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PRINCIPAL INVESTIGATOR: Jeffrey W. Harper, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

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Jeffrey W. Harper, Ph.D.

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

E-Mail: jahrper@bcm.tmc.edu

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Substrate selection in ubiquitination reactions is achieved by ubiquitin ligases, which simultaneously bind both the target protein and a ubiquitin conjugating enzyme. While recent data indicates that there are a large number of distinct ubiquitin ligases that are responsible for the destruction of hundreds or perhaps thousands of proteins, there are currently no general methods to identify proteins whose levels are controlled by this mechanism. The development of general methods to systematically identify proteins whose abundance is regulated has major implications for: (1) elucidating proteolytic components of signaling pathways such as those activated in response to oncogenes and growth inhibitory factors, (2) identifying candidate drug targets whose altered destruction leads to therapeutic benefit, and (3) identifying genes that are required for the proper destruction of particular proteins of interest. We propose to merge our expertise in gene discovery approaches in yeast and mammalian cells with our experience in the molecular biology of regulated proteolysis to devise techniques that will facilitate the systematic identification of proteins derived from breast cell cDNA libraries whose levels are regulated.

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FOREWORD

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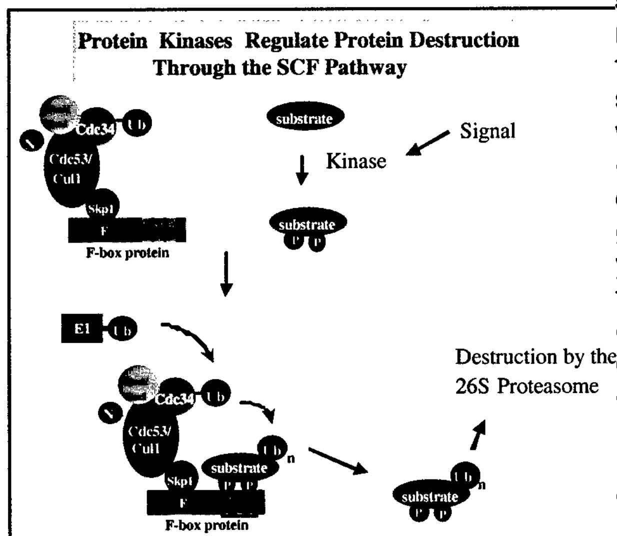
Introduction

Protein ubiquitination requires three components: E1, E2, and E3 (Hershko and Ciechanover, 1998). In the first step, a ubiquitin-activating enzyme (E1) is charged with ubiquitin through a thiol-ester linkage. This ubiquitin is then transferred to one of a dozen or so ubiquitin conjugating enzymes (E2) also as a thiol-ester. The ubiquitin is finally transferred from the E2 to one or more lysine residues in the substrate with the aid of an E3 ubiquitin ligase. In essence, E3s function as substrate-specific adaptors by simultaneously binding substrate and the E2, although in some cases, E3s may also serve as an intermediate in the ubiquitin transfer process.

Much of our knowledge of E3s has come from genetic dissection of signaling pathways that involve one or more ubiquitin-dependent events (reviewed in Koepp et al., 1999; Patton et al., 1998). These studies have revealed 3 broad classes of E3s that are likely to be responsible for targeting the ubiquitination of hundreds of proteins: 1) the HECT domain class which includes E6-AP, 2) the ring finger class which include Cbl and MDM2, and 3) the cullin-based ubiquitin ligases which include SCF, VBC, and APC complexes. Given the size of these different protein families, it is clear that many aspects of the biology of these E3s are unexplored. There are at least 40 HECT domain proteins in the human genome and more than 250 ring-finger containing proteins that may be involved in ubiqutination.

