AD NUMBER

ADB238945

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies only; Proprietary Info; Jul 98 Other requests shall be referred to Army Medical Research and Materiel Command, Fort Detrick, MD 21702-5012

AUTHORITY

US Army Med Research and Mat Cmd, MCMR-RMI-S [70-1y], ltr 6 Jul 2000, Ft Detrick, MD

AD_____

GRANT NUMBER DAMD17-96-1-6087

TITLE: Identification of Novel Targets of the Human Cell Cycle Regulatory Protein Cdc34

PRINCIPAL INVESTIGATOR: Debananda Pati, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030-3498

REPORT DATE: July 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1998	Annual (1 Jul 97 - 30 Jun 98)

4. TITLE AND SUBTITLE
Identification of Novel Targets of the Human Cell Cycle Regulatory Protein, CDC34

5. FUNDING NUMBERS
DAMD17-96-1-6087

6. AUTHOR(S)
Debananda Pati, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Baylor College of Medicine
Houston, Texas 77030-3498

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution authorized to U.S. Government agencies only (proprietary information, Jul 98). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Normal human cells have developed an elaborate system to control cell division and the response to genetic damage. A characteristic feature of human malignancy is the loss of normal cell cycle control. *CDC34,* a gene essential for mitotic cell division in yeast encodes an enzyme required for degradation of yeast cell cycle regulators. The goal of this project is to understand the role of human Cdc34 in cell cycle transition in normal and malignant mammary cells. We have used a genetic assay called two-hybrid cloning to identify proteins that interact with mammalian Cdc34, screening 1.5 million human cDNAs. 30 cDNA clones found to be active in this assay have been isolated and are currently being analyzed. Surprisingly, four of these clones are known to be involved in the control of meiosis and spermatogenesis, while two belongs to a family of transcriptional repressors, and one has a distinct role in DNA double strand break repair. Thus, it appears from our studies that human Cdc34 may have a novel role in meiosis, recombination, transcription and response to DNA damage by specifically targeting these regulators for ubiquitination. 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 breast cancer. This result will be critical to designing new therapeutic strategies that could modulate the activity of Cdc34 enzyme to prevent the growth and genesis of breast tumor.

14. SUBJECT TERMS
Breast Cancer, Cdc34, Ubiquitination, Two-hybrid, Cancer Genetics, Cell Cycle

15. NUMBER OF PAGES
40

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

____ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

JP In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

JP 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.

7/28/98

PI - Signature Date

Table of Contents

Introduction

The hall mark of human malignancies is the alteration of the normal cell division cycle including deregulated gene expression, uncontrolled growth in nutrient deprived conditions and absence of the normal cell cycle checkpoints in response to DNA damage. Therefore, understanding the regulation of mammalian cell cycle is essential to elucidate the mechanisms of carcinogenesis. A fundamental mechanism by which proteins control the mitotic cell cycle in yeast is by ubiquitin-mediated destruction of either positive or negative cell cycle regulators such as cyclins and CKIs (cyclin-dependent kinase inhibitors), respectively. *CDC34,* a gene essential for the early phases of the cell division cycle in yeast, encodes a ubiquitin ligase, an enzyme required for ubiquitination and proteolytic degradation of yeast cell cycle regulators (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). Isolation and sequencing of the *S. cerevisiae CDC34* gene, which encodes a 295 amino acid protein (Goebl et al., 1988), revealed amino acid similarities to the ubiquitin-conjugating enzyme (E2) family of proteins including the DNA repair gene *RAD6* (Jentsch et al., 1987, Haas et al., 1991), and was named UBC3 (Wiebl and Kunau, 1992, for review see Hochstrasser, 1995, Haas and Siepmann, 1997). The Cdc34 protein consists of a highly conserved catalytic domain common to all UBC enzymes and an unique carboxy terminal extension or tail (Goebl et al., 1988), which is essential for cell cycle function (Silver et al., 1992, Kolman et al., 1992). Possible functions of the tail include directing specific association with other regulatory proteins and Cdc34 self association (Ptak et al., 1994, Prendergast et al., 1995).

Highly related functional *CDC34* genes in the human and the mouse have been cloned and characterized for the first time in our laboratory (Plon et al., 1993, Hemzawi et al., 1995, Pati, D. and Plon, S.E., unpublished) and found to share significant homology at the protein level with *S. cerevisiae.* 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.

Ubiquitination is a highly conserved process among diverse eukaryotes, where ubiquitin, a small 8 Kd polypeptide of 76 amino acids, is covalently attached to the lysine residues of the target protein. This usually directs the protein into rapid proteolysis (for review see Hochstrasser, 1995, Haas and Siepmann, 1997). Ubiquitin forms a thiolester bond with the ubiquitin-conjugating enzyme (Ubc or E2). This enzyme alone or in conjunction with a third enzyme E3 (ubiquitin-protein ligase), catalyzes isopeptide bond formation between ubiquitin and the substrate and transfers the ubiquitin to the ε-amino group of a reactive lysine residue in the target. E3 activities which are thought to render the substrate specificity are often provided by diverse and sometimes large and complex protein assemblies (Hershko, 1997, and Patton et al., 1998 for review). Two of the most recently characterized E3s are the SCF (Skip1/Cullin/F-box protein) complex (Krek, 1998, Patton et al, 1998, for review) and the Anaphase Promoting Complex (APC) (for a review see Pagano, 1997, Hoyt, 1997) involved in the regulated proteolysis at the G1-S transition and at mitosis, respectively. The yeast Cdc34 E2 enzyme is

recruited to the SCF E3 by an interaction with the Cdc53 (cullin) subunit (Skowyra et al., 1997, Feldman et al., 1997). On the other hand M-phase proteolytic substrates possess a nine amino acid destruction box (D box) motif that targets them to the E3 activity of the anaphase promoting complex (King et al., 1996).

Cdc34 and some subunits of the SCF complex (G1/S ubiquitin ligases) of *S. cerevisiae* not only target both G1 cyclins (Cln2 and Cln3) and Cdk inhibitors p40Sic1, but also several other substrates (Deshaies et al., 1995, for a review see Deshaies, 1997, Krek, 1998) including, DNA replication protein Cdc6 (Piatti et al., 1996), Cln2/Cdc28 inhibitor Far1 (Mckinney et al., 1997), glucose synthesis repressor Grr1 (Kishi and Yamao, 1998) and transcription factor GCN4 (Kornitzer et al., 1994). Recent findings have indicated the 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 (Lanker et al., 1996; Schneider et al, 1996). Both Cdc34 and Rad6 appeared to have certain common targets such as yeast Gcn4 (Kornitzer et al., 1994) and human CKI p27, Kip1 (Tam et al., 1997). However, very little is known about the *in vivo* targets of Cdc34 and Rad6 in higher organisms, including humans. We have used a yeast-based *in vivo* genetic assay (two-hybrid cloning) to identify proteins that interact with mammalian Cdc34. cDNAs found to be active in this assay have been isolated and are currently being analyzed. Two of these Cdc34 targets obtained in this genetic assay belong to the family of cAMP-induced bZIP transcriptional repressors; ICER (Inducible cAMP early repressor) and ATFX (activating transcription factor X)

In eukaryotes, cAMP mediated transcription and signal transduction plays a crucial role in diverse physiological processes including gametogenesis, circadian rhythm , and neuro-endocrine functions (for review see Sassone-Corsi, 1998). 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 (CREBs), CRE modulators (CREMs) and Activating Transcription Factors (ATFs). Transcription factors responsive to cAMP belong to the bZIP family and bind as dimers to a 8 base pair palindromic DNA consensus sequence called CRE (Sassone-Corsi, 1995). Leucine zipper, an α-helical coil structure is required for parallel dimerization, and an adjacent basic domain, rich in lysine and arginine residues needed for the direct contact with DNA. In addition, CREB and CREM contain an unique amino-terminal phosphorylation domain (P-box, kinase inducible domain), flanked by two glutamine rich transcription activation domain. The CREM gene is controlled by two promoters and encodes a large family of alternately spliced transcripts of both activators and repressors of cAMP-dependent transcription in a tissue and developmentally regulated manner. The upstream promoter (P1) controls the activator τ and the repressors α, β, γ and S isoforms of CREM, while the downstream intronic promoter (P2) directs a family of potent repressors of cAMP-induced transcription, called inducible cAMP early repressor(ICER) (Lalli et al., 1996; Foulkes et al., 1991, 1992, Molina et al., 1993). A family of four types of ICER transcripts (ICERI, Iγ, IIγ, II) are generated by alternate splicing of the DNA binding domain and γ domain exons (Molina et al., 1993). CREMτ, a 43 kd activator form of CREM contains several phosphorylation sites and are potential substrates of a number of kinases. On the other hand, ICER proteins are small (estimated molecular weight ~13Kd) and unlike CREM lacks the phosphorylation box (P-Box) and activation domain and escapes from PKA-dependent phosphorylation. Thus, in contrast to the other CRE-binding proteins, the

principal determinant of ICER activity is not its degree of phosphorylation but its intracellular concentration (Molina et al., 1993), which depend upon the transcriptional rate of the P2 promoter and the degradation rate of the ICER polypeptide (Molina et al., 1993, Desdouets et al., 1995, Monaco et al., 1995).

Here, we report that a hICER isoform ICERIIγ and a new member of the ATF family (hATFX) cloned through yeast 2-hybrid methods using human CDC34 ubiquitin ligase as a bait are targeted by both hCdc34 and hRad6B and degraded *in vivo*. Following is our progress on 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, task #1-4 were performed over the first year of the grant period and Task #5-8 has been attempted in the 2nd year (see appendix1). Part of this results have been presented at the Keystone Symposium on Cell Cycle (Pati and Plon, 1998) and a manuscript is in preparation (Pati et al., 1998).

Body of the Report

Materials and Methods:

Two-hybrid Reagents: Reagents used in the two-hybrid screening include the Gal4-activation domain (AD) library, the Gal4-DNA binding (DB) vector (pPC97), the yeast host strain MV103 (Mat **a**, *leu2*, *trp1*, *his3*, Gal1:HIS3, Gal1:LacZ, Spal:URA3), and 5 constructs in MV103 for use as reference controls during screening (Vidal et al., 1996a,b). Control plasmids include 1) DB-pPC97+AD-pPC86, 2) DB-pRb+AD-E2F1, 3) DB-Fos+AD-Jun, 4) Gal4+AD, 5) DB-dDP-1+AD-dE2F.

Construction of the DNA Binding-Cdc34 Fusion ("bait"): The full length human *CDC34* cDNA (12) has been cloned into pPC97 (Leu^+) *GAL4* DNA-binding domain fusion vector (Vidal et al., 1996a, b). Human *CDC34* in the pBluescript KS^+ plasmid was cut with *Not*I followed by *Sma*I at the codon encoding the first methionine and inserted in frame into the vector. The ability of the 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 (Mat **a**, *cdc34-2*, *leu2-3*, *ura3*, *trp1-* B. Byers, University of Washington, Seattle) (Fig.1). After transformation, cells were spread onto plates containing leucine-deficient medium and kept at 23°C for 24h, after which they were shifted to either 30°C or 37°C and incubated for 3 days. Part of the DB-*CDC34* construct has also been verified by sequence analysis using an internal oligo 340 bp downstream of the 5'end of *CDC34*. We have also verified that the *GAL4*-Cdc34 fusion protein is expressed in the host two-hybrid strain (MV103) by Western blot using a monoclonal Cdc34 antiserum (Transduction Lab, Lexington, KY) (see below).

