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Award Number: DAMD17-99-1-9071

TITLE: Regulation of NF (kappa) B-dependent Cell Survival  
Signals Through the SCF (slimb) Ubiquitin Ligase Pathway

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REPORT DATE: July 2002

TYPE OF REPORT: Annual Summary

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

DISTRIBUTION STATEMENT: Approved for Public Release;  
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268

# REPORT DOCUMENTATION PAGE

Form Approved  
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<b>1. AGENCY USE ONLY (Leave blank)</b>		<b>2. REPORT DATE</b> July 2002	<b>3. REPORT TYPE AND DATES COVERED</b> Annual Summary (1 Jul 99 - 30 Jun 02)	
<b>4. TITLE AND SUBTITLE</b> Regulation of NF (kappa) B-dependent Cell Survival Signals Through the SCF (slimb) Ubiquitin Ligase Pathway			<b>5. FUNDING NUMBERS</b> DAMD17-99-1-9071	
<b>6. AUTHOR(S)</b> Jeffrey Harper, Ph.D.				
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> Baylor College of Medicine Houston, Texas 77030  <b>E-MAIL:</b> jharper@bcm.tmc.edu			<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b> report contains color				
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited			<b>12b. DISTRIBUTION CODE</b>	
<b>13. ABSTRACT (Maximum 200 Words)</b> NFkB is a transcription factor that functions to block the apoptotic response. Inappropriate activation of NFkB is thought to block apoptosis in breast cancer cells. NFkB activity is negatively regulated by a signaling pathway that responds to extracellular signals, including cytokines. Normally, NFkB is held in the cytoplasm by its inhibitor, Ikb. In response to extracellular signals, Ikb is destroyed by the process of ubiquitin mediated proteolysis. This process is activated through protein kinases that respond to cytokines such as TNFalpha. These kinases phosphorylate Ikb, thereby activating it for ubiquitination. Ubiquitination involves 3 activities: an E1 activating enzyme, an E2 ubiquitin conjugating enzyme, and an E3 ubiquitin-protein ligase. In work supported by this grant, we have identified the molecular components involved in Ikb ubiquitination. The ubiquitin ligase is composed of Skp1/Cull1/Rbx1 and the specificity factor beta-TRCP. We have also performed a series of biochemical experiments that have revealed a consensus sequence for association of TRCP with ubiquitination substrates and have identified residues in TRCP that function in substrate recognition.				
<b>14. SUBJECT TERMS</b> breast cancer, protein ubiquitination, phosphorylation, IKB, NF-kB			<b>15. NUMBER OF PAGES</b> 20	
			<b>16. PRICE CODE</b>	
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Unlimited	

FOREWORD

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20021114 268

## **Table of Contents**

<b>Cover.....</b>	<b>1</b>
<b>SF 298.....</b>	<b>2</b>
<b>Foreword.....</b>	<b>3</b>
<b>Table of Contents.....</b>	<b>4</b>
<b>Introduction.....</b>	<b>5-6</b>
<b>Body.....</b>	<b>6-10</b>
<b>Key Research Accomplishments.....</b>	<b>10</b>
<b>Reportable Outcomes.....</b>	<b>11</b>
<b>Conclusions.....</b>	<b>12</b>
<b>References.....</b>	<b>12</b>
<b>Appendices.....</b>	<b>13-18</b>

## Introduction

The process of apoptosis is critical to the development and homeostasis of multicellular organisms. It provides a mechanism for loss of cells during organogenesis and provides a pathway for removal of cells that are undergoing inappropriate proliferative events or have received unreparable DNA damage. A theme in cancer biology is that cellular transformation requires the establishment of survival pathways that limit the process of apoptosis. Recent studies have revealed that one such survival pathway is established through the action of the transcription factor NF $\kappa$ B. NF $\kappa$ B plays important roles in activation of genes in response to cytokines and other stimuli, and has been well characterized with respect to its role in inflammatory diseases. Cytokines such as TNF- $\alpha$  activate two pathways, one that activates a cell death response, and one that activates a survival response that is dependent upon NF $\kappa$ B. This finding has renewed interest in the development of inhibitors of the NF $\kappa$ B pathway that can be used therapeutically to block survival pathways while simultaneously allowing for agents such as TNF or chemotherapeutics to activate apoptotic pathways. The use of such combination therapy has the potential to allow proliferative control of established tumors. Because of the widespread nature of the NF $\kappa$ B survival pathway, such NF $\kappa$ B inhibitors would be expected to be useful in a wide variety of proliferative diseases and mammary cancer is no exception. Recent studies have revealed that mammary tumor cells utilize NF $\kappa$ B in an anti-apoptotic mechanism and display increased NF $\kappa$ B activity that correlates with estrogen-independence.