The complexity of these systems is perhaps best exemplified by the cullin-based ligases of which the SCF complex is the best understood. These are multicomponent E3s that dock substrates with a core ubiquitin conjugating system via modular substrate specific adaptors (reviewed in Koepp et al., 1999). The core components include a member of the cullin family of proteins, which contains 6 members in mammals, a RING finger protein typified by Rbx1 and APC11, and an E2 (Cdc34 or Ubc4). These core complexes interact with distinct families of substrate specific adaptors to generate a large number of ubiquitin ligases with distinct functions. Our work has focused on the SCF sub-family of cullin-based ubiquitin ligases, which refers to the three major components (Skp1, Cul1, and a member of the F-box family of proteins) (See scheme on left). Through genetic and biochemical studies in budding yeast, we identified Skp1 as a component of the SCF that links Cul1 to F-box proteins (Bai et al., 1996; Skowyra et al., 1997). We also discovered the F-box motif as a Skp1 binding element that is found in a large number of proteins that can bind to particular ubiquitination substrates in a phosphorylation dependent manner (Bai et al., 1996; Skowyra et al., 1997). The timing of ubiquitination and destruction of many proteins are controlled by protein phosphorylation, including cyclin-dependent kinase inhibitors such as p27 and Sic1, and G1 cyclins (cyclin E and Cln proteins). Through biochemical reconstruction of the SCF mediated ubiquitination of Sic1 and Cln1, we were able to demonstrate that distinct F-box proteins recognize distinct targets in a phosphorylation dependent manner and allow ubiquitination via an Rbx1/Cul1 dependent pathway (Skowyra et al., 1997, 1999; Kamura et al., 1999). In addition to the F-box motif that mediates interaction with Skp1, F-box proteins frequently contain C-terminal protein-protein interaction domains (Bai et al., 1996). The most common are WD40 and leucine-rich repeat domains. In an effort to understand the



complexity of mammalian F-box proteins, we have isolated a large number of cDNAs encoding F-box proteins (Winston et al., 1999a). In total, >68 distinct mammalian F-box proteins are now known to exist. We have shown that one of these, β-TRCP, is responsible for the ubiquitination of IkB, an inhibitor of the NFkB transcription factor complex required for the cytokine response as well as β-catenin, a transcription factor that functions as an oncogene when not properly destroyed by ubiquitin-mediated proteolysis (Winston et al., 1999b). The Cul2-based ubiquitin ligase has more than 20 known substrate adaptor proteins called SOCS-box proteins (Hilton et al., 1998), one of which is the von Hippel-Lindau tumor suppressor protein (Lisztwan et al., 1999). Although the functions of the vast majority of F-box and SOCS-box proteins are unknown, the finding that the limited number of F-box proteins that have been characterized all function to ubiquitinate multiple target proteins suggests that this family of E3s will be responsible for ubiquitination of possibly hundreds of proteins.

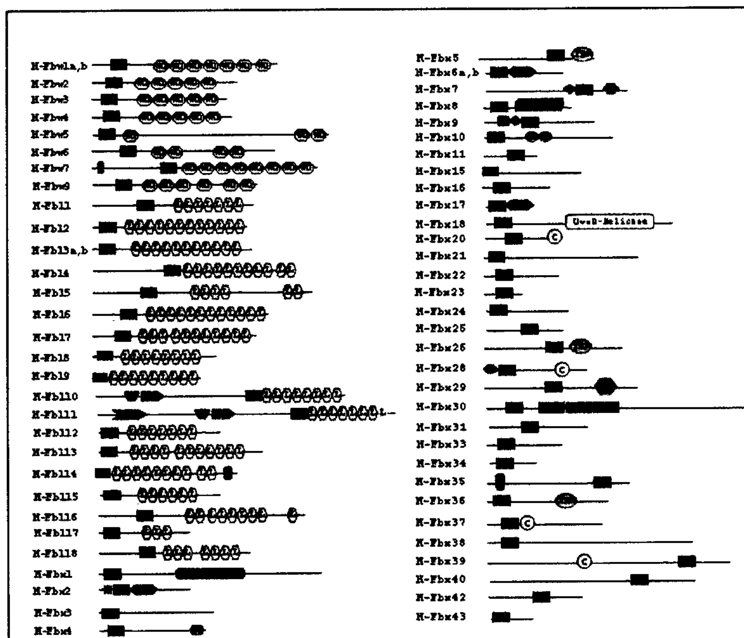
The challenge in the post-genome era will be: 1) to identify proteins whose abundance is regulated, 2) to determine what ubiquitin ligase pathways contribute to destruction of specific targets, and 3) to determine how the activities of particular ligases are controlled. Historically, the identification of ubiquitinated proteins has occurred on a case by case basis, and as such, we have a very limited view of the number and types of proteins in the cell that are controlled by this pathway. Moreover, we have little information that addresses how particular oncogenic events affect either the activities of different classes of ubiquitin ligases or the access of such ligases to their substrates. This is due, in part, to the fact that generally applicable methods are not available for identifying proteins that are destroyed in response to particular stimuli or in particular cellular contexts. In rare cases, it has been possible to use genetic screens in yeast to identify targets of ubiquitin ligases for which mutants were available. However, this approach is generally limited to yeast and even in cases where particular mutants in E3 components are available, substrates have been difficult to identify. In addition, approaches such as two-hybrid systems have not been particularly useful in identifying targets of ubiquitin ligase components such as F-box proteins. Given the large number of substrate adaptors that we and others have identified and that are likely to be identified in the future as a result of genome sequencing efforts, the identification of their important substrates will continue to be a major challenge.