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 (MGH Cancer Center, Charlestown, MA). The cDNAs were cloned into the *EcoRI* (5') and *SpeI* (3') sites. This library has approximately $2X10^6$ clones and the average insert size is 1kb. *hCDC34* is highly expressed in T-lymphocytes (E. Eleseva and S. Plon, unpublished) and the library appears to have a broad range of cDNAs and has been successfully used in two-hybrid screening (Vidal M. et al unpublished). This library was amplified once by electroporation using electrocompetent *E. coli*, JS4 cells (BioRad, Hercules, CA) followed by replica plating onto LB+Ampicillin plates. The DNA was prepared using a Plasmid Maxi kit from Qiagen (Valencia, CA).

Selection of *CDC34* interacting genes: The bait (Leu^+) and the library plasmid (Trp^+) were sequentially transformed into the yeast host strain MV103 using a modified Li-Acetate transformation protocol of Schiestl, and Giets (1989) and yeast total RNA and denatured salmon sperm DNA as carrier to achieve a transformation efficiency of 300,000 colonies per microgram plasmid DNA. 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-*CDC34* cells and 500,000 transformants from each pool were obtained.

The two-hybrid screen was performed by first selecting for growth of Cdc34 bait-library co-transformants on Sc-His-Leu-Trp+25mM 3AT. Subsequently additional reporter genes, *URA3* and *LacZ*, were selected for in the 3AT positive clones. The expression of the *URA3* gene was both selected for on media lacking uracil as well as counterselected against on media containing uracil and 0.1% 5-fluoroorotic acid (5FOA). Induction of the *LacZ* gene was assayed qualitatively in the presence of X-Gal for blue colonies. The phenotypes were then scored. Clones positive for all the reporters were PCR cloned into a pPCRII vector using TA-cloning kit from Invitrogen (Torry Pines, CA). Both strands of the DNA were then sequenced using a LI-COR automated sequencer.

Cell Cultures, Transfection and Expression Vectors: A panel of breast cancer cell lines including, MCF7, MDA-MB-157, MDA-MB-231, MDA-MB-136, BT-20, HBL100, SKBR-3 (all from ATCC, Rockville, MD) and normal human mammary epithelial cells, HMEC (Clonetics Corp, San Diego, CA) were maintained under similar conditions in DFCI media as previously described (Band and Sager, 1986). NIH-3T3 and human choriocarcinoma cell line JEG3 (both obtained from ATCC) were grown respectively in DMEM (Dulbecco's modified Eagle's medium) and MEM (modified Eagle's medium) supplemented with 10% fetal bovine serum (FBS) and maintained at 37 ^{0}C, 95% humidity and 5% CO_2. Cells used in the experiments were between 130-140 passages. Cells were transfected with appropriate plasmids in 100-mm dishes by the calcium phosphate method as previously described (Sambrook et al., 1989). A fixed amount of plasmid DNA was used in any given experiment. The amount of expression vectors was normalized by adding blank vectors to control for the promoter competition effect. When necessary, transfection efficiency was monitored by use of 1μg CMV-βgal plasmid per transfection, and calorimetric β-gal assay were performed using o-Nitrophenyl β-D-Galactopyranoside (ONPG) as a substrate (Sambrook, 1989).

The following plasmids were used for transfection: pCS2MT-ICERIIγ was constructed by ligation of the 500bp EcoRV/PmacI fragment bearing ICERIIγ cDNA, in frame at the end of the 6th myc epitope in pCS2MT (B. Kelley, Fred Hutchinson Cancer Center, Seattle, WA) that has been digested with StuI. pFLAGCMV2-CDC34 was generated by cloning the N-terminus end of the human CDC34 gene contained on a 1298bp NruI/KpnI fragment from pKS6110 (Plon et al, 1993) into pFLAGCMV2 (Kodak) that has been cut with EcoRV/KpnI. pFLAGCMV2-CDC34C-S, which contains the active site C155S mutation was also constructed analogously. pFLAGCMV2-RAD6B was constructed by cloning the N-terminal 766bp HincII/SspI fragment from pRR518 (L. Prakash, UTMB, Galveston, TX) in frame into pFLGCMV2 cut with SmaI/EcoRV. The sources of the following plasmids are as follows: pRR482(human Rad6A, L. Prakash, UTMB, Galveston, TX), pSomCAT (Paolo Sassone-Corsi, IGMC, Strasbourg, France), pSG5 (Promega, Madison, WI), pCαEV(mouse PKA catalytic subunit expression vector; G.S. McKnight, University of Washington School of Medicine, Seattle, WA), pMT133 and pMT107 (HA and His tagged Ubiquitin, respectively; D. Bohman, EMBL, Heidelberg, Germany).

The hCdc34 active site mutant was generated from pKSCDC34 by converting the cysteine at position 155 to a serine residue by standard PCR mutagenized methods (Ho et al.,

1989). The mutation was confirmed through sequencing. Mutant hCDC34 was then subcloned to the pFLAGCMV2 vector as described above (pFLAG-CDC34C-S).

Chloramphenicol Acetyltransferase (CAT) Assay: ICERIIγ, ATFX and hCDC34 sequences were cloned into the expression plasmid pSG5 (Promega, Green et al, 1988) for expression in mammalian cells. JEG3 cells were transiently transfected with pSG5- ICERIIγ, pSG5-ATFX alone or in combination with pSG5-CDC34. Reporter plasmids, β-D-galactosidase and pSOM-CAT were used to measure the transfection efficiency and the CAT activity, respectively. Reporter pSOMCAT has been used to score the effect of hCDC34 on the regulation of ICERIIγ and ATFX plasmids exerted on a canonical rat somatostatin CRE sequence inserted upstream from the herpes thymidine kinase prompter (tk) and the bacterial CAT gene (Foulkes et al., 1992). Activation of pSOMCAT transcription was obtained by the co-transfection of the mouse protein kinase A (PKA) subunit expression vector (pCαEV, Mellon et al., 1989) or treatment of 10 mM forskolin for 2-3h before harvest (data not shown). Forty-eight hours following transfection, protein extracts were made from freeze-thaw lysed cells. β-D-galactosidase activity in the lysate was measured using ONPG as a substrate (Sambrook, 1989). The amount of lysates to be used in subsequent CAT assay was normalized based on the β-D-galactosidase activity. CAT reaction was performed using extracts along with acetyl-CoA (Boehringer Mannheim, Indianapolis, IN) and ^{14}C-Chloramphenicol (NEN), incubated at 37 ^{0}C for 3-4 h and reaction products were extracted in ethyl acetate, separated on TLC plates (Whatman, Maidstone, England) in 95:5 Chloroform/Methanol. The CAT plates were visualized with a molecular dynamics Phosphoimager and the activity was quantified by measuring the percentage of Chloramphenicol acetylated using IMAGEQUANT™ software.

Antisera: The monoclonal antiserum against c-myc, Flag and HA epitopes were purchased from Oncogene Research product (Cambridge, MA), Sigma (St. Louis, MO) and BabCO (Richmond, CA) respectively. The polyclonal antisera against hRad6B, hICER, and Crem were a generous gift from Henk P. Roest & J. Hoeijmaker (Erasmus University, Rotterdam, The Netherlands), Carlos A. Molina (University of Medicine and dentistry of New Jersey, Newark, NJ), and Paolo Sassone-Corsi (IGBMC, Universte Louis Pasteur, Strasbourg, France), respectively. Monoclonal human Cdc34, mouse β-actin and bacterial trpE antibody were obtained from Transduction lab (Lexington, KY), Sigma (St. Louis, MO) and Oncogene Research Product, respectively. The polyclonal anti-pan CREM was purchased from Upstate Biotechnology (Lake Placid, NY).

Protein Analysis and Immunoprecipitation: Cells were lysed directly on 100-mm tissue culture dishes in RIPA buffer (1XPBS, 1% NP-40, 0.1% SDS, 0.5% Sodium deoxycholate) or PBSTDS buffer (1XPBS, 1% Triton X-100, 0.1% SDS, 0.5% Sodium deoxycholate) containing protease and phosphatase inhibitors (1mM EDTA, 0.2mM PMSF, 1μg/ml pepstatin, 30μl/ml aprotinin, 0.5μg/ml leupeptin, and 100 mM sodium orthovandate), followed by scraping and passing through a 21G needle. After protein quantification (using BioRad's protein dye and BSA as standards) and normalization, 10-40μg protein extracts were electrophoresed on SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) using BioRad mini Protein Blot apparatus as per manufacturer's protocol. The filters were initially blocked with 5% nonfat-dry milk in TBST (Tris buffer saline containing 0.1% Tween

20) for 1-2h at room temperature and then probed with 1.5μg/ml myc, 2.5μg/ml Flag, 1:100,000 β-Actin, 1:2000 Cdc34, 1:250 Rad6B, 1:1000 ICER antibodies. The bound antibodies were visualized by the enhanced chemiluminescence (ECL) detection system (Amersham, Buckinghamshire, England), in combination with the HRP conjugated anti mouse or anti rabbit secondary antibodies as appropriate. The intensity of the specific bands in the exposed films were quantified using a Molecular Dynamics Personal Densitometer and the IMAGE QUANT™ software.

Immunopreciptation was performed as follows: 1.0ml of cell lysates were precleared by incubating with 10μl of normal mouse IgG and 20μl protein G plus agarose (Oncogene Research Product, Cambridge, MA) at 4°C for 1h on a rotator. The precleared-lysate was collected after centrifugation at 3000 RPM for 15 min. 0.5-1.0ml of precleared lysates normalized for protein concentration were incubated at 4°C for 1h with appropriate dilution of the primary antibodies followed by addition of 20μl Protein G plus agarose. The mix were then incubated at 4°C for another 12-16h on a rotator. Precipitates were then washed four times with 1ml of icecold PBS before electrophoresis and Western blot analysis.

Pulse-Chase Assay: JEG3 cells at a passage of 138 and 140 were transiently transfected with pCS2MT-ICERII either in combination with the pFLAGCMV2 vector, or pFLAGCMV2-CDC34 or pFLAGCMV2-RAD6B were incubated for 2h in methionine- and cysteine- free DMEM. For metabolic labeling, cells were incubated for 1h with 125μCi/ml of [^{35}S]methionine (NEN, Boston, MA) in the same medium. Cells were harvested (time 0), or washed 4 times in PBS and incubated for 3, 6 and 9 hours respectively in complete medium (MEM) supplemented with 4mM methionine. At the end of each time period, cells were washed three times in ice-cold PBS and lysed in 2.5ml of PBSTDS buffer. Following centrifugation at 4000 RPM for 15 minutes, the supernatant were collected and analyzed for protein content using BIO-RAD (Hercules, CA) protein assay kit and the incorporation of the [^{35}S]-methionine by trichloro acetic acid (TCA) precipitation. The average TCA precipitation among samples was found to be 40 ± 2.0%. 25x10^6 TCA precipitable counts were immunoprecipitated with myc antibody as per the manufacturers instructions (Oncogene Research Products), resolved by SDS/PAGE, fixed in acetic acid (10%) and 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 10mM in DMSO. Approximately 36h after transfection and 5h before harvest, cells were treated with 0.025mM proteasome inhibitors. Cells were lysed as above with the addition of 5mM N-ethyl maleimide (Sigma) to the lysis buffer as previously described (Clurman et al., 1996). For the detection of HA-tagged ubiquitin-ICERIIγ conjugates, cells growing in 100mm dishes were co-transfected with ICERIIγ and either HA (pMT133)or His6 (pMT107) tagged ubiquitin expression plasmid, followed by treatment with proteasome inhibitor, lysis and immuno-precipitation and Western analysis as described above.

