments confirm the results seen with transfected ICERIIγ protein. Loss of either Rad6B or Cdc34 activity results in increased levels of endogenous ICER protein.

We have demonstrated that both the Cdc34 and Rad6B UBC enzymes are capable of targeting for degradation CREM, ICER, and ATF proteins. Although *S. cerevisiae CDC34* (6) was originally identified due to a cell cycle phenotype and *RAD6* (25) was identified through a DNA repair phenotype, the yeast enzymes also have common targets. For example, degradation of Gcn4, which contains a bZIP motif, also requires both Rad6 (UBC2) and Cdc34 (UBC3) (35). There are other examples of shared targeting, with Matα2 being targeted by UBC4, UBC5, UBC6, and UBC7 enzymes (7). In our experiments the targeting by multiple UBC enzymes (E2s) has different characteristics; e.g., hCdc34 has a more significant effect on the half-life of hICERIIγ than hRad6B does. The degradation kinetics with hRad6B was biphasic, with an initial lower rate. Similar differences in the kinetics have also been reported for the degradation of yeast Gcn4 by Cdc34 and Rad6 (35). Also consistent with the differential effect of Cdc34 and Rad6B, we detected an interaction in a mammalian two-hybrid assay with *hRAD6B* but not *hCDC34* cDNA as bait and *hICERIIγ* as the target construct (data not shown), presumably because the degradation of the target is too rapid in the presence of the transfected hCdc34.

It was suggested by Kornitzer et al. (35) that targeting of Cdc34 and Rad6 may be specific to proteins containing PEST sequences. Our analysis of hCdc34 interactants does not support that hypothesis. In ICER proteins, the unique ICER-specific domain and the γ exon are rich in PEST-containing sequences. ICERIIγ lacks the characteristic γ exon, and a construct containing only the ICER-specific domain was not destabilized by hCdc34. hICERIIγ also lacks the amino-terminal phosphorylation domain (P box or kinase-inducible domain) unique to CREM and CREB, which indicates that this specific phosphorylation is not required for hCdc34-mediated targeting. Further studies to identify specific domains, if any, in these bZIP transcription factors that are targeted by hCdc34 are in progress. However, extensive deletion studies of Gcn4 by Kornitzer et al. (35) were not able to demonstrate a single region of Gcn4 sufficient for efficient ubiquitination by both Cdc34 and Gcn4. Three targets (hICERIIγ, hATF5, and clone 30-17) isolated from the two-hybrid screen and CREM β and Gcn4 (35) all have a characteristic bZIP domain. In these targets, the basic domain, flanking the characteristic leucine zipper, is rich in lysine and arginine residues, and this lysine-rich region may form the target for ubiquitination.

Currently, there is uncertainty as to whether Cdc34-mediated ubiquitination requires an SCF complex for all targets. Michel and Xiong (47) have indicated that the SCF pathway, although similarly used by the mammalian Cullin-1, is not shared by other Cullin members, which may use a Skp1–F-box-independent pathway. From our studies, we cannot be conclusive about the involvement of the SCF complex in the Cdc34-mediated targeting of hICERIIγ and hATF5. hCdc34 apparently can complex with the SCF complex in yeast, based on complementation of a *cdc34-1* strain, and a carboxy-truncated mutant which cannot complex with the Cullin proteins is not capable of targeting hICERIIγ. However, we find that addition of exogenous hCdc34 or hRad6B protein alone in mammalian cells is sufficient to increase turnover of the ectopic hICERIIγ. Therefore, if the SCF complex is required for this degradation, it is found in excess and hCdc34 is limiting. At present it is also not known whether Rad6- and Cdc34-mediated ubiquitination of common targets requires shared proteins. Our development of a Cdc34-dependent in vivo assay for ubiquitination of these targets in mammalian cells should allow us to dissect these pathways and determine which components of the SCF complex are required for ubiquitination.

The expression pattern of mammalian Cdc34 and the SCF complex components has not been well described. We examined the expression profiles of both Cdc34 and Rad6B proteins along with the SCF subunit Cullin proteins (Cul-1 and Cul-2) during murine testicular development. In budding yeast, the Cdc34 protein level has been reported to remain constant throughout the cell cycle (20). We find that both UBC enzymes Cdc34 and Rad6B are expressed in a developmentally regulated manner in mouse testis and germ cells. The ratio of Cdc34 to the Cullin proteins does not remain constant during development, with maximal expression of Cul-1 and Cul-2 at day 20 in late pachytene and maximal expression of Cdc34 at day 30. Thus, at different points during spermatogenesis Cdc34 may form different complexes with unique targeting specificities. Cdc34 is maximally expressed late during spermatid differentiation when most intracellular proteins are being degraded and a complex series of chromatin modifications, including the ubiquitination of histone, takes place (for a review, see reference 9). We also reproducibly detect a higher-molecular-weight form of Cdc34 specifically in germ cells late in spermatogenesis, which may represent an additional form of regulation of the enzyme. Therefore, despite the evidence for Cdc34 function in mitotically growing cells, Cdc34 expression in the testis is highly regulated and is maximal in the postmeiotic phase of spermatogenesis. In addition, a second E1 protein, Ube1y, expressed in mouse testis is encoded by a gene on the Y chromosome (31). Therefore, a ubiquitin-activating (E1) enzyme plus multiple UBC (E2) enzymes are present in both X- and Y-containing gametes late in spermatogenesis when histone ubiquitination and massive protein degradation is occurring.