NF $\kappa$ B function is normally controlled by I $\kappa$ B, which holds NF $\kappa$ B in the cytoplasmic. Signals which activate NF $\kappa$ B lead to phosphorylation of I $\kappa$ B, which signals it for ubiquitin-mediated proteolysis thereby allowing NF $\kappa$ B to enter the nucleus. The importance of I $\kappa$ B in the survival function of NF $\kappa$ B is demonstrated by the fact that overexpression of non-phosphorylatable I $\kappa$ B blocks NF $\kappa$ B function, allowing for apoptosis.

Because of its critical position in the NF $\kappa$ B activation pathway, the I $\kappa$ B ubiquitin ligase represents an important therapeutic target. Blocking its activity would be equivalent to overexpression of non-phosphorylatable I $\kappa$ B and would be expected to lead to inhibition of NF $\kappa$ B. In the last progress report, we described our efforts to understand how the I $\kappa$ B protein is regulated in response to its phosphorylation by I $\kappa$ B kinase (IKK). It is now clear from our work and work of others that phosphorylation of I $\kappa$ B by IKK allows it to interact with a ubiquitin ligase, SCF $^{\beta\text{-TRCP}}$  (Winston et al., 1999; Maniatis, 1999). Ubiquitination of I $\kappa$ B by the SCF $^{\beta\text{-TRCP}}$  leads to the destruction of I $\kappa$ B by the proteasome. SCF complexes function as E3 ubiquitin ligases and are composed of Skp1, the Ring-H2 finger protein Rbx1, the Cul1 protein, and an F-box protein, in this case  $\beta\text{-TRCP}$ . This complex recognizes I $\kappa$ B in a phosphorylation-dependent manner and catalyzes I $\kappa$ B ubiquitination in vitro, in conjunction with an E2 ubiquitin conjugating enzyme and an E1 activating enzyme. Analysis of the role of  $\beta\text{-TRCP}$  in the destruction of I $\kappa$ B was a major goal of Aim 1. In the past year, we have explored the biochemistry of the interaction of  $\beta\text{-TRCP}$  with I $\kappa$ B using physical and mutagenic approaches. As described below, this has revealed a consensus sequence recognized by  $\beta\text{-TRCP}$ , which is found in additional targets of I $\kappa$ B, including  $\beta\text{-catenin}$ . In addition, we have identified residues on the surface of  $\beta\text{-TRCP}$  required for recognition of substrates. These studies were major goals of Aim 2.

During the initial stages of this work, we were interesting in the possibility that the  $\beta\text{-TRCP}$  complex might serve as a therapeutic target, as blocking its action might promote

apoptosis. A paper reported in the literature using peptides that mimic phosphorylated I $\kappa$ B and thereby block its ubiquitination were indeed found to promote apoptosis in particular settings, strongly suggesting that this approach will work. In principle, this is identical to blocking  $\beta$ -TRCP function, at least with respect to NF $\kappa$ B signaling. However, we and others also found that  $\beta$ -TRCP also is responsible for destruction of  $\beta$ -catenin. This creates a problem with respect to the use of  $\beta$ -TRCP as a therapeutic target.  $\beta$ -Catenin is an oncogene and blocking its turnover would be expected to cause cell proliferation and possibly lead to transformation. In fact, mutations in the  $\beta$ -catenin phosphorylation sites that allow it to bind to  $\beta$ -TRCP are oncogenic. Thus, we felt that this strategy of blocking I $\kappa$ B turnover would might be of clinical value. Therefore, we refocused our efforts in the last year on understanding substrate recognition through crystallographic studies in collaboration with Nikola Pavletich and on understanding the role of the related  $\beta$ -TRCP protein,  $\beta$ -TRCP2. These new studies are outlined below.