Extraction of DNA and RNA, and Northern Analysis: Total RNA was extracted from transfected JEG3 cells using total RNA isolation kit from Qiagen (Valencia, CA). DNAse-I treated RNAs were Northern blotted from 1% formaldehyde gels with Hybond N+ sheet

(Amersham). The blots were hybridized with the nicktranslated γ^{32}P-dCTP labeled full length ICERIIγ probe. Human GADPH, a house keeping gene was used as a control to compare equal loading. The probes were hybridized overnight at 65^{0}C in 10% dextran sulphate, 2xSSC, 1%SDS, and 250μg/ml salmon sperm DNA. The final wash was in 0.1XSSC-0.1%SDS at 65^{0}C. A multiple tissue Northern blot was purchased from Clontech (Palo Alto, CA) and hybridized with the ICERIIγ probe as per the manufacturer's protocol.

Total RNA and genomic DNA were also extracted from the exponentially growing cultures of breast cancer cell lines using kits from Qiagen. The Northern blots containing the RNA from the breast cancer cell line were probed with random primed γ^{32}P-dCTP labeled ATFX, clone #28C and #42-2 and nicktranslated ICERIIγ cDNA.

Results

Technical Objective #1: Identification of Cdc34 Target Proteins

Task #5

Isolation and Sequencing of Activation Domain Fusion Plasmids From True Positives of *hCDC34* two-hybrid screen: Successes 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 evident by its ability to complement a temperature sensitive *cdc34^{ts}* mutant yeast strain, SJ1098-3d (Fig.1A). In this assay, unlike the control plasmid (pPC97), the pPC97-*hCDC34* 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 can form a functional complex with the 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 (Fig.1B). The bait protein has no autonomous effect on His3, Ura3 and LacZ reporters, due for example to the presence of cryptic transcriptional activation sequences. 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. 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. 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 hATFX are Targets of Cdc34: Two of the clones found to interact genetically with hCdc34 in the above 2-hybrid screen are members of bZip family namely, ICERIIγ (Fig. 2A) and ATFX (Fig. 2B). ICERIIγ, an ICER isoform generated by alternate splicing, encodes a 108 aa protein and lacks the characteristics γ-axon and one of the DNA binding and dimerization domain. ICERIIγ has 97.2% homology with the mice isoform ICERIIγ and 76.7% with the human ICERI, the 120aa full length ICER protein. hATFX is a novel clone, a partial sequence of its homolog in mouse has recently been reported (Nishizawa and Nagata, 1992). hATFX 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 ATFX are 96.6% homologous in their available sequence. In the leucine zipper domain, hATFX, 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.

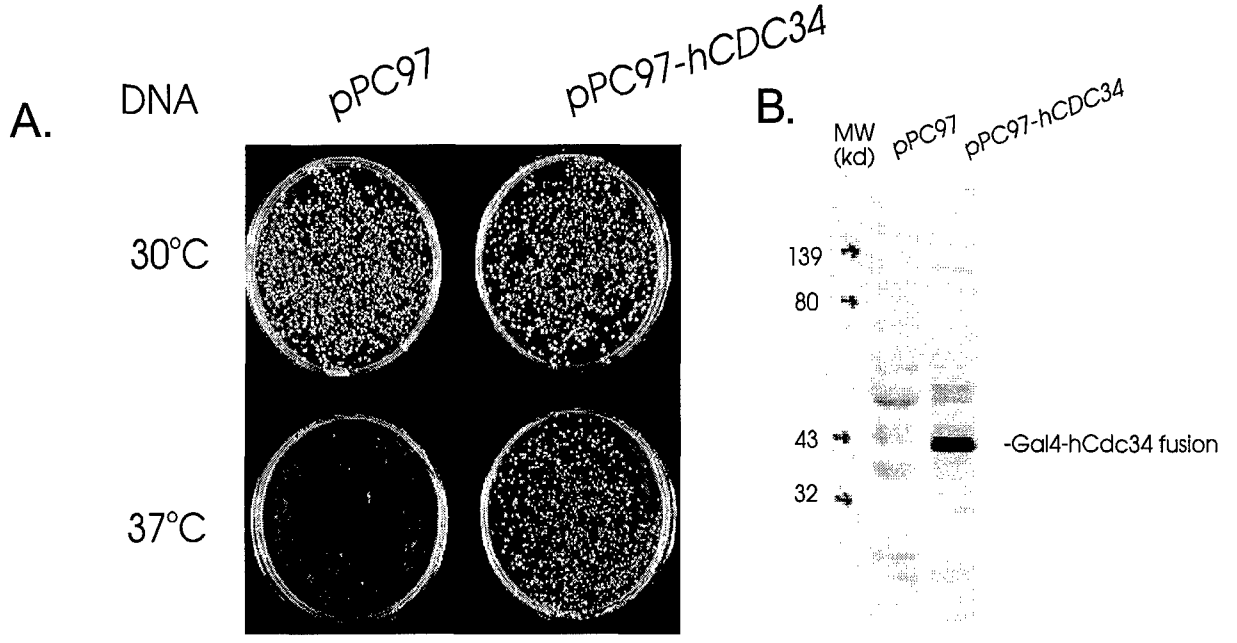


Fig.1 A. PC97-*hCDC34* bait encodes the functional Cdc34 protein. This was tested in yeast by its ability to complement a temperature sensitive *cdc34*^{ts} mutant yeast strain, SJ1098-3d (Mat **a**, *cdc34-2*, *leu2-3*, *ura3*, *trp1*). After transformation with the plasmid DNA, pPC97 or the pPC97-*hCDC34*, cells were spread onto plates containing leucine-deficient medium and kept at 23°C for 24h, after which they were shifted to either 30°C or 37°C and incubated for 3 days. Unlike the control plasmid (pPC97), the pPC97-*hCDC34* construct was able to completely suppress the *cdc34*^{ts} mutation, allowing growth at 37°C.

B. Western blot analysis of the *GAL4*-Cdc34 fusion protein, expressed in the host two-hybrid strain (MV103). Protein extracts made from overnight cultures of yeast bearing the control plasmid (pPC97) or the bait (pPC-97-hCDC34) were electrophoresed on a 12% SDS-PAGE gel and transferred onto a Immobilon-P membrane (Millipore) using BIO-RAD Mini-Trans blot apparatus. The membrane was then probed using a monoclonal anti hCdc34 antiserum (Transduction Lab). The left hand panel shows the molecular weight marker (in kd).

A.

```
ICERIIγ::  MAVTGDDT------------AATGDMPTYQIRAPTAALPQ
(108aa)    :::::::.:            :::::::::::::::.::::
mICER::    MAVTGDET------------AATGDMPTYQIRAPTTALPQ
(108aa)    ::::::::             :::::::::.:::::.::::
hICER::    MAVTGDETDEETELAPSHMAAATGDMPTYRIRAPTAALPQ
(120aa)

ICERIIγ ::  GVVMAASPGSLHSPQQLAEEATRKRELRLMKNREAARECR
            :::::::::::::::::::::::::::::::::::::::::
mICERIIγ::  GVVMAASPGSLHSPQQLAEEATRKRELRLMKNREAARECR
            ::::::::::::::::::::::::::::::::::::::.:::
hICERI::    GVVMAASPGSLHSPQQLAEEATRKRELRLMKNREAAKECR

ICERIIγ::   RKKKEYVKCLENRVAVLENQNKTLIEELKALKDLYCHKVE
            :::::::::::::::::::::::::::::::::::::::.:
mICERIIγ::  RKKKEYVKCLENRVAVLENQNKTLIEELKALKDLYCHKAE
            :.:::::::::.:::::: ::: :::::..:::.    :..
hICERI::    RRKKEYVKCLESRVAVLEVQNKKLIEELETLKDICSPKTD
```

		%Identity
ICERIIγ	vs hICERI	76.7%
ICERIIγ	vs mICERIIγ	97.2%
hICERI	vs mICERIIγ	77.5%

B.

```
hATFX::            RHEIYCRNEAGQEEVGMPPLPPPQQPPPPSPPQP
(131AA,Part)
mATFX::   ------------------------------------------
(84AA, Part)                                .  : :.: .:
mATF4::   ----------------------------------RGSPRPKPYDP
(349AA, 250-349)                             .: : .::
hATF4::   ----------------------------------RPKPYDPPGE
(351AA, 260-351)

hATFX::   SRLAPYPHPATTRGDRKQKKRDQNKSAALRYRQRKRAEGEALEG
              ::: ::.:::::::::::::::::::::::::::::::::
mATFX::   ----PYPSPASTRGDRKQKKRDQNKSAALRYRQRKRAEGEALEG
            ..   .  : . :.: :: .::::.:: ::::.::::::::::
mATF4::   PGVSLTAKVKTEKLDKKLKKMEQNKTAATRYRQKKRAEQEALTG
          . .:   .    . :.: :: .::::.:: ::::.:::: ::: :
hATF4::   KMVAA--KVKGEKLDKKLKKMEQNKTAATRYRQKKRAEQEALTG

hATFX::   ECQGLEARNRELKERAESVEREIQYVKDLLIEVYKARSQRTRSC
          ::::::::::::.:::::::::::::::::::::::::::::::
mATFX::   ECQGLEARNRELRERAESVEREIQYVKDLLIEVYKARSQRTRST
          :. :: ..:. :::.:.:. .::::.:::. :: :::.:.
mATF4::   ECKELEKKNEALKEKADSLAKEIQYLKDLIEEVRKARGQK----
          ::  :: .:. ::::::.:. .::::.:::. :: :::...
hATF4::   ECKELEKKNEALKERADSLAKEIQYLKDLIEEVRKARGKK----
```

	%Identity
hATFX vs. mATFX	96.4%
hATFX vs. mATF4	50.9%
hATFX vs. hATF4	52.1%

Fig.2. Sequence alignment between human and mouse Inducible cAMP Early Repressor (ICER) isoforms (**A**), and between human activating transcription factor-X (ATFX) and human/mouse ATF-4 (**B**). Alignment was performed using the ClustalW alignment program (Thompson et al., 1994). Amino acids in boldface represent the conserved leucines in the bZIP domain. **:**, identical amino acids, **.**, conservative changes. Conservative changes were defined as a relative value of >1 in the Dayhoff PAM matrix (George et al., 1988).

Task #6
Independent Biochemical Conformation of Authentic Two-Hybrid Interactions

Co-expression of Cdc34 relieves the cAMP-induced Transcriptional Repression by ICER and ATFX in JEG3 Cells: As previously reported by Molina et al., (1993), we have found that ICERIIγ is a powerful repressor of PKA(cAMP)-induced transcription of somatostatin (Fig.3). In this assay for the activity of somatostatin-CAT, ATFX also shows a strong repressor activity on this reporter. However, co-expression of hCDC34 with ICERIIγ or ATFX completely relieved the transcriptional repression of cAMP-induced accumulation of somatostatin-CAT, providing strong evidence for an *in vivo* interaction between hCdc34 and the ICERIIγ and ATFX proteins in mammalian cells.