The transcriptional activity of ICER, including the repression of its own promoter, has been reported to be determined by the intracellular concentration of the ICER proteins (48, 49). Thus, turnover of ICER and its degradation via the ubiquitin-proteasome pathway may act as a regulatory mechanism to relieve transcriptional repression and to control the negative effect of ICER on the cAMP-inducible transcriptional response. Although we were unable to show a simple inverse correlation between the expression of ICER and Cdc34 and Rad6B proteins in germ cells, this may be for several reasons: (i) ICER protein expression is a sum of transcriptional regu-

lation, alternative splicing, and protein stability; (ii) there may be a requirement for other regulatory proteins, including subunits of the SCF complex, to mediate ubiquitination of ICER isoforms; and (iii) ubiquitin-mediated proteolysis of ICER proteins may occur in only a specific subset of cells in the testis. On the other hand, a clear negative correlation was observed between the repressor CREM isoform CREM β and Cdc34 and Rad6B proteins during testicular development and in the haploid germ cells, indicating a potential targeting of CREM β by Cdc34 in late spermatids. This finding was further supported by the destabilization of CREM β by Cdc34 in transfection assays and warrants further investigation.

ICER isoforms regulate a number of biological processes, including spermatogenesis, circadian rhythm of transcription in the pineal gland, and cell proliferation (for a review, see reference 60). For example, ICER isoforms negatively regulate the expression of cyclin A mRNA, potentially resulting in specific induction of cyclin A from mid-G_1 to early S, when the level of ICER is low (13) and the Cdc34-SCF complex is active (36). Therefore, Cdc34 may regulate cyclin A both at the transcriptional level and by the previously documented association of the cyclin A-CDK2 complex with the Cdc34-SCF complex (41). Conversely, a recent report has demonstrated that overexpression of ICERIIγ inhibits tumor cell growth and results in G_2 arrest at a point in the cycle when the Cdc34-SCF complex may be less active (56). The cyclin A promoter is also suppressed by ATF4, which has considerable sequence homology to hATF5, a protein identified as a target of Cdc34. Thus, knowledge gained about mammalian Cdc34 function will be important in understanding how selective destabilization of proteins through ubiquitination regulates diverse processes, including both meiotic and mitotic cell cycles.

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IDENTIFICATION OF NOVEL TARGETS OF THE HUMAN CELL CYCLE REGULATORY PROTEIN Cdc34, AN UBIQUITIN LIGASE USING YEAST REVERSE TWO-HYBRID SYSTEM

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Loss of cellular growth control and derangement of normal cell cycle regulation are hallmarks of human malignancy. A fundamental mechanism of cell cycle control is the coordinated destabilization of either positive or negative regulatory molecules by ubiquitination. *CDC34,* a gene essential for the G1/S phase transition in yeast is a ubiquitin ligase, an enzyme required for ubiquitination and proteolytic degradation of specific cellular proteins. Recently, highly conserved human and murine homologues of yeast *CDC34* gene have been cloned and characterized in our laboratory. Our goal is to understand the role of human Cdc34 in the cell cycle transition in normal and malignant mammary cells. We hypothesize that Cdc34 is a cell cycle regulatory protein in mammalian cells which modulates the stability of specific cellular proteins by targeting them for degradation. The physiological targets of mammalian Cdc34 are not presently known. We have used a yeast-based *in vivo* genetic assay called two-hybrid cloning to identify proteins that interact with mammalian Cdc34. cDNAs found to be active in this assay have been isolated and currently being analyzed.

Reagents used in the following two-hybrid screen include the Gal4-activation domain (AD) library, the Gal4-DNA binding (DB) vector (pPC97), and the yeast host strain MV103. The full length human *CDC34* cDNA (Plon et al., 1993, *PNAS*, 90,10484) has been cloned into pPC97 (Leu+) *GAL4* DNA-binding domain fusion vector (Vidal et al., 1996, *PNAS*, 93,10315). The ability of pPC97-*hCDC34* construct to encode the functional Cdc34 protein was tested in yeast by its ability to complement a temperature sensitive *cdc34*^{ts} mutant yeast strain, SJ1098-3d (B. Byers, University of Washington, Seattle).

Key words: Cell Cycle, Ubiquitin Ligase, Cdc34, Two-Hybrid, Cancer Biology

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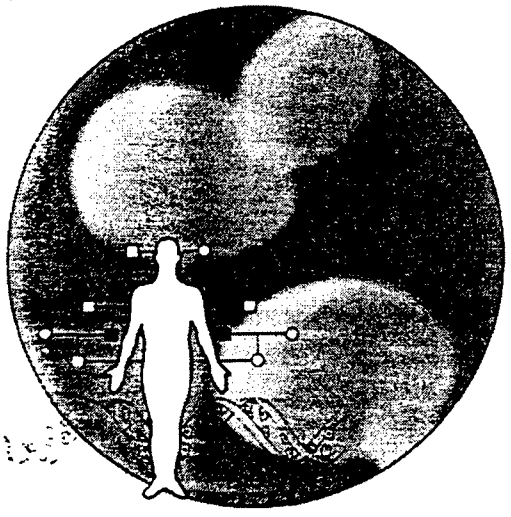
The pPC97-*hCDC34* construct was able to completely suppress the cdc34^{ts} mutation allowing growth at 37°C. We have also verified that the *GAL4*-Cdc34 fusion protein is expressed in the host two-hybrid strain (MV103) in Western blot using a monoclonal Cdc34 antiserum (Transduction Lab) and has no autonomous effect on the His3, Ura3 and LacZ reporters, due for example to the presence of cryptic transcriptional activation sequences. We also determined the minimal concentration of 3 amino triazole (3AT; 10-25 mM) which prevented growth of the pPC97-*hCDC34* bait. A human T-cell cDNA fusion library in the activation domain vector pPC86 (trp+) was kindly provided by J. La Baer (MGH Cancer Center). The library appears to have a broad range of cDNAs and has been successfully used in two-hybrid screening (Vidal et al., in preparation).