Three complementary approaches are being undertaken to identify ubiquitination targets. In one approach, we are taking advantage of the facile genetics in budding yeast to identify targets of known ubiquitin ligase. Yeast has provided many of the important insights into ubiquitination pathways that have been shown to be general to all eukaryotes. Progress in this area was described in the previous progress report, including a publication in **Science** wherein we reported the identification of an F-box protein – Fbw7 - for human cyclin E. Human cyclin E is a prognostic marker for breast cancer and there is evidence that Fbw7 is mutated in human cancer, consistent with the possibility that it is a tumor suppressor. We have followed up this study by performing a series of mutagenesis experiments that help define the defects in Fbw7 mutations found in cancer, as described below. We are also continuing to pursue the general use of yeast as a system for finding ubiquitination targets (Aim 1). Tasks 1 and 2 have been completed and were described in detail in the previous report. We are currently working on Task 3 (aim 1) but have encountered technical difficulties, which we hope to sort out in the near future. In stead of using flow cytometry, we are employing a yeast sectoring assay to look for targets. There was too much background in the flow cytometry method but yeast sectoring assays may get around this. This effort is too preliminary to describe in detail here. In a second approach, we are using a collection of F-box proteins as biochemical and genetic reagents to identify substrates. Our progress on the identification of E3s for Cdc25A will be presented below. Finally, in a third approach, we are attempting to develop general methods to identify substrates in mammalian cells. For the reasons described below, we have modified our initial objective (Aim 2) in this regard to take advantage of new technology that has emerged since this grant was submitted. These new approaches, we feel, have a much better likelihood of yielding important results.

Body

Development of a library of F-box proteins

We previously reported the identification of 33 human F-box proteins. Through subsequent work, we have now expanded this to more than 68 family members in humans and 70 family members in the mouse. These F-box proteins fall into three classes: Fbws, which contain WD40 repeats, Fbls which contain leucine rich repeats, and Fbxs which contain either no recognized domain or have other classes of protein interaction domains. These C-terminal protein interaction domains are thought to mediate interaction with substrates. We have cloned and sequenced a number of these (more than 50 to date) and have made reagents that allow us to express these genes in human cells. This panel of reagents provides a unique opportunity to employ both yeast genetic systems and biochemical approaches to the identification of ubiquitination targets, the focus of this proposal.



Identification of an F-box protein important for degradation of the breast cancer oncoprotein Cdc25A. Previous work has demonstrated that Cdc25A is an oncogene and is overexpressed in breast cancer (Cangi et al., 2000; Evans, 2000). Cdc25A catalyzes the dephosphorylation of Cdk2, leading to its activation and thereby promoting S-phase entry. Cdc25A is regulated by multiple proteolytic mechanisms, including the anaphase promoting complex. Moreover, recent data indicate that Cdc25A is destroyed in response to DNA damage in a manner that depends upon the Chk1 kinase. Previous data suggested the involvement of an SCF complex in Cdc25A regulation but the identity of the F-box protein involved was not known.