ICERIIγ and ATFX Protein is degraded by the Ubiquitin-proteasome machinery: In order to determine the nature of the above interaction we expressed epitope tagged versions of hCDC34 and its targets. Expression of myc-tagged ICERIIγ resulted in production of a 24Kd protein recognized by the anti-myc epitope antibody. Co-transfection of the CMV promoter driven myc-epitope tagged ICERIIγ with Flag-hCDC34 into JEG3 (Fig. 4B) and NIH3T3 cells (data not shown) resulted in considerable and some cases complete loss of the ICERIIγ fusion protein. Northern analysis of the mRNA isolated from ICERIIγ transfected cells demonstrated no significant difference in the steady state ICERIIγ transcripts compared to the cells co-transfected with hCDC34 (Fig. 4A). This loss of protein was found in multiple experiments.

In view of the role of Cdc34 (UBC3) in ubiquitin mediated proteolysis, a likely explanation for the loss of ICERIIγ protein is the involvement of 26S proteasome. To explore this possibility, we used potent inhibitors of 26S proteasome MG115 and MG132 to investigate their effect on ICERIIγ protein stability. These peptide aldehydes inhibit proteasome-mediated proteolysis and result in the accumulation of proteins that are degraded by this pathway (Rock et al, 1994, Maki and Howley, 1997). Incubating the transfected cells for 5-6h in the presence of 0.025mM MG115 or MG132 prevented the loss of ICERIIγ protein in cells co-transfected with hCDC34 (Fig. 4C). This strongly suggest that the destabilization of ICERIIγ protein may involve ubiquitin-proteasome machinery. The specificity was further explored by using a mutant hcdc34 protein where the active site cysteine of the enzyme was replaced with a serine. In co-transfection assays, compared to the wild type Cdc34, mutant enzyme had no effect on the steady state level of ICERIIγ protein (Fig. 4d), again indicating the targeting of ICERIIγ protein by hCdc34, required a functional ubiquitin ligase.

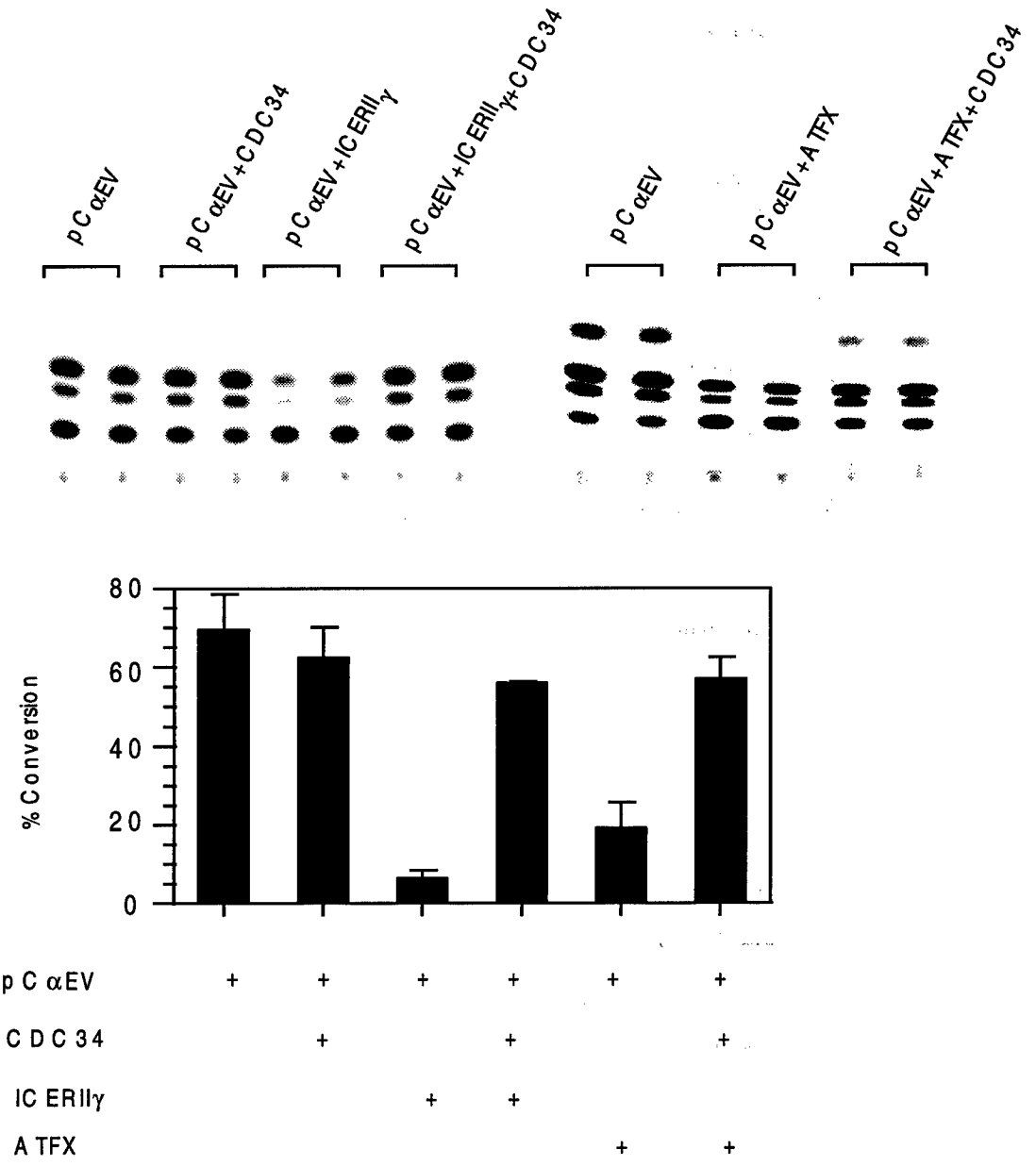


Fig. 3. hCDC34 antagonizes ICERIIγ and ATFX-induced transcriptional repression of the reporter pSomCAT in JEG3 cells. Cotransfection of hCDC34 with ICERIIγ or ATFX completely relieved the transcriptional repression of pCαEV (PKA expression plasmid)-induced accumulation of somatostatin-CAT. Transfection were performed with a total of 15μg DNA per 10cm plate, including 1μg of reporter CAT plasmid and 2μg of pCαEV. Experiments were repeated 3 times, and variability was less than 20%.

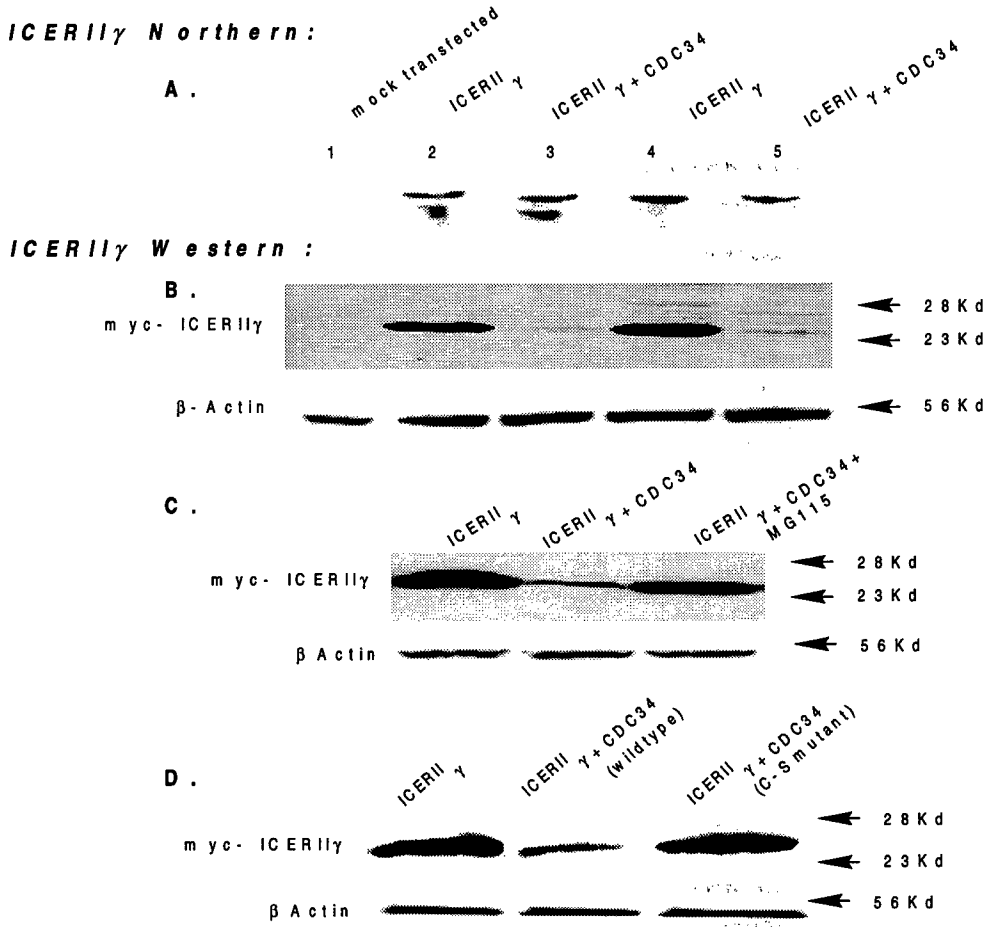


Fig. 4. Cotransfection of hCDC34 destabilizes ICERIIγ protein but not the transcripts in JEG3 cells; involvement of ubiquitin-proteasome machinery. **A.** Northern blot analysis of the mRNA isolated from the cells cotransfected with pCS2MT-ICERIIγ and the blank vector (pFLAGCMV2) or the pFLAGCMV2-hCDC34 as described in the methods. The RNA was probed with the full length ICERIIγ cDNA. First lane represents the untransfected control. Experiment was repeated twice. **B.** Western blot analysis of the protein collected from the identical sets of experiments described above. Protein lysates in PBSTDS buffer were prepared as described in the methods and electrophrosed on a 5-15% SDS-PAGE gel (BIORAD), transferred onto the PVDF membrane and probed with the anti-myc epitope antibody. Blots were reprobed with the mouse β-actin antibody to compare loading. The numbers in the right hand panel represent the molecular weight marker in kd. Experiments were repeated 11 times. **C.** Effect of a proteasome inhibitor, MG115 on the hCDC34-induced destabilization of ICERIIγ protein. Transfected JEG3 cells were incubated for 5-6h in the presence or absence of 0.025mM peptide aldehyde MG115, before harvest. Protein were analyzed as before. **D.** Mutant hcdc34 enzyme failed to destabilize ICERIIγ protein in a co-transfection assay. hCDC34 enzyme was mutated by replacing the active site cysteine at position 155 to serine using PCR based mutagenized techniques described in the methods. The mutant or the wild type enzyme was cotransfected with the ICERIIγ onto JEG3 cells and protein was analyzed as before. Experiments were repeated 3 times.