A key to the success of a two-hybrid screening is sufficient transformation efficiency to allow screening of at least one million clones. We have optimized the transformation protocol using lithium acetate method (Schiestl and Giets, 1989, *Curr. Genet.*, 16, 339) which is highly efficient and reproducible. We have developed a modified procedure which achieve transformation efficiency of 300,000 colonies per microgram plasmid DNA, a substantial improvement over previous protocols (Pati, D. and Plon, S.E., unpublished). With this transformation protocol we have sequentially transformed the bait (Leu+) and the library plasmid (Trp+) into the yeast host strain MV103. The transformants containing the bait and library plasmids were selected on media lacking Leucine and Tryptophan. Three separate pools of library DNA were used to transform the MV103+pPC97-*hCDC34* cells and 500,000 transformants from each pool was obtained.

The screen was carried out by first selecting for growth of Cdc34 bait-library co-transformants on Sc-His-Leu-Trp+25mM 3AT. Subsequently the 3AT positive clones were selected for the additional reporter genes, *URA3* and *LacZ*. The expression of *URA3* gene was both selected for on media lacking Uracil as well as counter selected against on media containing Uracil and 0.1% 5-fluoroorotic acid (5FAO). Induction of *LacZ* gene was assayed qualitatively in the presence of X-Gal for blue colonies. The phenotypes were then scored.

In the screen out of the 1.5 million transformants 30 clone were found positive for all the reporters (His/3AT+, Ura+, FOA-, X-Gal+) whereas 139 others were partially positive (3AT+, Ura+, FOA?, X-Gal?). Out of these 30 clones 18 clones have been sequenced and fourteen are known genes and four are novel clones. One group of these positive clones are found to be involved in control of meiosis and spermatogenesis, one of which also has a distinct role in DNA double strand break repair. Further studies are currently in progress to characterize the Cdc34-mediated regulation of these interactors and their physiological role in the cell cycle progression and development of malignancy. It appears from our studies that hCdc34 may have a novel role in meiosis in higher organisms.

Knowledge gained about mammalian Cdc34 function and its interacting partners will be instrumental in understanding how selective destabilization of proteins through ubiquitination regulates the cell division cycle and its role in the development of breast cancer and oncogenesis in general.



YEAST GENETICS AND HUMAN DISEASE II

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Human Cdc34 and Rad6B Ubiquitin-Conjugating Enzymes Target Repressors of cAMP-induced Transcription for Proteolysis

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Ubiquitin mediated proteolysis controls diverse physiological processes in all eukaryotes. A conserved Cdc34 ubiquitin conjugating enzyme (UBC)- Skip1/Cullin/F-Box (SCF) ubiquitin protein ligase complex functions in both yeast and human cells to mediate ubiquitin-mediated proteolysis. Our goal was to identify specific *in vivo* ubiquitination targets of mammalian Cdc34. A yeast-based two hybrid screen of a human T cell library with human Cdc34 as bait resulted in three cDNAs encoding bZip DNA binding motifs. Two of these are repressors of cAMP-induced transcription, hICERIIγ, a product of the CREM gene and hATF5, a novel ATF homolog. Assays in mammalian cell lines demonstrate both Cdc34- and the related UBC Rad6B-dependent ubiquitin-mediated proteolysis of hICERIIγ and hATF5. This degradation results in loss of the biologically active ICERIIγ and ATF5 transcriptional repressors. Addition of exogenous hCdc34 or hRad6B protein alone is sufficient to increase turnover of hICERIIγ and therefore the UBC is rate limiting in these cells. Consistent with these results endogenous ICER protein is elevated in *mHR6b-/-* (murine Rad6B) fibroblasts and in human cells transfected with dominant negative and anti-sense *CDC34* constructs. There are substantial parallels between ubiquitination of ICER/ATF5 and stability of the yeast bZip transcription factor Gcn4 which is also ubiquitinated by both Cdc34 and Rad6 with similar degradation kinetics (impact of Cdc34>Rad6). ICER/CREM isoforms regulate a number of important biological processes including spermatogenesis, circadian transcription in the pineal gland and cyclin A transcription in proliferating cells. In particular, both *crem-/-* and *mHR6b-/-* mice have defects in spermatogenesis. Analyis of murine testis and isolated germ cells demonstrate (1) the level of Cdc34, Rad6B and Cullin proteins are developmentally regulated (2) expression of Rad6B is limited to germ cells and a germ cell specific form of Cdc34 is detected on Western blots and (3) Cdc34 and Rad6B protein is significantly elevated in meiotic and post meiotic haploid germ cells undergoing late stages of spermatogenesis. Thus, in diverse eukaryotes the stability of specific transcription factors is the result of complex targeting by multiple UBC's and this process impacts on cAMP-inducible gene regulation during both meiotic and mitotic cell cycles. Our data also suggests that for ubiquitination of some proteins, although specificity of targeting is mediated by the E3 complex the rate of targeting is dependent on the availability of the UBC enzyme.