β-TRCP Recognition Motif:

```
                     P    P
H-Cdc25A:    GSSEST     FCLD P
M-Cdc25A:    GSSEST     FCLD P
Rat-Cdc25A:  GSSEST     FCLD P
H-Emi1:         LYE     YS
M-Emi1:         LYE     YS
X-Emi1:          LQ     YS
F-Rca1:        LEMN     YT
IκBα:           DRH     LD
β-Catenin:      SYL     IH
                  /    φX
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To address this, we first confirmed the previous finding that dominant negative inhibitors of Cul1 lead to stabilization of Cdc25A. Interestingly, this occurs independently of DNA damage. Moreover, we demonstrated that Cul1 associates with Cdc25A in vivo. To search for F-box proteins that interact with Cdc25A, we performed a series of transfection experiments using various F-box proteins and then performed immunoprecipitations using Cdc25A antibodies. We found one F-box protein – β-

TRCP - that consistently interacted with Cdc25A. We previously discovered β-TRCP as an F-box protein that interacts with destruction motifs in IκB and β-catenin. β-TRCP interacts with sequences containing the consensus: DSGIXS where both serine residues are phosphorylated. In the absence of phosphorylation, neither IκB nor β-catenin interact with β-TRCP. We scanned the sequence of Cdc25A for sequences that look like those found in IκB and found a sequence centered on Ser-81 that has similarities to the sequence in IκB. We have constructed a number of point mutants in Cdc25A in serines that represent potential interaction sites and are currently testing these for interaction with β-TRCP. Two additional experiments are underway. In one series of experiments, we are using RNAi against β-TRCP1 and β-TRCP2 to determine whether Cdc25A is stabilized either in cycling cells or in response to DNA damage. If β-TRCP is the requisite E3 for Cdc25A, then we would expect this to be the case. To accomplish this, we have made retroviruses expressing hairpin RNAis for the TRCP genes. Second, we have made baculoviruses for β-TRCP in order to assemble SCFβ-TRCP complexes with the hope of reconstituting Cdc25A ubiquitination. We expect to be in a position to write a paper on this work in the near future.

Development of retroviral systems for identification of ubiquitination substrates in mammalian cells (Aim 2)

The goal of aim 2 is to develop a retroviral based system for identifying unstable proteins in mammalian cells. Our original plan was to employ GFP-fused cDNA libraries to develop a flow-cytometry based screen that would allow us to identify candidate substrates of particular E3s. Our progress on this was described in the previous report. However, the recent development of RNAi strategies in mammalian cells has radically changed the types of experiments that we can perform. We are now incorporating loss of function type experiments into a new approach to identify substrates that has features of the previously proposed system but is more likely to provide important results. In this approach, we will generate libraries of mammalian cells expressing CFP-tagged cDNAs via integrating retroviral vectors wherein the CFP epitope splices into endogenous genes, creating protein fusions. The CFP-tagged cDNA will be linked via an IRES to GFP such that sub-libraries of cells with defined GFP/CFP ratios can be isolated by live cell sorting. Ablation of particular ubiquitin ligases by RNAi would be expected to lead to increased levels of target proteins, resulting in a shift in the ratio of GFP to CFP in individual cells where the target gene is CFP tagged. Cells displaying altered ratios will be isolated by cell sorting and the identities of recipient genes determined.

The strategy is built upon previous work employing retroviral based tagging of genes (29) using enhanced retroviral mutagen (ERM) vectors. This system has been used to identify dominant proliferative genes. The primary features of these vectors relative to this proposal is that the tagging epitope (CFP for example) under control of its own promoter (CMV for example) is physically linked with splice donor sequences and upon integration into a cellular gene, CFP coding sequences may be spliced into the recipient gene, creating a protein fusion between CFP and the coding sequence of the recipient. Hundreds of thousands of independent integration events occurring in individual cells can be selected by virtue of