## Body

### Identification of a consensus substrate recognition motif for $\beta$ -TRCP.

As a first step towards understanding how  $\beta$ -TRCP interacts with I $\kappa$ B and  $\beta$ -catenin destruction motifs, we wanted to search for sequences that are able to bind to  $\beta$ -TRCP. One approach to this problem, which we proposed, is the use of peptide libraries containing a large number of diverse sequences. Sequence analysis of peptides that bind to a particular protein provides a consensus sequence for binding which can then be followed up with more detailed experiments, depending upon the degeneracy observed. Together with Dr. Songyang who developed the peptide library approach, we performed an analysis of  $\beta$ -TRCP. Because large amounts of immobilized protein is required for this technique, we had to develop a system for expressing large quantities of functional  $\beta$ -TRCP. Preliminary experiments indicated that expression of  $\beta$ -TRCP in bacteria was sub-optimal and the protein that could be expressed as incapable of binding to I $\kappa$ B (data not shown). Therefore, we developed an insect cell expression system wherein we co-express untagged  $\beta$ -TRCP with GST-Skp1. Complexes are then purified using GSH-sepharose. Using this approach we were able to generate sufficient amounts of essentially homogeneous GST-Skp1/ $\beta$ -TRCP complexes for binding studies.

Because we already knew that  $\beta$ -TRCP interacts with phospho-serine containing destruction motifs, we used a peptide library containing two fixed phosphoserine residues with the first phosphoserine preceded by an aspartate. Peptide library was incubated with immobilized GST-Skp1/ $\beta$ -TRCP and the beads washed extensively. Peptide was eluted by treating the complex with acid (pH2) and released peptides subjected to Edman degradation to determine the collection of peptide sequences. The data are shown in Fig. 1 (appendix). The consensus was  $\phi$ - $\phi$ -[A,N]-D-pS-[G,E,N,Y]-[Y,E]-[A,F,Y,E]-pS-[Y,F]-[Y,F] (where  $\phi$  = a hydrophobic amino acid). Some aspects of this consensus conform to the sequences of I $\kappa$ B and  $\beta$ -catenin while other aspects do not. For example selection of Y and F residues in the last two positions was not expected, based on the I $\kappa$ B sequence. This suggests that it might be possible to generate a specific inhibitor of  $\beta$ -TRCP. Several

other libraries were tried, including single phosphoserine libraries but these did not bind, suggesting that two phospho-serines are required for binding.

We are continuing to search emerging protein sequence data bases to identify proteins that contain potential  $\beta$ -TRCP recognition sequences. Recently, we found that Emi (early mitotic inhibitor) contains a candidate  $\beta$ -TRCP interacting motif. Emi is a very interesting cell cycle regulator. It acts as an inhibitor of the anaphase promoting complex, a ubiquitin ligase that functions to control the levels of a variety of proteins in the cell, including mitotic cyclins and the Pds1 protein required for maintenance of sister chromatid cohesion. We are currently very excited about the possibility that Emi is regulated by  $\beta$ -TRCP. Emi is known to be an unstable protein and is destroyed as cells go into mitosis (allowing the APC to become active). Emi is regulated by E2F and is required to facilitate accumulation of cyclin A as cells enter S-phase. We are currently in the process of testing whether Emi is targeted by  $\beta$ -TRCP using biochemical and genetic techniques.

### **Identification of residues involved in recognition of I $\kappa$ B and $\beta$ -catenin by $\beta$ -TRCP.**

As a second step towards identifying small molecules that interact with  $\beta$ -TRCP and block association with I $\kappa$ B and  $\beta$ -catenin, we sought to identify residues in  $\beta$ -TRCP that are required for this association.  $\beta$ -TRCP is a member of the WD40 repeat family of proteins and contains 7 WD40 repeats. Proteins containing 7 WD40 repeats, such as  $\beta$ -transducin, form a  $\beta$ -propeller structure in which each WD40 repeat forms a blade of the 7-blade propeller. We hypothesized that basic residues (lysine and arginine) located on the surface of  $\beta$ -TRCP might function in the recognition of phosphorylated I $\kappa$ B and  $\beta$ -catenin destruction motifs. To examine this question, we developed a model of  $\beta$ -TRCP based on the known structure of  $\beta$ -transducin. Using this model, we identified lysine and arginine residues that are conserved among  $\beta$ -TRCP family members but not other family WD40 containing F-box proteins. Residues were classified as either being on the surface of the face of the propeller that binds  $\alpha$ -transducin, the face of the propeller that binds  $\gamma$ -transducin, or buried in the central core of the propeller. Residues identified by this exercise are shown in Fig. 2 (appendix).