109) Yeast Dam1p Interacts With Mps1p Protein Kinase And Has A Role In Spindle Integrity And The Mitotic Checkpoint.

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We have identified a mutant allele of the $_{AM1}$ gene in a screen for mutations that are lethal in combination with the *mps1-1* mutation. *MPS1* encodes an essential protein kinase that is required for duplication of the spindle pole body (SPB) and for the spindle assembly checkpoint. DAM1 also displays genetic interactions with *STU1*, *CIN8*, and *KAR3* genes encoding proteins involved in spindle function. DAM1 is an essential gene that encodes a protein recently described as a member of a microtubule binding complex (Hofmann *et al.*, 1998, J Cell Biol. 143:1029-1040). We have used immunofluorescence and EM, to show that cells harboring the Dam1-1 mutation fail to maintain spindle integrity during anaphase at the restrictive temperature. These cells show a mitotic cell bias that requires checkpoint genes *MAD1-3*. Interestingly, although the spindle assembly checkpoint is activated, it is not functioning correctly; instead of observing the typical spindle assembly checkpoint state with a short spindle and one mass of duplicated DNA, we observe two separated masses of DNA flanking the ends of the broken spindle. We are currently analyzing which step in the checkpoint pathway is defective. We have observed that a Dam1p-Myc fusion protein expressed at endogenous levels and localized by immunofluorescence microscopy, appears to be evenly distributed along short mitotic spindles but at later times in mitosis, is found at the spindle poles, peripheral to the spindle pole body. This pattern is reminiscent of several kinetochore proteins and may indicate that Dam1p interacts specifically with kinetochore microtubules. We will pursue this hypothesis by using immuno-EM to localize Dam1p at higher resolution, and by testing for interactions with a variety of kinetochore associated proteins.

110) INTRAGENIC SUPPRESSOR ANALYSIS OF MUTATIONS LOCATED IN THE NUCLEOTIDE BINDING DOMAINS OF THE YEAST ABC TRANSPORTER YCF1

ERASO, P. (1), FALCON-PEREZ, J.M. (1), MOLANO, J. (2), MAZON, M.J. (1)
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ATP-binding casette (ABC) transporters form a superfamily of structurally related membrane proteins responsible for the translocation of a variety of molecules across biological membranes. Eukaryotic ABC transporters include the yeast proteins a-factor transporter (Ste6p) and the yeast cadmium factor protein (Ycf1p) and many proteins of medical significance, such as the cystic fibrosis (CF) transmembrane conductance regulator (CFTR), the multidrug resistance protein (MDR1) or the multidrug resistance-associated protein (MRP1). The main distinguishing feature of this superfamily is a highly conserved nucleotide binding domain (NBF). The importance of the NBFs is further emphasized by the large number of CF-associated mutations that have been found in these domains. One way to understand the mechanism by which mutations alter the function of a protein is to isolate intragenic suppressor mutations that compensate the defects caused by the primary mutations. We have chosen the yeast Ycf1p to perform an intragenic suppressor analysis of mutations located in the NBFs. Ycf1p is a vacuolar membrane protein that transports cadmium ions and several drugs as glutathion complexes and shows strong sequence similarity with CFTR and MRP1. The sequence identity of these proteins suggests that the information obtained in the yeast system could provide valuable insights into the molecular mechanism of transport by the human proteins. A search for second-site suppresors was undertaken for five mutations, G663V, G756D, D777N, G1306 and G1311R, located in the highly conserved Walker A, Walker B and signature regions of Ycf1p NBFs. These changes correspond to CF-associated mutations in CFTR and severely affect Ycf1p function but not biogenesis of the protein (see also Abstract by). Random mutagenesis of the mutant genes was performed and revertants were selected by their ability to detoxify cadmium ions in the growth medium. We were unable to isolate any intragenic suppressor of mutations G663V, G756D, G1306 and G1311R, only full revertants were obtained. In the case of D777N, we identified 30 second-site suppressors. Mapping and sequencing revealed 13 different mutations located in the transmembrane and NBF domains. Partial characterization of the suppressor mutants will be presented.

111) Analysis of the GMxCxxCIE copper-binding motifs in the copper transport function of ATP7B.

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Medical Genetics, Univ. of Alberta, Edmonton, Alberta, Canada

Using a yeast complementation assay, we have analyzed the functional effect of site-directed mutations and deletions in the copper-binding domain of ATP7B (the copper transporting P-type