selectable markers on the retrovirus, allowing the creation of libraries of cells containing a particular gene fused with CFP. An added feature of this system is that even genes that are not normally expressed in the target cell line can come under control of the retroviral derived CMV promoter, thereby allowing expression of otherwise silent genes. We have constructed the tagging tagging vector that expresses two spectrally separated GFP coding sequences from the same CMV driven transcript using an intervening IRES (internal ribosomal entry sequence) (Figure 1). The first GFP is expressed as an intact protein while the second variant GFP (CFP) lacks a stop codon but contains a splice-donor sequence. Upon integration into a recipient gene, GFP and the CFP fusion proteins should be produced at a constant ratio since they are derived from the same mRNA, although their individual stabilities may be different. Additional

versions of this vector have been generated, including CD20 instead of GFP and vectors that splice in two other reading frames. CD20 is a cell surface protein that can be detected immunologically in live cells.

Because the ratio of GFP to CFP-fusion protein should be stable in each recipient cell, groups of cells with particular ratios can be isolated by fluorescence activated cell sorting and then expanded for experimental manuipulation. We are currently in the process of generating MCF7 cells and related breast cancer cell lines that are stably transduced with this vector. Our initial goal is to generate pools of cells that each display a particular ratio of GFP (or CD20) to CFP-fusion protein. We will isolate 10 pools of cells that reflect GFP/CFP ratios ranging from 10 to 0.1. Each pool is expected to contain hundreds of thousands of different integration events. A substantial fraction of these integration events will lead to the production of a fusion protein between CFP and recipient coding sequences. The resulting CFP fusion proteins may represent full-length sequences or may represent fragments of the recipient gene, possibly containing sequences important for regulated turnover. In principle, by making cells with vectors in all three reading frames prior to sorting all cells together, it should be possible to have a larger number of recipient genes represented in the collection of pools. This effort represents Aim 2, tasks7 and 8.

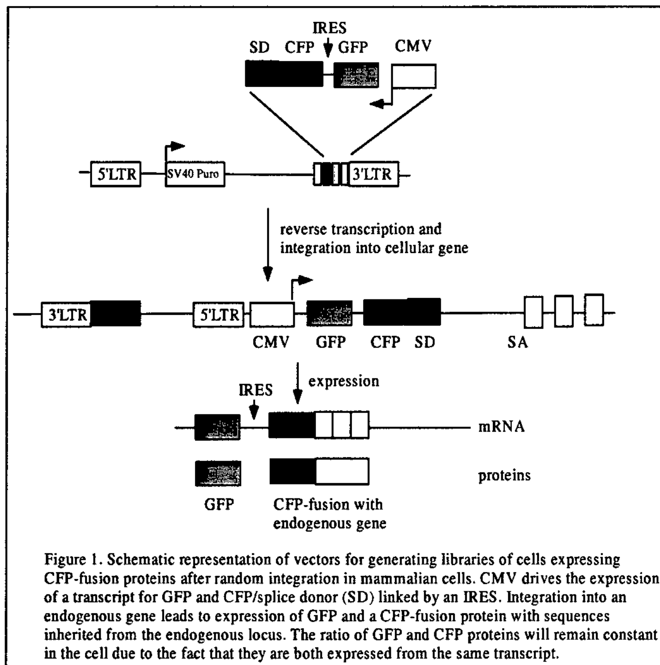

Figure 1. Schematic representation of vectors for generating libraries of cells expressing CFP-fusion proteins after random integration in mammalian cells. CMV drives the expression of a transcript for GFP and CFP/splice donor (SD) linked by an IRES. Integration into an endogenous gene leads to expression of GFP and a CFP-fusion protein with sequences inherited from the endogenous locus. The ratio of GFP and CFP proteins will remain constant in the cell due to the fact that they are both expressed from the same transcript.

A second part of this approach involves the development of RNAi for E3s. As part of a collaboration with Dr. Greg Hannon at Cold Spring Harbor, we have developed retroviral vectors expressing shRNA (short hairpin RNAs) for a large number of ubiquitin ligase components. These shRNAs are made against specific sequences in the E3 component and we have three different sequences per gene, thereby increasing the likelihood that knock-down can be achieved. Each of the shRNAs are under control of the U6 promoter. We are currently preparing DNA for shRNA vectors directed against ~50 F-box proteins which we have identified.

Depletion of a particular ubiquitin ligase by RNAi would be expected to lead to an increase in the abundance of requisite CFP-tagged substrates and this should be reflected in the alteration in the ratio of GFP to CFP. Thus, once pools of cells are established, RNAi will then be used to knock-down particular F-box proteins and cells that display increased levels of CFP-recipient fusion proteins relative to GFP isolated using FACS. Given the window of fluorescence ratios chosen for each pool, we anticipate to be able to identify cells in which the GFP/CFP ratio is decreased by as little as 3 fold. These cells contain tagged candidates for ubiquitination targets of particular F-box proteins. Based on our previous experience with RNAi against other ubiquitin ligases (Fbw7 for example) we easily see a 3-fold increase in cyclin E levels after RNAi (27), in the range we expect to be able to seen by FACS. If necessary, we will reconstruct the system using cyclin E as target to standardize parameters.