Given the likelihood that ligands bind  $\beta$ -TRCP from the  $\alpha$ -face, we made point mutants in all of the conserved basic residues on this surface. These residues in  $\beta$ -TRCP were changed to alanine by site-directed mutagenesis and the mutant cDNAs cloned into expression vectors. Proteins were expressed and tested for binding to I $\kappa$ B and  $\beta$ -catenin destruction motif peptides in either the phosphorylated or unphosphorylated forms. As shown in Figure 3 (appendix), mutations of most of the residue had no effect on the interaction of  $\beta$ -TRCP with I $\kappa$ B and  $\beta$ -catenin destruction motifs. However, mutation of two residues - Arg306 and Lys289 - led to dramatic decreases in binding affinity. These two residues are located in the first WD40 repeat, adjacent to the F-box motif. Previous deletion studies have revealed that this WD40 repeat is important for interaction with  $\beta$ -TRCP targets. As described below, we have recently taken a structural approach to begin to define how this interaction occurs in a direct fashion.

### **Specificity of ligand binding by WD40 repeat-containing F-box proteins.**

As described in the previous progress report, we have cloned a family of mammalian F-box proteins, including WD40 and leucine rich repeat containing proteins. Previously, we had found 5 WD40 repeat containing proteins. In the last year, we have identified 2 additional WD40 repeat containing F-box proteins, Fbw6 and Fbw7. Given this rather large number of F-box proteins, we wondered whether there was a common theme to substrate recognition or whether different F-box proteins used different structural elements to interact with targets. To date, the only other WD40-containing F-box protein whose substrate has been identified is Fbw7. We recently found that Fbw7 is responsible for ubiquitin-mediated destruction of cyclin E. Like  $\beta$ -TRCP, Fbw7 interacts with a short phosphorylated destruction motif in cyclin E, Leu-Leu-phosphoThr-Pro-Pro. We used a similar strategy as described for  $\beta$ -TRCP to identify residues involved in binding of cyclin E to Fbw7. This analysis revealed that three arginine residues were important for the cyclin E interaction (Fig. 4, appendix). Unlike the situation with  $\beta$ -TRCP, these residues were located in WD40 repeats 3, 4, and 5. Mutation of these residues independently to alanine either abolished or greatly reduced the interaction of Fbw7 with cyclin E. These data suggest that different WD40 repeat elements confer substrate specificity upon different F-box proteins. This work is currently being written for publication.

#### **WD40 elements alone are insufficient for destruction motif recognition.**

Given the results with  $\beta$ -TRCP and I $\kappa$ B/ $\beta$ -catenin destruction motifs, we were interested in determining whether isolated WD40 elements could interact with phosphopeptides. We generated  $\beta$ -TRCP proteins that were truncated after each WD40 repeat and tested these for binding to I $\kappa$ B destruction motifs. Only the full length protein containing all 7 WD40 repeats was capable of binding (data not shown). It is likely that this reflects the absence of structural stability of the WD40  $\beta$ -propeller structure when one or more repeats are missing.

#### **Crystallographic analysis of a $\beta$ -TRCP/Skp1/phosphopeptide complex.**

Although the mutagenesis studies described above begin to address how  $\beta$ -TRCP recognizes substrates, there is no substitute for having an X-ray structure of the complex. We have had a continuing collaboration with Nikola Pavletich's lab at Sloan Kettering. Thus far, we published two papers on SCF function; one involving the structure of the Skp1/Skp2 complex and one involving the structure of a Skp1/Cul1/Rbx1/Skp2 complex. These structures have provided important insight into how the SCF functions but we still do not have a good idea concerning how it interacts with substrates and positions them for ubiquitination. In a continuation of this collaboration, we have solved the structure of the  $\beta$ -TRCP/Skp1/I $\kappa$ B peptide complex to 3 angstroms resolution (Fig. 5). The structure of Skp1 and the associated  $\beta$ -TRCP F-box match closely to that found with the Skp1/Skp2 complex. The WD40 repeats of  $\beta$ -TRCP form a  $\beta$ -propeller, as expected. The I $\kappa$ B phosphopeptide binds to the alpha face of the  $\beta$ -propeller, consistent with our mutagenesis studies outlined above. In fact, the residues we identified as being involved in binding the phosphopeptide are located close to the first phosphoserine of the Asp-phosphoSer-Gly-Ile-His-phosphoSer recognition motif as well as Asp at position 1 of the recognition motif.