D. Pati

Abstracts of papers presented
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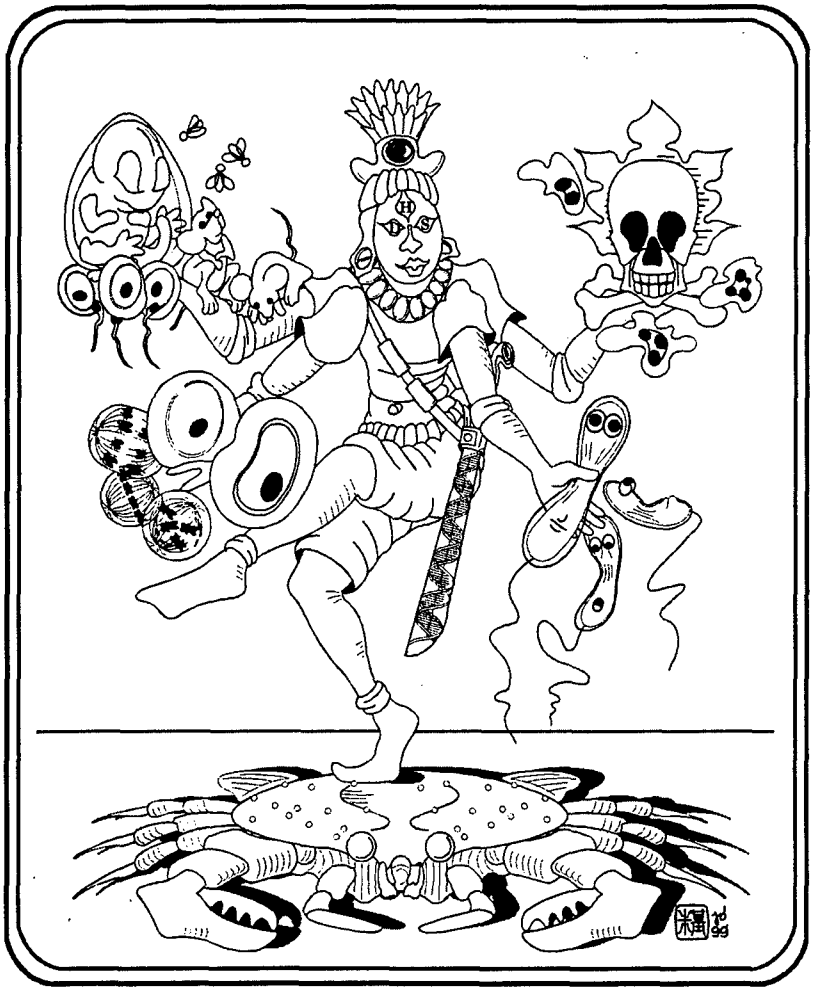

Cold Spring Harbor Laboratory
Cold Spring Harbor, New York

HUMAN Cdc34 AND Rad6 UBIQUITIN-CONJUGATING ENZYMES TARGET bZIP TRANSCRIPTIONAL REPRESSORS FOR PROTEOLYSIS: IMPLICATIONS FOR SPERMATOGENESIS