Functional analysis of point mutations in Fbw7 found in human cancer

In our previous report, we described our identification of Fbw7 as the F-box protein responsible for ubiquitination of cyclin E. There is now clear evidence of mutations in Fbw7 in human cancer, including ovarian, breast, and endometrial cancers (Moberg et al., 2000; Koepp et

al., 2000; Spruck et al., 2002). Our work initially found defects in expression of Fbw7 in breast cancer cell lines relative to normal cell lines. Several point mutations were found in the WD40 repeats of Fbw7, including R385, R425, and R463. We thought that it may be worthwhile to follow up our studies by examining the consequence of these mutations on function. To determine the effect of these mutations on interaction with cyclin E, we introduced these mutations into Fbw7 and examined their interaction with cyclin E. Prior to performing these experiments, we determined the phosphorylation status of cyclin E in mammalian cells and identified several phosphorylation sites that we thought might be important to interaction with Fbw7. We identified phosphorylation at S372, T380, and S384. Peptides containing various phospho-forms of this region of cyclin E were synthesized and tested for binding to Fbw7. Also, the sequence around T62 is similar to the Fbw7 recognition sequence centered at T380, so various phosphopeptides in this region were made as well. Using binding reactions with in vitro translated Fbw7 and phosphopeptides immobilized on agarose, we found that phosphorylation of T380 is sufficient to interact with Fbw7. Phosphorylation of S384 or S372 in the context of T380 phosphorylation had no impact on binding. In addition, phosphorylation of T62 was sufficient to allow for binding but phosphorylation of S58 had no effect on this binding. We next tested the point mutants seen in Fbw7 in cancer for binding to both the T62 peptide and the T380 peptide. We found that cancer derived mutations either did not bind at all to these peptides (R385A) or bound poorly relative to WT Fbw7 (R425A, R463A). The ability of Fbw7 to interact with two different cyclin E derived phosphopeptides could reflect either the presence of two different binding sites or the presence of a single binding site, wherein binding is mutually exclusive. To examine this issue, we performed a competition experiment wherein association of Fbw7 with phospho-T380 peptide was competed with either T62 peptide or with phosphorylated T62 peptide. We found that phosphorylated T62 peptide (but not unphosphorylated peptide) competed for binding to phospho-T380. Therefore, it would appear that a single site is responsible for binding to both of the peptides. Therefore, phosphorylation of either of these sites would be expected to affect turnover of cyclin E by Fbw7. Experiments to examine this are underway, as are experiments that test the interaction between Fbw7 mutants and full-length cyclin E.

Figure 2. (A) Binding of Fbw7 to immobilized cyclin E derived peptides. (B) Binding of Fbw7 mutants found in cancer to cyclin E derived phosphopeptides. (C) Phospho-T62 peptides compete with phospho-T380 peptides for binding to Fbw7.

Key Research Accomplishments:

Year 1
*Development of a yeast system for identification of proteins whose stability is regulated by a specific ubiquitin ligase

*The use of a related system to identify F-box proteins involved in cyclin E turnover

*Demonstration that Fbw7 in mammalian cells controls cyclin E levels

Year 2

* Completed an analysis of F-box proteins in the human and mouse genomes, leading to the identification of 68 human and 70 mouse F-box proteins

* Cloned and sequenced 50 human F-box proteins. Each F-box protein was placed into mammalian expression plasmids, facilitating the discovery of substrates

*Identified β-TRCP as a candidate ubiquitin ligase for the breast cancer oncoprotein Cdc25A using a panel of F-box proteins that we identified and cloned

*Developed new retroviral vectors that can be used in a dual fluorescence system to identify ubiquitination targets in combination with RNAi-dependent gene ablation