The Ile residue at position 4 is buried in the WD40 fold. Interestingly, mutation of residues located near phospho-serine at position 6 (Lys430 and Arg431) do not affect binding to phosphopeptides *in vitro*, although we cannot rule out the possibility that these do affect binding to I $\kappa$ B *in vivo*.

### Functional studies on $\beta$ -TRCP2.

$\beta$ -TRCP is a member of a larger family of WD40-containing F-box proteins and its closest homolog is  $\beta$ -TRCP2. Both of these proteins are capable of functioning to ubiquitinate I $\kappa$ B, suggesting functional redundancy. A major question concerns the relative importance of these genes both during normal biology and during transformation. There are data in the literature that indicate that  $\beta$ -TRCP is mutated in a subset of prostatic tumors. In contrast,  $\beta$ -TRCP is not mutated in colon cancer. There is likely to be tissue specificity in this pathway and effects on transformation will most likely occur in situations where  $\beta$ -TRCP2 is not expressed. Clearly, it is important to understand the similarities and differences between these two genes. Recently,  $\beta$ -TRCP knock-out mice have been generated (personal communication from Michele Pagano). However, these mice do not have strong developmental phenotypes. This may reflect the action of  $\beta$ -TRCP2. To address this issue, we initiated during the last year, an experiment to address this. We are generating mice that lack  $\beta$ -TRCP2. Once available, these mice will be analyzed for phenotypes and will be mated with  $\beta$ -TRCP mice to look for the effects of redundancy.

We are well on our way to generating these mice and currently have mouse embryonic stem cells that contain a conditional allele of  $\beta$ -TRCP2 (Fig. 6). We cloned genomic fragments for  $\beta$ -TRCP2 and flanked exon 3 encoding the F-box motif with loxP sites. This will allow us to use cre recombinase to remove the F-box encoding segment of the  $\beta$ -TRCP2 gene, thereby generating an inactive allele. At this stage, the cells are waiting to be injected into blastocysts to create chimeric mice. Ultimately, we will be able to employ mice expressing cre in particular cell populations such as breast epithelial cells to determine the role of  $\beta$ -TRCP and  $\beta$ -TRCP2 in proliferation, apoptosis and transformation.

Work in *Drosophila* (which has a single  $\beta$ -TRCP protein) indicates that mice lacking both  $\beta$ -TRCP and  $\beta$ -TRCP2 proteins will have detectable phenotypes. In flies, this gene is essential for life and controls a large number of processes. A major goal of our work will be to generate cell lines that lack  $\beta$ -TRCP. Because we have generated a conditional allele, we will be able to make cells even if the mice die *in utero* prior to the day that fibroblast can normally be isolated (day 13). Mutations in the *Drosophila* protein cause defects in centrosome function and we can easily test whether this is the case in mammalian cells as well.

### Discussion

Activation of NF $\kappa$ B involves an extensive signal transduction pathway that culminates in the destruction of the NF $\kappa$ B inhibitor I $\kappa$ B. We have demonstrated that I $\kappa$ B is ubiquitinated by an SCF <sup>$\beta$ -TRCP</sup> ubiquitin ligase complex. In principle, molecules that block I $\kappa$ B destruction could act as pro-apoptotic agents. F-box proteins such as  $\beta$ -TRCP function by binding to destruction sequences, and in I $\kappa$ B, the destruction motif is created upon