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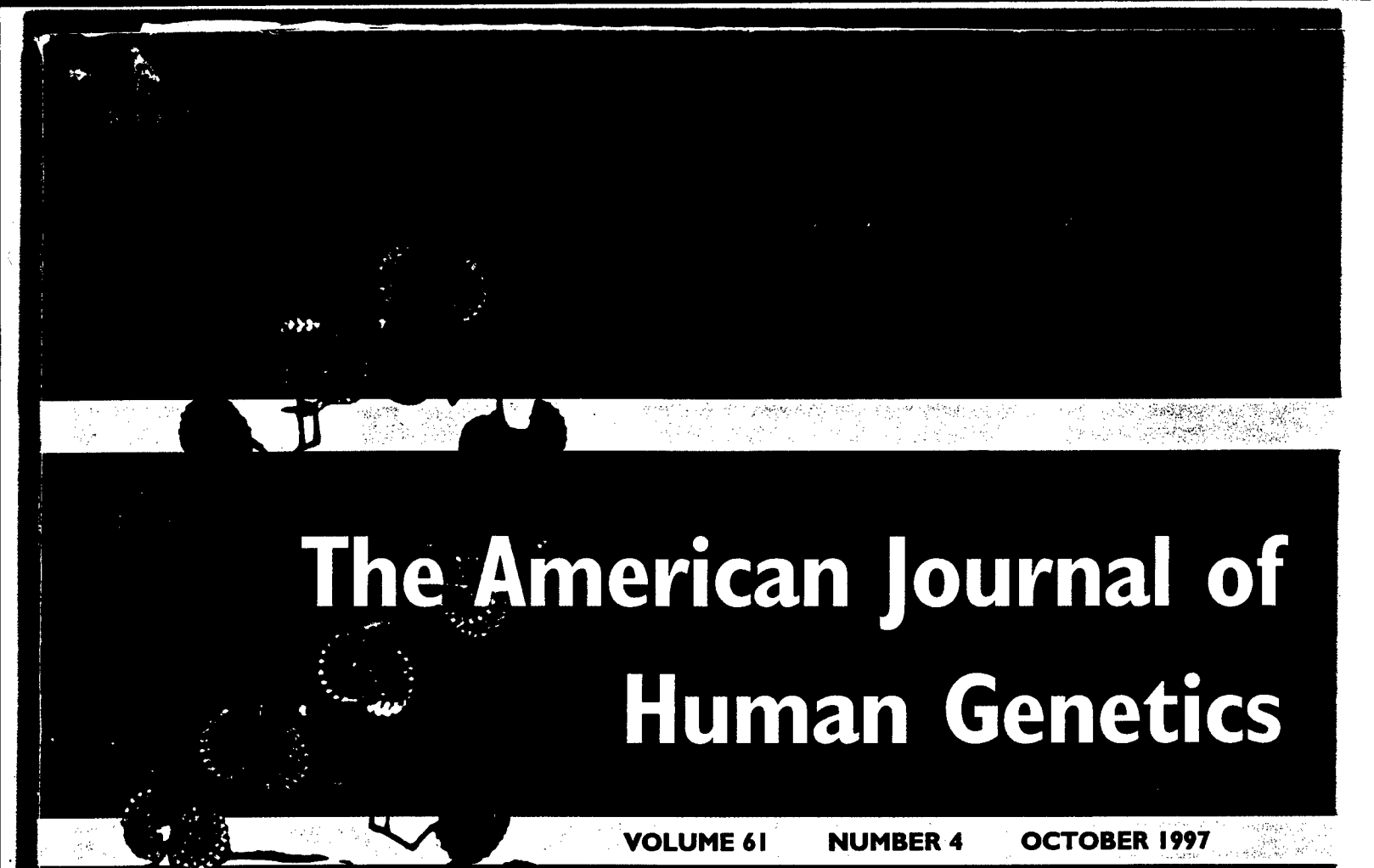
Ubiquitin-mediated proteolysis controls diverse physiological processes in eukaryotes. However, few *in vivo* targets of the mammalian Cdc34 and Rad6 ubiquitin-conjugating enzymes are known. A yeast-based genetic assay to identify proteins that interact with human Cdc34 resulted in 18 cDNAs positive for all three reporters. Three of these positive clones encode bZip DNA binding motifs; two are repressors of cAMP-induced transcription, hICERIIγ, a product of the CREM gene and hATF5, a novel ATF homolog, and a novel cDNA. Assays in mammalian cells demonstrate both hCdc34-dependent and the closely related E2, hRad6B-dependent ubiquitin-mediated proteolysis of hICERIIγ and hATF5. This degradation requires an active ubiquitin-conjugating enzyme and results in abrogation of ICER and ATF5-mediated repression of cAMP induced transcription. These findings taken together demonstrate that Cdc34/Rad6B co-expression results in the loss of the biologically relevant repressor protein, which parallels the Cdc34/Rad6B-mediated degradation of Gcn4 in yeast. A mini-ICER construct with just the ICER specific exon (including the PEST sequences) was not targeted for degradation. The targeting data suggests that the lysine rich region juxtaposed to the bZip domain may be required for ubiquitination. Based on the requirement for CREM/ICER and Rad6B proteins in spermatogenesis, we determined expression of Cdc34, Rad6B, CREM/ICER isoforms and the Skip1/Cullin/F-Box (SCF) subunits Cul-1 and Cul-2 during murine testicular development. Cdc34, Rad6B and the Cullin proteins are expressed in germ cells in a developmentally regulated manner with distinctly different patterns for Cdc34 and the Cullins. Cdc34 and Rad6B proteins are significantly elevated in meiotic and post-meiotic haploid germ cells when a complex series of chromatin modifications takes place. Thus, the stability of specific mammalian transcription factors is the result of complex targeting by multiple ubiquitin-conjugating enzymes and may impact on cAMP-inducible gene regulation in meiotic, mitotic and non-dividing cells. Furthermore, identification of these protein targets using *hCDC34* cDNA as the bait demonstrates that this methodology is capable of detecting the potentially transient interaction between hCdc34 and its substrates.



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Significance of borderline fragile X premutations. *R. C. Osthus, M. S. DiMaio, M. J. Mahoney and A. E. Bale.* Dept. of Genetics, Yale Univ. School of Medicine, New Haven, CT.

Large expansions of the CGG repeat region (> 200 repeats) in FMR1 result in fragile X syndrome. Women bearing premutations (60 - 200 repeats) are at high risk for producing affected offspring, and the risk of expansion when an allele is transmitted from mother to child is directly related to the number of repeats (Fisch et al., Am J Hum Genet 56:1147-1155, 1995). Alleles in the range of 50 to 60 repeats do not confer a high risk of transmitting fragile X syndrome, but affected offspring have been reported in women with these "borderline premutations."

Over the past year, Fragile X carrier screening has been offered to 2350 women undergoing prenatal genetic counseling for other reasons, and 339 women were tested. DNA was analyzed by PCR utilizing a strongly denaturing buffer system (2M betaine) and Klentaq I/Pfu polymerase instead of Taq polymerase (Baskaran et al. 1996). This method does not require 7-deaza-guanidine, and PCR products can be visualized by standard ethidium bromide staining in an agarose gel. This protocol gives highly reproducible results and can be used for direct mutation detection from amniotic fluid or buccal mucosa samples.

We found no carriers of full mutations, three carriers of premutations (90 repeats in one and 65 repeats in two) and 10 carriers of borderline premutations, the most difficult class to counsel. Of eight pregnancies tested among the latter 10 women four fetuses inherited their mother's normal allele and four inherited their mother's at risk allele with no change in repeat number. Direct sequencing of the borderline premutation alleles has consistently shown imperfect CGG repeats interrupted by three to five AGGs.

These data indicate that population screening detects a significant number of women with FMR1 expansions of unknown significance but that these expansions are probably benign in most cases. Direct sequencing to differentiate perfect repeats from those with interruptions may prove to be a useful adjunct to methods that determine allele size, only.

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Maternal weight and first trimester Down syndrome screening. *G.E. Palomaki, L.M. Neveux, G.J. Knight, J.E. Haddow.* Foundation for Blood Research, Scarborough, Maine. Intro. by L.A. Bradley.