*Determined the requirements for interaction of cyclin E with Fbw7 and demonstrated that cancer-prone mutations in Fbw7 loose binding with cyclin E phospho-degrons.

Reportable outcomes.

Publications directly supported by this grant:

Koepp, D., Schaffer, L., Ye, X., Keyomarsi, K., Chu, C., Harper, J.W., and Elledge, S.J. (2001) Phosphorylation-dependent ubiquitination of cyclin E by a conserved SCF^{Fbw7} ubiquitin ligase. **Science** 294, 173-177.

Jin, J., Elledge, S.J., and Harper, J.W. (2003) Regulation of Cdc25A by the $SCF^{\beta\text{-TRCP}}$ ubiquitin ligase. Manuscript in preparation.

Xin, Y., and Harper, J.W. (2003) Recognition of human Fbw7 by phospho-degrons in cyclin E. Manuscript in preparation.

Reviews and papers related to protein ubiquitination published by this lab during the last year:

Zheng, N., Schulman, B.A., Miller, J.J., Wang, P., Jeffrey, P.D., Chu, C., Koepp, D.M., Elledge, S.J., Pagano, M., Conaway, R.C., Conaway, J.W., Harper, J.W., and Pavletich,

N.P. (2002) Structure of the Cul1–Rbx1–Skp1–F box^{Skp2} SCF Ubiquitin Ligase Complex. **Nature,** 416, 703-709.

Jin, J. and Harper, J.W. (2002) Ring-finger specificity in SCF driven protein degradation. **Developmental Cell** 2, 685-687.

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Harper, J.W., Burton, J.L., and Solomon, M.J. (2002) The anaphase promoting complex: It's not just for mitosis anymore. **Genes and Development**, 16, 2179-2206.

Passmore, L., McCormack, E.A., Au, S.W.N., Paul, A., Willison, K.R., Harper, J.W., and Barford, D. (2003) Doc1/Apc10 mediates the E3 ubiquitin ligase activity of the anaphase promoting complex by contributing to APC-substrate recognition. **EMBO J.** 22, 786-796.

Wu, G., Schulman, B., Harper, J.W., and Pavletich, N. (2003) Structure of a β-TrCP1–Skp1–β-catenin complex: destruction-motif binding and lysine specificity of the SCF ubiquitin ligase. **Molecular Cell**, in press.

Jin, J., and Harper, J.W. (2003) A license to Kill: Transcriptional activation and enhanced turnover of Myc by the SCF^{Skp2} ubiquitin ligase. **Cancer Cell**, in press.

Invited seminars to discuss the outcome of work funded by this grant:

Control of cell cycle transitions by the SCF ubiquitin ligase. The Cell Cycle, Cold Spring Harbor Laboratory, May 16, 2002

Protein ubiquitination and control of cell division. Division of Medical Oncology, University of Colorado Health Science Center, Denver, CO, July 15, 2002.

Control of cell division by protein phosphorylation and ubiquitin mediated proteolysis. Department of Pathology, Harvard Medical School, September 18, 2002.

Control of cell division by protein phosphorylation and ubiquitin mediated proteolysis. Fred Hutchison Cancer Research Center, November 12, 2002.

Novel targets upstream of the proteasome. Continuing Medical Education Program on Novel Anticancer Agents in the Proteasome Pathway, American Society of Hematology, Philadelphia, PA, December 6, 2002.

Substrate recognition by Cullin-based ubiquitin ligases. NCI Workshop of Ubiquitin Pathways, Rockville MD, December 8, 2002.

The SCF ubiquitin ligase pathway, University of Chicago, February 19, 2003

Substrate specificity of cullin-based ubiquitin ligases, Cold Spring Harbor Laboratory, The ubiquitin Family meeting, April 24, 2003

The ubiquitin-Proteasome Pathway, Karolinska Institute/Baylor College of Medicine Symposium, May 12, 2003

Funding applied for using preliminary data from this grant:

National Institutes of Health, R01 – "Functional anatomy of cullin-3 ubiquitin ligases"; submitted June 1, 2003

Employment/Research Opportunities impacted by the grant:

I was recruited to Harvard Medical School to join the Department of Pathology as the Bert and Natalie Vallee Professor of Molecular Pathology, a position I will assume on August 1, 2003. Our research funded by the grant contributed significantly to this recruitment.

Conclusion

The SCF pathway is widely used to control the levels of regulatory proteins. We are using multiple systems – both biochemical and genetic – to identify ubiquitin ligases for important cell cycle regulators as well as to identify substrates of particular ubiquitin ligases. The new system we are developing to identify substrates has the potential to help uncover numerous proteins whose levels are controlled by the ubiquitin pathway and may be involved in the genesis of breast cancer.

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