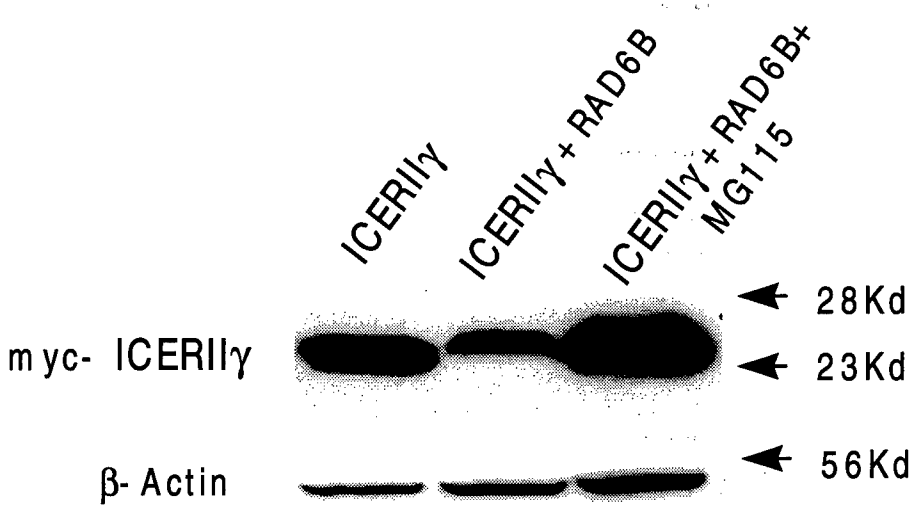


Fig. 5. Cotransfection of hRAD6B and ICERIIγ results in the loss of ICERIIγ protein in JEG3 cells, which can be prevented by treating the cells with a proteasome inhibitor, MG115. The treatment and Western analysis of the protein is same as mentioned in Fig.4. β-actin is shown to compare loading. Experiments were repeated 3 times.

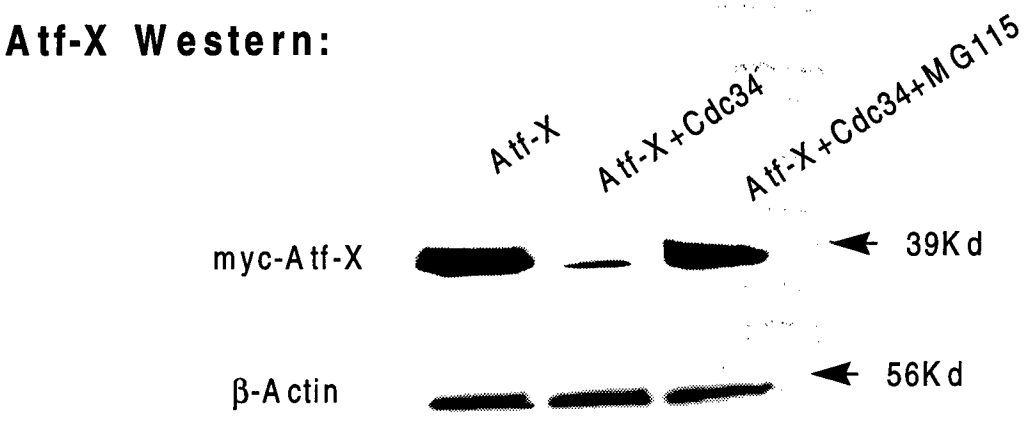
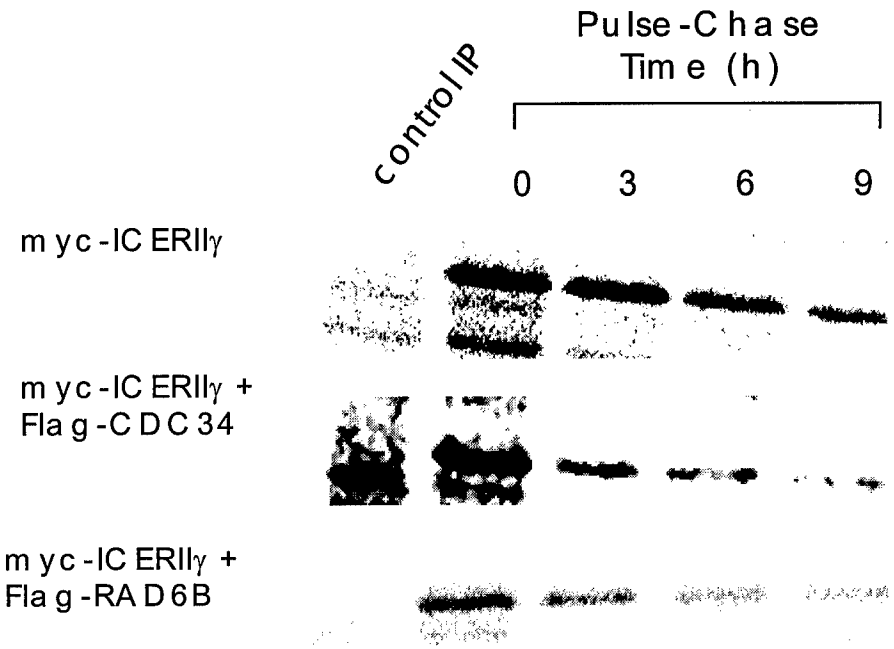


Fig. 6. Cotransfection of hCDC34 and ATFX results in the loss of ATFX protein in JEG3 cells; which can be prevented by treating the cells with proteasome inhibitor, MG115. The treatment and Western analysis of the protein is same as mentioned in Fig.4. β-actin is shown to compare loading. Experiments were repeated 4 times.

In view of the considerable sequence homology between hCDC34 and hRAD6B in their active site region and role of murine Rad6B and ICER proteins in spermatogenesis, we performed co-transfection assays with hRad6B and the targets obtained in the Cdc34 screen. Co-expression of Flag-Rad6B with myc-ICERIIγ can mimic the loss of ICERIIγ protein to a similar degree as hCDC34. This loss of ICERIIγ can also be reversed in the presence of poteasome inhibitors (Fig. 5). Similar to ICERIIγ , co-transfection of ATFX with either hCDC34 (Fig. 6) or hRAD6B (data not shown) resulted in significant loss of ATFX protein, which can be reversed by incubation with MG115. However, in comparison to Cdc34, the loss of ATFX in the presence of hRad6B is lower.

To determine whether change in steady-state levels of protein upon co-transfection with hCDC34 and hRAD6B is due to degradation, we performed pulse-chase experiments to examine the stability of the ICERIIγ protein in JEG3 cells (Fig. 7). The cells were transfected with myc-epitope tagged, pCS2MT ICERIIγ in combination with flag tagged pFLAG-CMV2 vector, pFLAGCMV2-CDC34 or pFLAGCMV2-RAD6B. Forty hours following transfection, cells were metabolically labeled with [^{35}S]methionine. The labelling period was followed by a chase period with excess of cold methionine. Immunoprecipitation studies carried out using the myc antibody on samples collected at various time points during the chase demonstrated the half-life of ICERIIγ protein is 3.82h. However, consistent with our earlier findings, in the presence of hCDC34 or hRAD6B, ICERIIγ degraded more rapidly with an estimated half-life of 2.3h and 2.9h, respectively. It is interesting to note that unlike that of hCDC34, the kinetics of ICERIIγ protein in the presence of hRAD6B suggests a biphasic pattern of instability with the increased half life later in the pulse. (Fig.7). This may explain the overall slower rate of degradation of the ICERIIγ protein in the presence hRAD6B.



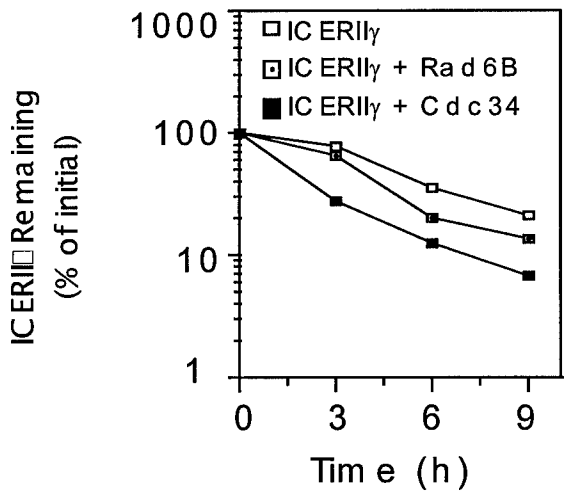


Fig.7. Pulse-chase analysis of the stability of ICERIIγ protein in the JEG3 cells alone or co-transfected with either hCDC34 or hRAD6B. ^{35}S labelling and chase with the complete medium containing excess unlabelled methionine for indicated period of time were performed as described in the methods. Cell lysates were immunoprecipitated with monoclonal anti myc-tagged antibody, and the immunoprecipitates were analyzed by a 15% SDS/PAGE and a STORM imager. The control IP was performed using a mouse monoclonal antibody raised against the bacterial trpE protein.

Accumulation of Ubiquitinated Intermediates of ICERIIγ : As ubiquitination is not a prerequisite for the degradation of proteins via 26s proteasome (Ciechanover, 1994), we studied the polyubiqutination of ICERIIγ protein in the presence of hCdc34. The approach that was used is essentially similar to that described for the ubiquitination of cJun (Trier et al., 1994) , cyclin E (Clurman et al., 1996), and p27 (Pagano et al., 1996) involving the detection of Ub conjugated ICERIIγ protein ladders in the presence of peptide aldehyde MG115 and isopeptidase inhibitor n-ethyl malaeimide (NEM, Sigma). To demonstrate the formation of the polyubiquitin-ICERIIγ conjugates in the presence of hCDC34, an immunoprecipitation-Western assay was used in which a HA tagged ubiquitin construct was co-transfected along with the myc-tagged ICERIIγ in the presence and absence of pFLAG-CMV2-CDC34 into JEG3 cells. Five hours before harvest cells were treated with MG115. Extracts were made in the presence of NEM and immunoprecipitated with either the anti myc or anti HA monoclonal antisera, followed by Western blotting with either anti-myc or anti-HA antisera. High molecular weight species of ICERIIγ protein were detected with either antibody and in cells transfected with both HA-Ub, and myc-ICERIIγ or cells transfected with hCdc34, myc-ICERIIγ and HA-Ub (Fig. 8). However, no Ub conjugates were observed in the presence of His_6-tagged ubiquitin as opposed to HA-Ubiquitin. Thus the slower migrating species contain both myc-ICERIIγ protein and HA ubiquitin and represent multiubiquitinated form of ICERIIγ protein. The formation of ladders in the presence and absence of exogenous hCDC34, indicate the involvement of endogenous E2s in the process, conforming an earlier report (Falco and Koren et al., 1997). Evidence for this is also found in the increased accumulation of ICERIIγ protein in the presence of proteasome inhibitor MG115 (data not shown). Collectively, these findings indicted the targeting of ICERIIγ and ATFX by both hCdc34 and hRad6B, for ubiquitin-mediated proteolysis.