Adjusting second trimester screening markers for maternal weight is now routine. The possibility of first trimester screening for Down syndrome is now being considered, using either the free beta subunit of hCG (free-β) or hCG, and pregnancy-associated plasma protein A (PAPP-A). We have analyzed the relationship between maternal weight and these first trimester markers using serum measurements from 4,168 women between 9 and 15 weeks gestation. The table shows the unadjusted free-β, hCG and PAPP-A MoM levels, stratified by maternal weight.

Maternal Weight	Number of Women	Median free-B	Median hCG	Median PAPP-A
80-99	19	1.21	1.38	1.57
100-119	425	1.26	1.18	1.23
120-139	1373	1.08	1.10	1.12
140-159	1186	1.00	0.99	0.99
160-179	623	0.91	0.91	0.88
180-199	286	0.79	0.86	0.74
200-219	118	0.79	0.80	0.70
220-239	80	0.77	0.72	0.55
240-259	34	0.71	0.86	0.67
260-350	24	0.77	0.75	0.47

The maternal weight effect is present for all three analytes, but is most pronounced for PAPP-A (the largest of the molecules). The effect (data not shown) is least for unconjugated estriol (the smallest of the molecules). Regression of the median MoM versus the reciprocal maternal weight fits the observed data well for all three, with a slope and intercept of 102.0074, 0.29836 for free-β, 94.0095, 0.35675 for hCG and 148.0965,-0,03133 for PAPP-A. Adjusting for maternal weight reduces population variability and improves screening performance. At a false positive rate of five percent, the modeled detection rate for free-β/PAPP-A increases from 66 to 70 percent with weight adjustment. The corresponding increase for hCG/PAPP-A is 62 to 68 percent.

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Hippocampus-Independent Deficits in the Fragile X Mouse. *W Paradee, D Rasmussen, H Melikian, PJ Conn, and ST Warren.* Emory University School of Medicine, Atlanta, GA 30322.

Fragile X syndrome is a common form of inherited neurodevelopmental disability that is due to the absence of the FMR-1 protein. FMRP is a nucleocytoplasmic shuttling protein that binds RNA and is found, at steady-state, associated with polyribosomes. A mouse model for Fragile X syndrome has been described (Dutch-Belgian Fragile X Consortium, 1994), and these mice show an increased exploratory behavior that parallels hyperactivity seen in Fragile X patients. They only display a subtle deficit in spatial learning, as tested in the Morris water maze, but it is enhanced significantly when the conditions of the maze are altered; suggesting these mice have a problem learning variations of a task they have already mastered. Since spatial learning in the water maze is believed to be a function of the hippocampus, we chose to investigate the impact of FMRP on neuronal plasticity, by measuring long term potentiation in the Shaffer collateral/CA1 synapse of the hippocampus. Using a theta burst protocol to induce activity, we saw no difference in the protein synthesis-dependent late phase of LTP between knockout (n=5) and control littermates (n=6). Therefore, in order to examine hippocampal-independent behavior, we next tested these animals using hippocampus-independent conditioned fear response. When compared with control littermates, we found a significant effect when knockout animals were exposed to the conditioned stimulus (auditory tone; $p<0.005$) but not when subjected to the unconditioned stimulus ($p>0.1$). Since FMRP is widely expressed throughout the brain, these results suggest that regions other than the hippocampus may be involved with cognitive deficits observed in Fragile X patients.

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Application of FISH in clinical practice: analysis of maternal cell contamination (MCC). *E.Pergament, S. Hockstein, P. Chan, M. Thangavelu, R. Tamura, M. Fiddler.* Reproductive Genetics. Northwestern University Medical School, Chicago, IL.

Potential diagnostic errors due to MCC have been highlighted in a series of reports on FISH applied to uncultured amniotic fluid (AF) cells (NuB et al, 1994; Christensen et al, 1993; Winsor et al., 1996). MCC may vary with patient factors such as body mass index (BMI), placental location, and gestational age or with procedure-related factors such as operator experience, placental penetration, number of passes and gross appearance of AF. Three operator groups were designated: (1) "most experienced" (one operator performing >1000 amniocenteses annually for 10 years); (2) "intermediate experienced" (five private practice physicians performing amniocentesis in their offices); and (3) "inexperienced operators-in-training" (3rd year residents). 130 samples from male fetuses were analyzed: group (1) 50; (2) 50; (3) 30. Based on a pilot study of 50 specimens, the initial aliquot of AF, usually discarded to minimize MCC, was deliberately selected as the study sample. A control system consisting of 8 different levels of MCC (peripheral female blood mixed in known quantities with male blood) and analyzed in a blinded fashion, validated the methods used to count cells and calculate MCC: the detected MCC vs actual MCC were highly correlated ($r=0.99$; $p=<.0001$). The overall mean MCC in 130 specimens was 5.7%; the median, 2.0%; standard deviation, 9.2%; MCC ranged from 0 to 55%. MCC did not correlate with body mass index (BMI) ($p=0.59$); gestational age ($p=0.13$); placental location ($p=0.55$). MCC was correlated with placental penetration ($p=.0005$); number of passes ($p=.002$); fluid color ($p=.0004$) and operator experience ($p=.026$). The median MCC for (1) "most experienced," (2) "intermediate experienced" and (3) "inexperienced" operators was 1%, 4.5% and 2.0%, respectively; the difference between (1) and (2) was statistically significant whereas it was not between (1) and (3). In conclusion, the frequency and incidence of MCC in uncultured AF cells are directly dependent on operator performance and are not a function of laboratory technique such as FISH preparation and scoring.