phosphorylation at Ser32 and Ser34. The sequences we have identified by peptide library analysis (Fig. 1) correspond to the sequence of I $\kappa$ B. These sequences also occur in  $\beta$ -catenin and both biochemical and genetic evidence indicate a role for  $\beta$ -TRCP in interacting with  $\beta$ -catenin or armadillo in flies (Jiang and Struhl, 1998).  $\beta$ -catenin is a component of the Wingless/Wnt signaling pathway and functions with Tcf/Lef transcription factors to regulate patterning and other developmental decisions. Recent work in *Xenopus* has revealed that expression a  $\beta$ -TRCP protein lacking the F-box leads to accumulation of  $\beta$ -catenin and ectopic activation of the Wnt pathway (Marikawa and Elinson, 1998). This, together with our data linking  $\beta$ -TRCP to direct recognition of the phosphorylated  $\beta$ -catenin destruction motif strongly implicates SCF $\beta$ -TRCP as the  $\beta$ -catenin ubiquitin ligase. Further studies are required to determine whether any of the many proteins containing the DSG $\phi$ XS motif are also substrates for SCF $\beta$ -TRCP.

Thus far, we have identified a total of 10 human F-box proteins that contain WD40 repeats. These motifs are likely to bind to phosphor-substrates as we have shown for Fbw7 and  $\beta$ -TRCP. Together with the Pavletich lab, we have generated the first structure of an F-box protein bound to a substrate. This provides molecular insight into how phosphopeptides are recognized by WD40 motifs and sets the stage for additional studies aimed at understanding the underlying principle behind phosphopeptide recognition. The crystal structures of other WD40 containing F-box proteins associated with their targets will help clarify how destruction motifs are recognized and will facilitate the identification of agents that can block interactions with destruction motifs.

## **Research Accomplishments:**

### **Year 1**

- \* Identification of the I $\kappa$ B ubiquitin ligase
- \* Demonstration that the SCF $\beta$ -TRCP complexes recognizes I $\kappa$ B in a phosphorylation dependent manner
- \* Identification of the  $\beta$ -catenin ubiquitin ligase
- \* Identification of a second  $\beta$ -TRCP gene in the human genome

### **Year 2**

- \* Identification of a consensus sequence for  $\beta$ -TRCP substrates
- \* Identification of residues located in WD40 repeat 1 required for interaction of  $\beta$ -TRCP with substrates
- \* Identification of a  $\beta$ -TRCP homolog and a determination of the residues in this protein required to interact with its substrate cyclin E

### **Year 3**

- \* Determination of the structure of  $\beta$ -TRCP/Skp1 complexes bound to a target phosphopeptide (collaboration with Nikola Pavletich)
- \* Cloning of the gene for mouse  $\beta$ -TRCP2, construction of a conditional knock-out construct, and generation of mouse ES cells containing a conditional allele of  $\beta$ -TRCP2.

## **Reportable outcomes.**

Publications supported by this grant:

Winston, J.T., Strack, P., Beer-Romero, P., Chu, C., Elledge, S.J., and Harper, J.W. (1999) The SCF <sup>$\beta$ -TRCP</sup> ubiquitin ligase specifically associates with phosphorylated destruction motifs in I $\kappa$ B and  $\beta$ -catenin and stimulates I $\kappa$ B ubiquitination in vitro. **Genes and Development**, 13, 270-283. (recognized as a "Hot Paper" by ISI, ranked 5th among all papers for citations in 1999)

Winston, J.T., Koepp, D.M., Zhu, C., Elledge, S.J., and Harper, J.W. (1999) A family of mammalian F-box proteins. **Current Biology** 9, 1180-1182.

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<sup>Fbw7</sup> ubiquitin ligase. **Science** 294, 173-177. (contains data in Fig. 4 related to the specificity of the interaction of related WD40 containing F-box proteins such as with their substrates)

Wu, G., Winston, J.T., Harper, J.W., and Pavletich, N. Mechanism of substrate recognition by the F-box protein  $\beta$ -TRCP. manuscript in preparation.

Publications related to the SCF pathway from this lab but not supported directly by this grant:

Jin, J. and Harper, J.W. (2002) Ring-finger specificity in SCF driven protein degradation. **Developmental Cell**, 2, 685-694. (preview)

Nalepa, G., and Harper, J.W. (2002) Linking estrogen-independent breast tumor growth to the proteolysis of a cell cycle regulator. **Nature Medicine**, in press. (News and Views)

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<sup>Skp2</sup> SCF Ubiquitin Ligase Complex. **Nature**, 416, 703-709.