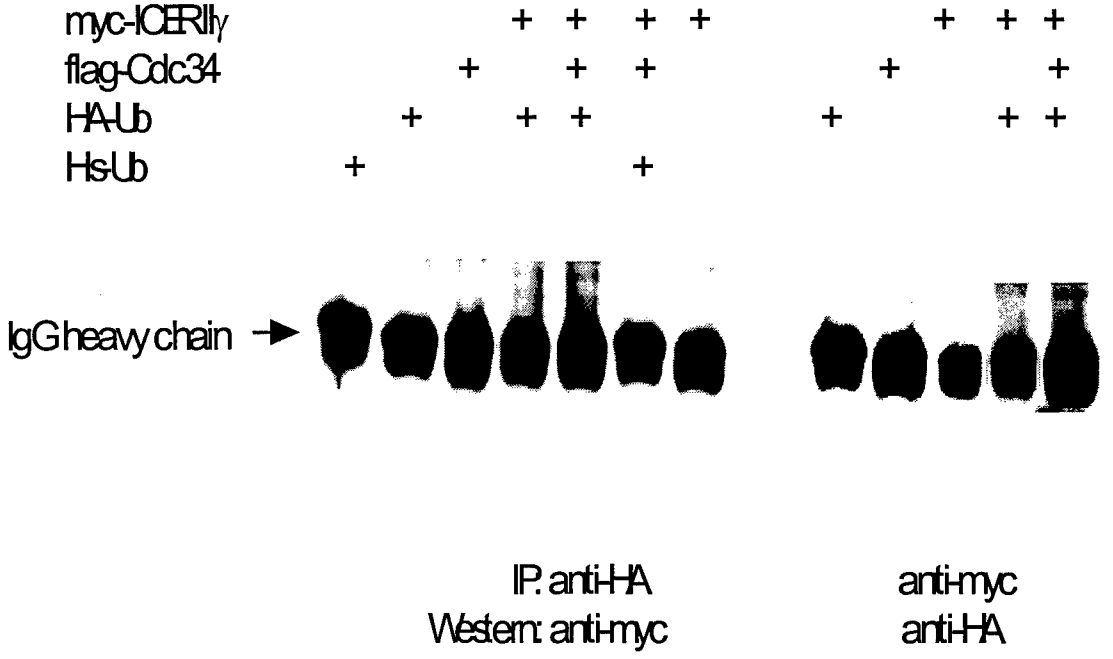


Fig.8. Ubiquitination of ICERIIγ *in vivo*. Identification of ICERIIγ-ubiquitin conjugates by an immunoprecipitation-Western blot analysis. JEG3 cells were transfected with the indicated plasmids followed by treatment with 0.025mM MG115, five hours before harvest. *Left panel*: Cell lysates were immunoprecipitated with anti-HA antibody, followed by Western blotting with the anti-myc antibody (which recognizes the myc tag on ICERIIγ). *Right panel*: Cell lysates were immunoprecipitated with the anti-myc-tagged antibody, and the Western blot was probed with anti-HA. Ubiquitin-ICERIIγ conjugates are detected only in cells transfected with both myc and HA-epitope tagged constructs and in the presence of hCDC34. His_6 tagged ubiquitin construct (His-Ub) is used as a negative control. IgG heavy chain is visualized in this analysis.

ICER-specific domain is not required for degradation: All four isoforms of human ICER proteins are highly conserved and posses the same 9 amino acid N-terminal domain specific to this family. The isoforms differ based on the presence or absence of two exons, exon γ and exon Ia. To analyze the role if any of the ICER-specific domain in the stability of ICERIIγ *in vivo*, we constructed a mini-ICER construct. A myc epitope-tagged construct (pCS2MT-ICERIIγ1–33) bearing the N-terminal 33 amino acids encoding the ICER-specific domain and Exon γ of the ICERIIγ protein was made. Co-transfection of pCS2MT-ICERIIγ1–33 with pFLAGCMV2-CDC34 into either JEG3 cells (Fig. 9) or NIH3T3 (data not shown) resulted in no significant loss of the truncated protein, indicating that the ICER-specific domain and Exon γ may not be essential for hCdc34 targeting and subsequent degradation via ubiquitination.

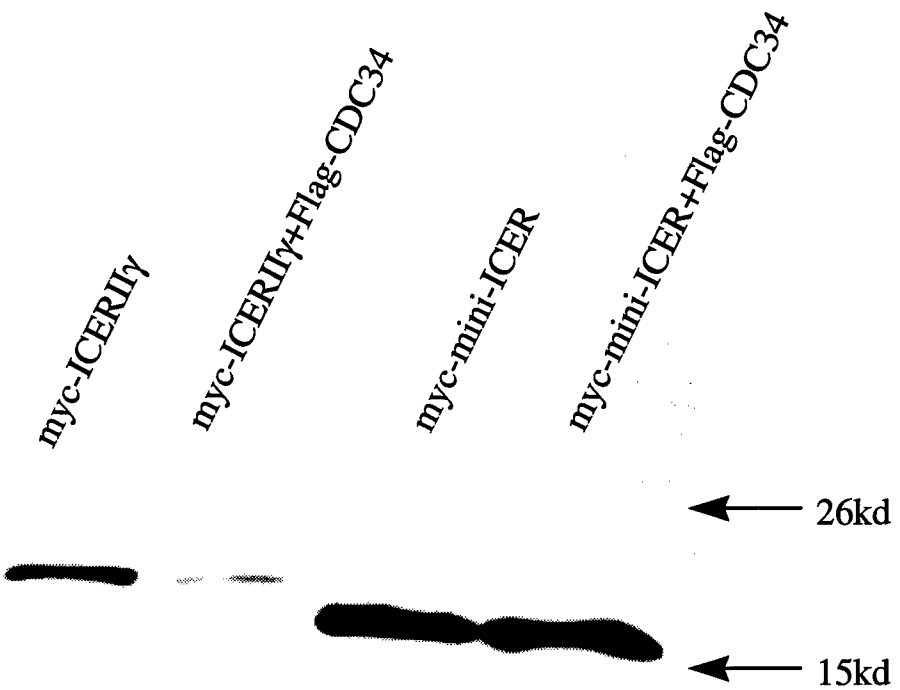


Fig. 9). ICER specific domain is not essential for the hCDC34-mediated destabilization of the ICERIIγ protein. A myc epitope-tagged construct (pCS2MT-ICERIIγ1–33) bearing the N-terminal 33 amino acids encoding the ICER-specific domain and Exon γ of the ICERIIγ protein was made as described in the methods section. Co-transfection of pCS2MT-ICERIIγ1–33 with pFLAGCMV2-CDC34 into either JEG3 cells and Western analysis were performed as described in the legends for Fig.4. The first two lanes represent the stability of the full length ICERIIγ protein in the presence and absence of hCDC34.

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

Task# 7 & 8

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 physiological 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 (Keyomarsi et al., 1993, 1994, Gudas et al., 1996, Swift et al., 1991, Sharan et al., 1997). This panel includes both estrogen receptor positive and negative lines which 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 are being 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 (Fig. 10, 11). 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.

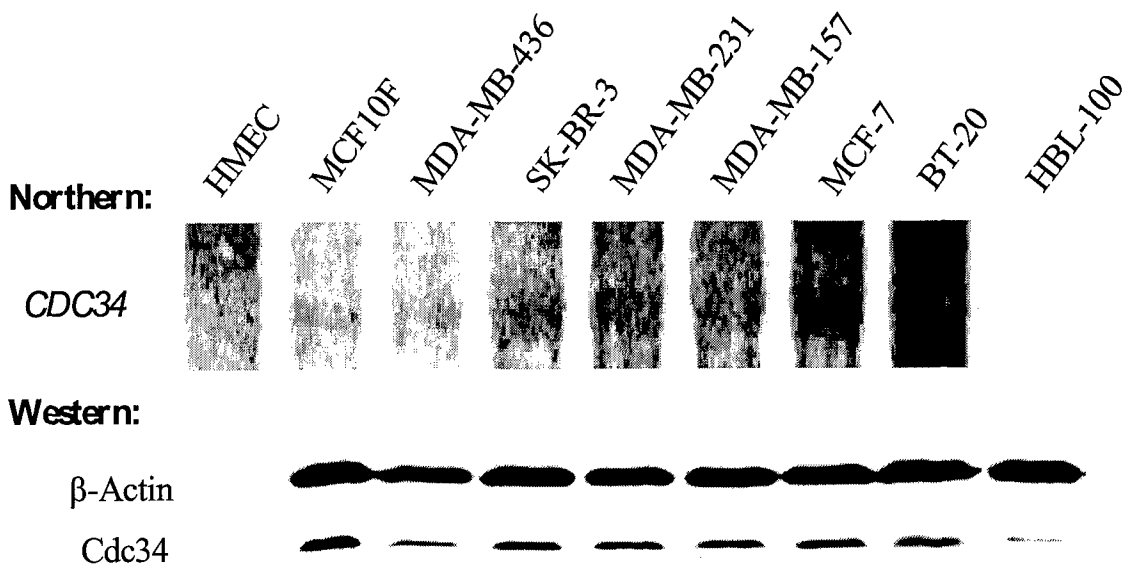


Fig.10. Expression of hCDC34 transcripts (upper panel) and protein (lower panel) in a panel of breast cancer-derived cell lines. Northern and Western analysis were formed as described in the methods. β-actin is shown to compare the loading. HMEC represents the normal breast epithelial cells.

In a recent set of studies we have examined the expression pattern of four hCdc34 interactors, ICERIIγ, ATFX, Clone #28C (Fission yeast Rad21 homolog), and #42-2 (Leukemia cell differentiation factor) in these breast cancer cell lines (Fig. 11). 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 ATFX appear to have altered expression pattern (Fig. 11). Compared to the normal HMEC 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, ATFX 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 (Fig. 10, bottom panel) and clone #28C transcripts are up regulated (Fig. 11). 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.

As described in task #7, we will shortly begin to study the structure of hCDC34 and a few selected interactors in breast cancer tissues and cell lines using Southern analysis. In this context, we intend to procure samples of primary breast tumors through the Methodist Hospital Breast Tumor Tissue Bank (Houston, TX). This bank contains over two hundred primary breast tumors including a wide range of tumor types and stages of presentation. For many samples matched pairs of normal breast and tumor cells are available for mutational analysis. We will use these reagents to study further the gene structure, and expression of the human CDC34 and its interacting proteins using Southern, Northern and Western blot analysis.