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Rapid prenatal diagnosis by primed *in situ* labeling (PRINS). *O.P. Phillips, G.V.N. Velagelati, L.P. Shulman, S.A. Tharapel, P.R. Martens, S.S. Wachtel, A.T. Tharapel.* Depts. of Ob-Gyn and Pediatrics, Univ. of Tennessee, Memphis TN.

PRINS is a diagnostic technique that combines features of fluorescence *in situ* hybridization (FISH) and polymerase chain reaction. Because PRINS has demonstrated high efficiency for discriminating alpha satellite sequences and analysis can be completed within a few hours, PRINS would be a desirable adjunct in prenatal diagnosis. We therefore sought to evaluate PRINS in analysis of amniotic fluid (AF) and chorionic villus (CV) cells for fetal aneuploidy. Forty unselected prenatal specimens were collected: 6 AF specimens (2-3 ml.) and 34 cell suspensions from direct preparation of CV cells. PRINS was carried out according to published protocols using oligonucleotide primers specific for chromosomes 18, 21, X and Y. Fluorescent signals were scored for a minimum of 50 nuclei per primer by 2 observers. Results were compared to those obtained by conventional cytogenetic analysis. In all cases, including a case of trisomy 21, PRINS correctly predicted fetal sex and chromosome 18 and 21 complements. Our results showed a higher hybridization efficiency with PRINS (86-87%) compared to published data using FISH. The percentage of nuclei showing 0,1,3 or 4 signals in samples with diploid karyotypes was less than 5% for each probe and less than the average observed with FISH. Moreover, PRINS is faster than FISH and less labor intensive. We conclude that PRINS is a reliable technique that could prove useful in the prenatal diagnosis of common aneuploidies when rapid results are required.

[redacted]

Identification of meiotic cell cycle regulators as potential targets of the Human Cdc34 ubiquitin ligase. *S. E. Plon[1,2] and D. Pati[1].* Texas Children's Cancer Center, Departments of Pediatrics[1] and Molecular and Human Genetics[2], Baylor College of Medicine, Houston, TX.

One mechanism by which proteins control mitotic cell cycles is by ubiquitin-mediated destruction of cell cycle regulators including Cyclins and Cdk inhibitors. *CDC34*, a gene essential for the early phases of the cell division cycle in yeast encodes an enzyme required for degradation of yeast cell cycle regulators. Highly related functional *CDC34* genes in human and mouse have been cloned and characterized in our laboratory. Our goal is to understand the role of human Cdc34 in mammalian cell cycle transitions. The specific targets of the mammalian Cdc34 ubiquitin ligase are not presently known. We have used a genetic assay called two-hybrid cloning to identify proteins that interact with human Cdc34 by screening 1.5 million human cDNAs from a T lymphocyte library, a cell type where *CDC34* is highly expressed. Of 30 clones which activate all three reporter constructs in a *CDC34*-dependent manner, 18 clones have been sequenced. Fourteen encode known genes and four are novel clones. Surprisingly, four of these 18 positive clones represent genes which have been previously identified in the regulation of meiosis and spermatogenesis, one of which also has a distinct role in DNA double strand break repair. These genes are also known to be expressed in lymphocytes presumably due to the requirement for recombination of immunoglobulin and T cell receptor genes in lymphocytes. Thus, it appears from our studies that human Cdc34 may have a novel role in meiosis and recombination in higher organisms by specifically targeting meiotic regulators for ubiquitination. Knowledge gained about mammalian Cdc34 function will contribute to understanding how selective destabilization of proteins through ubiquitination regulates both meiotic and mitotic cell division cycles.

Cell Cycle Meeting, Keystone Symposia, Keystone, CO, March 27-April2, 1998.

327REGULATORS OF MEIOSIS AND SPERMATOGENESIS AS POTENTIAL TARGETS OF HUMAN Cdc34 UBIQUITIN LIGASE

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One mechanism of cell cycle control is ubiquitin-mediated destruction of cell cycle regulators. *CDC34,* a gene essential for mitotic cell division in yeast encodes an ubiquitin-ligase required for degradation of yeast cell cycle regulators. The goal of this project is to understand the role of human Cdc34 in the cell cycle transition of normal and malignant cells. The targets of mammalian Cdc34 are not presently known. We first used two-hybrid cloning to identify proteins that interact with mammalian Cdc34. After screening 1.5 million human cDNAs from a T cell library, 30 cDNA clones were found to be active in this assay and are currently being analyzed. Surprisingly, four of these clones are known to be involved in the control of meiosis and spermatogenesis, one also has a distinct role in DNA double strand break repair. Crem2, one of the target proteins is one of the newly discovered and least well characterized member of the Crem family of proteins, known to be involved in meiosis and spermatogenesis. Crem2 protein has very high sequence homology to the mouse and human inducible cAMP early repressor (ICER) and may act as a transcriptinonal repressor. Co-expression in mammalian cells of Cdc34 with Crem2, has resulted in the destabilization of the Crem2 protein. The authenticity of the Cdc34 interaction with Crem2 is further being explored using Cdc34 dominant negative and Cdc34 active site mutant constructs. Presently we are investigating the ontogenic expression of Cdc34 and Crem2 protein during mouse testicular development. It appears from our studies that human Cdc34 may have a novel role in meiosis, recombination, and response to DNA damage in higher organisms by specifically targeting these regulators for ubiquitination.

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