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## Conclusion

This report concludes the Department of Defense sponsorship of this research project. We have learned a significant amount about how  $\beta$ -TRCP functions. However, we are embarking on new areas of research as a result of this proposal, including an analysis of  $\beta$ -TRCP2 deficient mice and funding from other sources will be sought for this work. In addition, our efforts at understanding how  $\beta$ -TRCP recognizes substrates will no doubt facilitate a more complete understanding of WD40-containing F-box proteins in the future.

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- \*Tom Maniatis (1999) A ubiquitin ligase complex essential for the NF-kB, Wnt/Wingless, and Hedgehog signaling pathways. *Genes & Dev.* 13: 505-510.
- \*Marikawa, Y., and R.P. Elinson. 1998.  $\beta$ -TRCP is a negative regulator of Wnt/ $\beta$ -catenin signaling pathway and dorsal axis formation in *Xenopus* embryos. *Mech. Dev.* **77**: 75-80.
- \*Winston, J.T., Strack, P., Beer-Romero, P., Chu, C., Elledge, S.J., and Harper, J.W. (1999) The SCF <sup>$\beta$ -TRCP</sup> ubiquitin ligase specifically associates with phosphorylated destruction motifs in I $\kappa$ B and beta-catenin and stimulates I $\kappa$ B ubiquitination in vitro. *Genes and Development*, 13, 270-283.

Appendix:

Award Number: DAMD17-99-1-9071

TITLE: Regulation of NF (kappa) B-dependent Cell Survival Signals Through the SCF (slimb) Ubiquitin Ligase Pathway