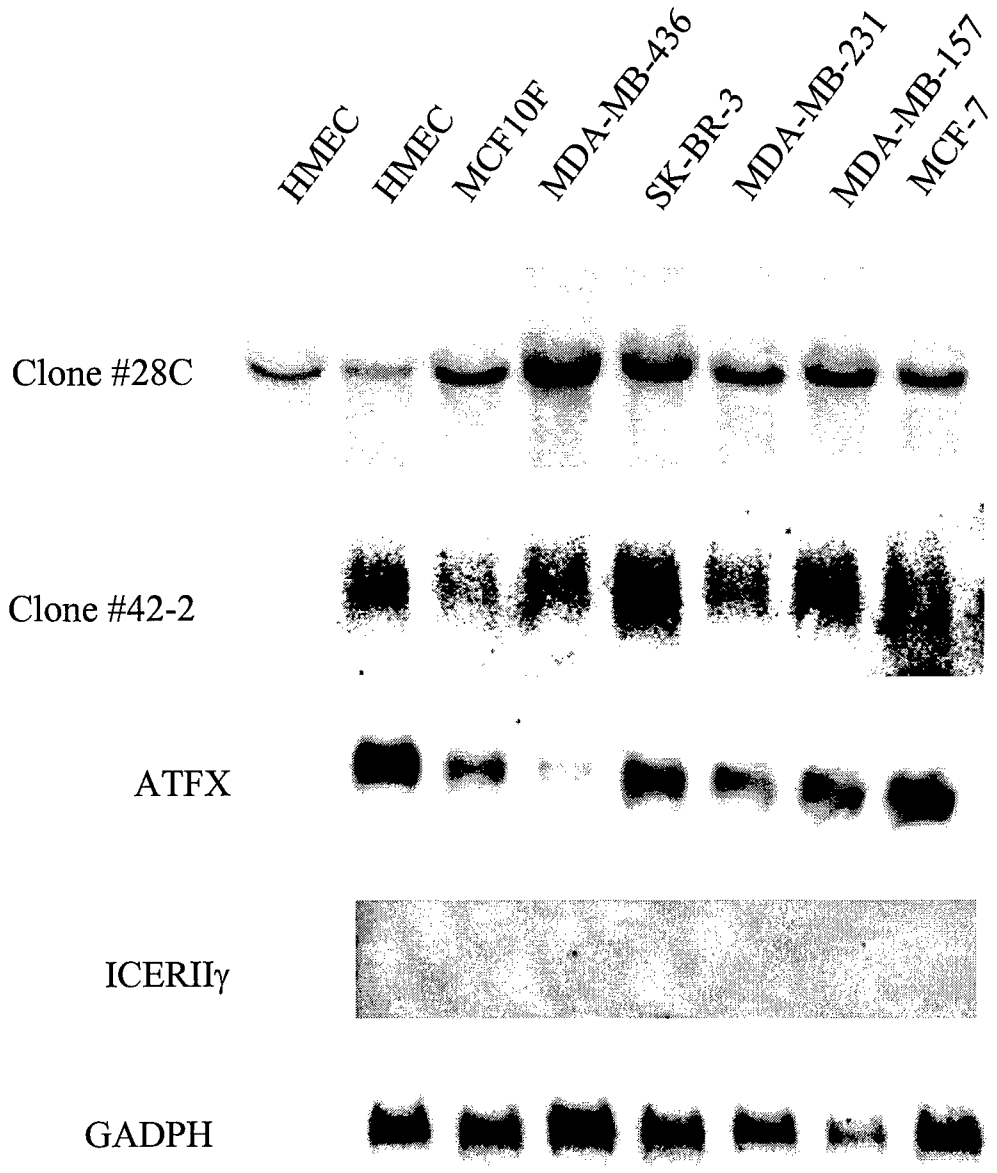


Fig. 11. Northern blot analysis of the hCDC34 interacting cDNAs in a panel of breast-cancer derived cell lines. Expression of the house keeping glyceraldehyde-3-phosphate dehydrogenase (GADPH) gene is shown to compare loading. HMEC represent normal breast epithelial cells.

Discussion

So far in this project we have demonstrated that mammalian Cdc34 ubiquitin ligase, a gene essential for cell cycle progression in yeast, has a wide variety of potential targets in human, including transcriptional factors, regulators of meiosis, spermatogenesis, cell proliferation 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 physiological 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 ligases for degradation. ICER IIγ, a known ICER isoform, and hATFX, a novel gene isolated in the present study are potent repressors of cAMP-induced transcription. Both ICERIIγ and ATFX, interact genetically 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 hATFX 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. Among the CREM/ATF family of transcription factors, ICER is best known for its rapid inducibility by cAMP and lack of the characteristic phosphorylation domain distinct to this family (Lalli and Sassone Corsi, 1994; Molina et al., 1993). The induction of ICER transcript is rapid and independent of *de novo* protein synthesis. 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 (Molina et al, 1993; Monaco et al., 1995). 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 ATFX 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.

The finding that Cdc34 and Rad6B are involved in the degradation of transcriptional repressors and regulators of diverse neuroendocrine functions is surprising, because CDC34 was

first identified as a gene required for cell cycle progression (Gobel et al., 1988) and RAD6 was a gene primarily characterized for its requirement in DNA repair (Haynes and Kunz, 1981). In addition, it is also interesting to note that both hCDC34 and hRad6 appear to target the same molecules. Once again, this is analogous to the findings in yeast where transcription factors GCN4 and MATα2 are targeted by multiple ubiquitin conjugating enzymes (Kornitzer et al., 1994, Chen et al., 1993). For example, degradation of GCN4 requires Rad6 (UBC2) and Cdc34 (UBC3) while Mat2α requires UBC4, UBC5, UBC6 and UBC7 (Chen et al., 1993). However, the targeting by multiple UBCs does have different characteristics, e.g. hCDC34 has a more significant effect on half life of ICERIIγ and kinetics of its degradation are different. This difference is also evident by the finding that in a mammalian two hybrid assay hRad6 but not hCdc34 was found to interact with ICERIIγ (D. Pati, S. Plon, unpublished observation) presumably because the degradation of the target is too rapid in the presence of ectopic hCDC34. Similar differences have also been reported in the degradation of yeast Gcn4 by Cdc34 and Rad6 (Kornitzer et al., 1994).

It was suggested by Kornitzer et al. (1994) that targeting of Cdc34 and Rad6 may be specific to proteins containing PEST sequences. Our analysis of hCDC34 interactors did not support that hypothesis. A number of the targets do not posses specific PEST sequences. In ICER proteins, the unique ICER specific domain and the γ exon is rich in PEST containing sequence. ICERIIγ lacks the characteristic γ exon but is targeted by hCdc34 and hRad6B for ubiquitination. In addition, a mini ICER construct containing the ICER-specific domain was not destabilized in the presence of hCDC34, suggesting a lack of specificity for targeting this region by hCdc34. Further studies are in progress to identify specific domains if any in these b-Zip transcription factors that are targeted by hCDC34.

From our studies involving the expression of Cdc34 and its targets it is evident that Cdc34 and some of the targets (e.g. ATFX and Clone#28C) 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 its 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.

Acknowledgements

We thank L. Prakash, Paolo Sassone-Corsi, G.S. McKnight, B. Kelly, B. Clurman, and D. Bohman, for stains and plamids, J. La Baer for the cDNA library, C. Molina, H. P. Roest, J. Hoeijmaker, P. Sassone-Corsi for antisera, and to Shirong Luo for assistance in the Northern analysis.

References

Band, V. , Sager, R., Distinctive traits of normal and tumor-derived human mammary epithelial cells expressed in a medium that supports long-term growth of both cell types. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 1249-1253 (1986).

Byers, B., Goetsch, L., Duplication of spindle plaques and integration of the yeast cell cycle. *Cold Spring Harb Symp Quant Biol*, **38**, 123-31 (1974).

Ciechanover, A., The ubiquitin-proteasome proteolytic pathway, *Cell*, **79**, 13-21 (1994).

Clurman, B. E., Sheaff, R. J., Thress, K., Groudine, M., Roberts, J. M., Turnover of cyclin E by the ubiquitin-proteasome pathway is regulated by cdck2 binding and cyclin phosphorylation. *Genes and Developmen*, **10**, 1979-1990 (1996).

Desdouets, C., Matesic, G., Molina, C. A., Foulkes, N. S., Sassone-Corsi, P., Brechot, C., Sobczak-Thepot, J., Cell cycle regulation of cyclin A gene expression by the cyclic AMP-responsive transcription factors CREB and CREM. *Mol Cell Biol,* **15**, 3301-3309 (1995).

Deshaies, R. J., Phosphorylation and proteolysis: partners in regulation of cell division in budding yeast. *Current opinion in Genetics and development*, **7**, 7-16 (1997).

Deshaies, R.J., Chau, V., Krischner, M., Ubiquitination of the G1 cyclin Cln2p by a Cdc34p-dependent pathway. *EMBO J.*, **14**, 303-312 (1995).

Durfee, T., Becherer, K., Chen, P.L., Yeh, S.H., Yang, Y., Kilburn, A.E., Lee, W.H., Elledge, S.J., The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes Dev.*, **7**, 555-569 (1993).

Feldman, R. M. R., Correll, C. C., Kaplan, K. B., Deshaies, R., J., A complex of Cdc4p, and cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. *Cell*, **91**, 221-230 (1997).

Folco, E. J., and Koren, G., degradation of the inducible cAMP early repressor (ICER) by the ubiquitin-proteasome pathway. *Biochem. J.*, **328**, 37-43 (1997).

Foulkes, N. S., Borrelli, E., Sassone-Corsi, P., CREM gene: use of alternativeDNA binding domains generates multiple antagonists of cAMP-induced transcription. *Cell*, **64**, 739-749 (1991).

Foulkes, N. S., Mellstorm, B., Benusiglio, E., Sassone-Corsi, P., Developmental switch of CREM function during spermatogenesis: from antagonist to transcriptional activator. *Nature*, **355**, 80-84 (1992).

George, D., Hunt, L., Barker, W., Macromolecular sequencing and synthesis. Alan R. Liss, New York, N.Y. (1988).

Glotzer, M., Murray, A.W. and Kirschner, M.W., Cyclin is degraded by the ubiquitin pathway. *Nature*, **349**, 132-138 (1991).

Goebl, M., Yochem, J., Jentsch, S., McGrath, J.P., Varshavsky, A., Byers, B., The yeast cell cycle gene *CDC34* encodes a ubiquitin-conjugating enzyme. *Science*, **241**, 1331-1335 (1988).

Green, S., Issemann, I., Sheer, E., A versatile *in vivo* and *in vitro* eukaryotic vector for protein engineering. *Nuc. Acid Res.*, **16**, 369 (1988)

Gudas, J.M., Li, T., Nguyen, H., Jensen, D., Rauscher III, F.J., Cowan, K.H., Cell cycle regulation of BRCA1 messanger RNA in human breast epithelial cells. *Cell Growth Diff*, **7**, 717-723 (1996).

Haas, A.L., Reback, P.B., Chau, V., Ubiquitin conjugation by the yeast *RAD6* and *CDC34* gene products. *J. Biol. Chem.*, **266**(8), 5104-5112 (1991).

Haas, A. L., Siepmann, T. J., Pathways of ubiquitin conjugation. *FASEB J.*, **11**, 1257-1268 (1997).

Haynes, R. H., and Kunz, B. A., In Strathern, J., Jones, E., and Broach, J. (eds), *The Molecular Biology of the Yeast Saccharomyces cerevisiae: Life Cycle and Inheritance.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 371-414 (1981).

Hemzawi, Z., Mooney, E.E., Plon, S.E., Characterization of the mammalian CDC34 cell cycle gene. *American Association of Cancer Research,* **36**, A191 (1995).

Hershko, A., Role of ubiquitin-mediated proteolysis in cell cycle control. *Current Opinion in Cell Biology,* **9**, 988-799 (1997).

Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K., Pease, L. R., Site-directed mutagenesis by overlap extension using the polymerase chain reaction.*Gene*, **77**, 51-59 (1989).

Hochstrasser, M., Ubiquitin, proteasomes, and the regulation of intracellular protein degradation. *Currrent Opinion in Cell Biology*, **7**, 215-223 (1995).

Holloway, S.L., Glotzer, M., King, R.W., Murray, A.W., Anaphase is initiated by proteolysis rather than by the inactivation of maturation-promoting factor. *Cell*, **73**, 1393-1402 (1993).

Hoyt, M. A., Eliminating all obstacles: regulated proteolysis in the eukaryotic cell cycle. *Cell,* **91**, 149-151 (1997).

Hunt, T., Destruction's our delight. . . . *Nature*, **349**, 100-101 (1991).

Hunter, T., Pines, J., Cyclins and cancer II: cyclin D and CDK inhibitors come of age. *Cell*, **79**, 573-582 (1994).

Jentsch, S., McGrath, J.P., Varshavsky, A., The yeast DNA repair gene *RAD6* encodes a ubiquitin-conjugating enzyme. *Nature*, **329**, 131-4 (1987).

Keyomarsi, K., O'leary, N., Molnar, G., Lees, E., Gingert, H., Pardee, A., Cyclin E, a potential prognostic marker for breast cancer. *Cancer Research*, **54**, 380-385 (1994).

Keyomarsi, K., Pardee, A., Redundant cyclin over expression and gene amplification in breast cancer cells. *Proc Natl Acad Sci USA*, **90**, 112-116 (1993).

King, R.W., Deshaies, R. J., Peters, J-M., Krischner, M. W., How proteolysis drives the cell cycle. *Science*, **274**, 1652-1659 (1996).

Kishi, T., Seno, T., Yamao, F., Grr1 functions in the ubiquitin pathway in *Saccharomyces cerevisiae* through association with Skp1, *Mol Gen Genet*, **257**, 143-148 (1998).

Kolman, C.J., Toth, J., Gonda, D.K., Identification of a portable determinant of cell cycle function with the carboxyl-terminal domain of the yeast *CDC34* (UBC3) ubiquitin conjugating (E2) enzyme. *EMBO J*, **11**, 3081-3090 (1992).

Kornitzer, D., Raboy, B., Kulka, R.G., Fink, G.R., Regulated degradation of the transcription factor GCN4. *EMBO J.*, **13**, 303-312 (1994).

Krek, W., Proteolysis and the G1-S transition: the SCF connection. *Current Opinion in Genetics and Development*, **8**, 36-42 (1998).

Lalli E., Lee, J. S., Lamas, M., Tamai, K., Zazopoulos, E., Nantel, F., Penna, L., Foulkes, N.S., Sassone-Corsi, P. The nuclear response to cAMP: role of transcriptionfactor CREM, Philos *Trans R Soc Lond B Biol Sci*, **351**, 201-209 (1996).

Lalli, E., Sassone-Corsi, P., Signal transduction and gene regulation: the nuclear response to cAMP. *J Biol Chem*, **269** (26), 17359-17362 (1994).

Lanker S., Valdivieso M.H., Wittenberg C. Rapid degradation of the G1 cyclin Cln2 induced by CDK-dependent phosphorylation. *Science***15**; 271(5255), 1597-1601 (1996).

Lisztwan, J., Marti, A., Sutterluty, H., Gstaiger, M., Wirbelauer, C., Krek, W., Association of human CUL-1 and ubiquitin-conjugating enzyme CDC34 with the F-box protein $p45^{SKP2}$: evidence for evolutionary conservation in the subunit composition of the CDC34-SCF pathway. *EMBO J.*, **17**, 368-383 (1998).

Maki, C. G., and Howley, P., Ubiquitination of p53 and p21 is differentially affected by ionizing and UV radiation. *Mol. Cell. Biol.*, **17**, 355-363 (1997).

Mellon, P. L., Clegg, C. H., Correll, L., A., McKnight, G. S., Regulation of transcription by cyclic AMP-dependent protein kinase. *Proc. Natl. Acad. Sci, USA*, **86**, 4887-4891 (1989).

Molina, C. A., Foulkes, N. S., Lalli, E., Sassone-Corsi, P. Inducibility and negative autoregulation of CREM: an alternative promoter directs the expression of ICER, an early response repressor. *Cell*, **75**, 875-886 (1993).

Monaco, L., Foulkes, N. S., Sassone-Corsi, P., Pituitary follicle-stimulating hormone (FSH) induces CREM gene expression in Sertoli cells: involvement in long-term desensitization of the FSH receptor. *Proc Natl Acad Sci U S A*, **92**(23), 10673-10677 (1995).

Nishizawa, M., Nagata, S., cDNA clones encoding leucine-zipper proteins which interact with G-CSF gene promoter element 1-binding protein. *FASEB Lett*, **299**(1), 36-38 (1992).

Pagano, M., Cell cycle regulation by the ubiquitin pathway. *FASEB J.*, **11**, 1067-1075 (1997).

Pati, D., Plon, S. E., Regulators of meiosis and spermatogenesis as potential targets of human Cdc34 ubiquitin ligase. The Cell Cycle, Keystone CO, Abstract# 327, (1998).

Pati, D., Vidal, M., Meistrich, M. L., Plon, S. E., Human Cdc34 and Rad6B ubiquitin ligases target members of the basic domain/leucine zipper transcriptional repressors ICER and ATF family for proteolysis: role in meiosis and spermatogenesis. *(In Preparation).*

Patton EE, Willems AR, Tyers M Combinatorial control in ubiquitin-dependent proteolysis: don't Skp the F-box hypothesis. *Trends Genet* **14**(6), 236-243 (1998)

Plon, S.E., Leppig, K.A., Do, N.-H. and Groudine, M., Cloning of the human homolog of the CDC34 cell cycle gene by complementation in yeast. *Proc. Natl. Acad. Sci. USA*, **90**,10484-10488 (1993).

Prendergast, J.A., Ptak, C., Arnason, T.G., Ellison, M.J., Increased ubiquitin expression suppresses the cell cycle defect associated with the yeast ubiquitin conjugating enzyme, Cdc34 (UBC3). *J. Biol. Chem.*, **270**(16), 9347-9352 (1995).

Ptak, C., Prendergast, J.A., Hodgins, R., Kay, C.M., Chau, V. and Ellison, M.J., Functional and physical characterization of the cell cycle ubiquitin-conjugating enzyme *CDC34* (UBC3). *J. Biol. Chem.*, **269**(42), 26539-26545 (1994).

Roset, H. P., Klaveren, J. V., Wit, J. de, vanGurp, C. G., Koken, M. H. M., Vermey, M., van Roijen, J. H., Hoogerbrugge, J. W., Vreeburg, J. T., M., Baarends, W., M., Bootsma, D., Grootegoed, J. A., Hoeijmakers, J. H. J., Inactivation of the HR6B ubiquitin-conjugating

DNA repair enzyme in mice causes male sterility associated with chromatin modification. *Cell*, **86**, 799-810 (1996).

Sambrook, J., Fritsch, E. F., Maniatis, T., Molecular cloning a laboratory manual, 2nd edition. Cold Spring harbor, New York: Cold Spring Harbor Laboratory Press (1989).

Sassone-corsi, P., Coupling gene expression to cAMP signalling: role of CREB and CREM, *Int. J. Biochem. Cell Biol.*, **30**, 27-38 (1998).

Schiestl, R.H., Giets, R.D., High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr. Genet.*, **16**, 339-346 (1989).

Schneider, B.L., Yang, Q.H., Futcher, A.B., Linkage of replication to start by the Cdk inhibitor Sic1. *Science*, **272**, 560-562 (1996).

Schwob, E., Böhm, T., Mendenhall, M.D. and Nasmyth, K., The B-type cyclin kinase inhibitor $p40^{SIC1}$ controls the G1 to S transition in S. cerevisiae. *Cell*, **79**, 233-244 (1994).

Silver, E.T., Gwozd, T.J., Ptak, C., Goebl M., Ellison, M.J., A chimeric ubiquitin conjugating enzyme that combines the cell cycle properties of *CDC34* (UBC3) and the DNA repair properties of *RAD6* (UBC2): implications for the structure, function and evolution of the E2s. *EMBO J*, **11**, 3091-3098 (1992).

Skowyra, D., Craig, K. L., Tyres, M., Elledge, S., J., Harper, J. W., F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. *Cell*, **91**, 209-219 (1997).

Spataro, V., Norbury, C., Harris, A. L., The ubiquitin-proteasome pathway in cancer. *British J. Cancer*, **77**, 448-455 (1998).

Tam SW, Theodoras AM, Pagano M, Kip1 degradation via the ubiquitin-proteasome pathway. *Leukemia* Suppl **3**:363-366 (1997).

Thompson, J.D., Higgins, D.G., Gibson, T.J., CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matix choice. *Nucleic Acid Res.*, **22**, 4673-4680 (1994).

Treier, M., Staszewski, L., Boh,ann, D., Ubiquitin-dependent c-Jun degradation *in vivo* is mediated by the g domain. *Cell*, **78**, 787-798 (1994).

Vidal, M., Brachmann, R., Fattaey, A., Harlow, Ed., Boeke, J.D., Reverse two-hybrid and one-hybrid systems to detect dissociation of protein-protein and DNA-protein interactions. *Proc. Natl. Acad. Sci., USA*, **93**, 10315-10320 (1996a).

Vidal, M., Braun, P., Chen, E., Boeke, J.D., Harlow, Ed., Genetic characterization of a mammalian protein-protein interaction domain by using a yeast reverse two-hybrid system. *Proc. Natl. Acad. Sci., USA,* **93**, 10321-10326 (1996b).

Wiebl, F.F., Kunau, W.H., The Pas2 protein essential for peroxisome biogenesis is related to ubiquitin-conjugating enzymes. *Nature*, **359**, 73-6 (1992)

Sharan, S.K., Morimatsu, M., Albretch, U., Lim, D.S., Regal, E., Dinh, C., Bradley, A., Embryonic lethality and radiation hypersensitivity mediated by RAD51 in mice lacking BRCA2. *Nature*, **386**, 804-810 (1997).

Swift, M., Morrel, D., Massey, R., Chase, C., Incidence of cancer in 161 families affected by ataxia-telangiectasia. *N Eng J of Med*, **325**, 1831-1836 (1991).

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..

Tasks in Progress:

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.

Rec'd
7/19/2000

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 6 Jul 00

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-OCA, 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statements

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for the following awards:

DAMD17-94-C-4068	ADB218322
DAMD17-94-V-4036	ADB232944
DAMD17-94-J-4481	ADB222571
DAMD17-95-C-5054	ADB227112
DAMD17-96-1-6016	ADB228823
DAMD17-96-1-6073	ADB248567
DAMD17-94-J-4057	ADB221437, ADB247857
DAMD17-96-1-6069	ADB230256, ADB239319
DAMD17-95-1-5067	ADB236775, ADB249592
DAMD17-94-J-4308	ADB225776, ADB234457, ADB249935
DAMD17-96-1-6087	ADB232086, ADB238945, ADB250354
DAMD17-96-1-6075	ADB228777, ADB238338, ADB249653
DAMD17-95-1-5008	ADB225250, ADB236089, ADB243691
DAMD17-94-J-4310	ADB222453, ADB235860, ADB247801

Request the limited distribution statement for Accession Document Numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Virginia Miller at DSN 343-7327 or by email at Virginia.Miller@det.amedd.army.mil.

FOR THE COMMANDER:

Phyllis Rinehart

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management