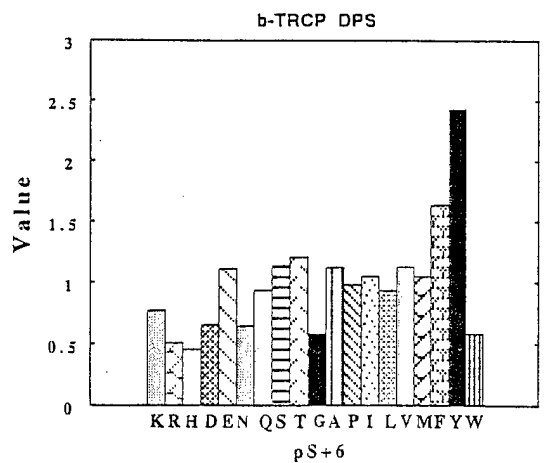
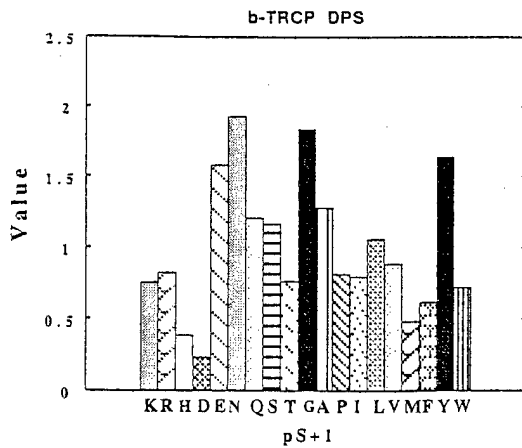
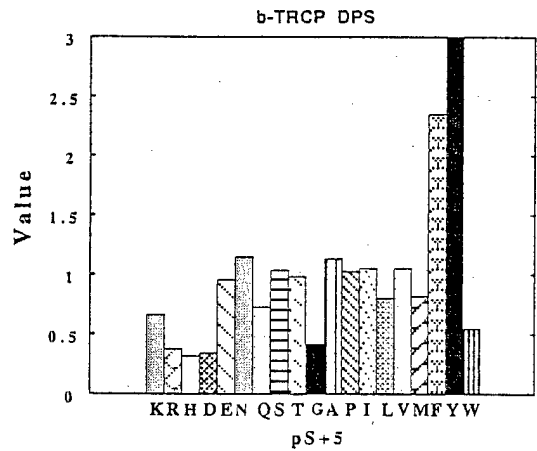
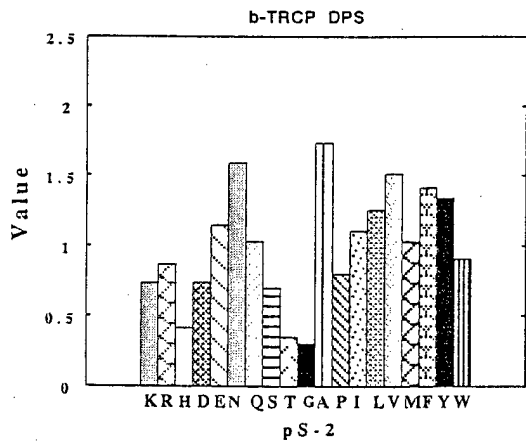
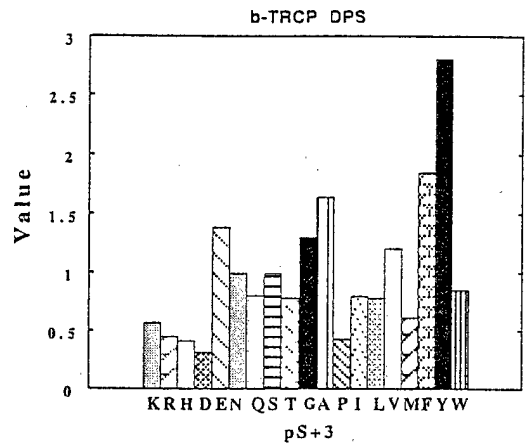
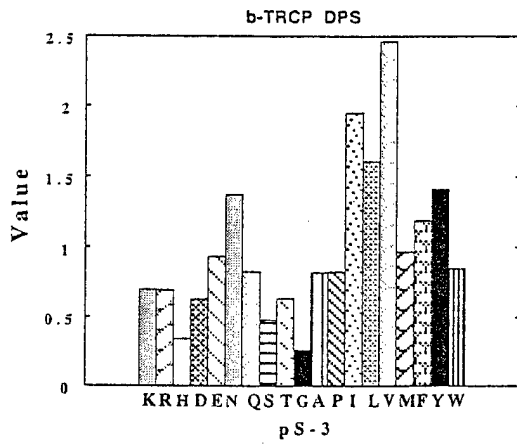
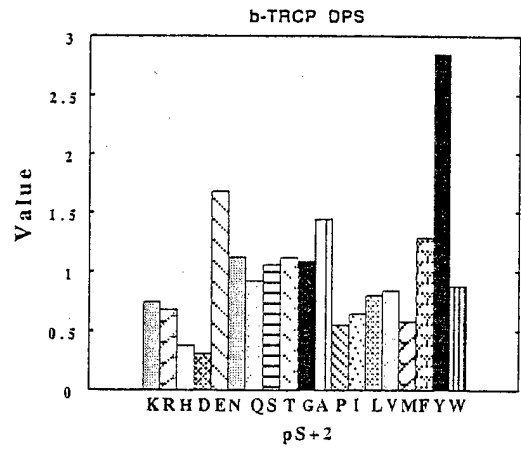
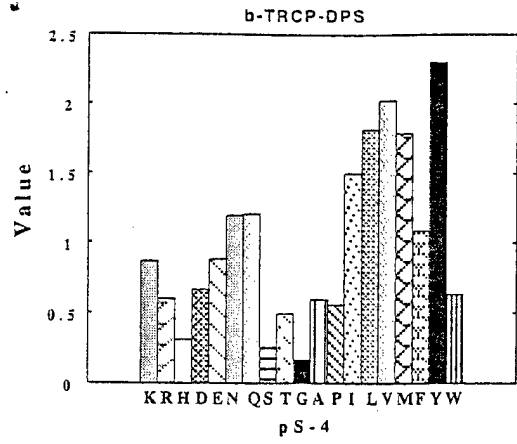
PRINCIPAL INVESTIGATOR: Jeffrey Harper, Ph.D.

Figs 1-6

Figure 1 (legend)

Determination of consensus sequences for interaction of phospho-peptides with b-TRCP. The relative abundance of amino acids at each position in the peptide library KNXXDpSXXpSXXAK where the first pS is residue zero is indicated. These relative levels were determined by Edman degradation of peptides after selection on GST-Slkp1/ $\beta$ -TRCP.

Figure 1



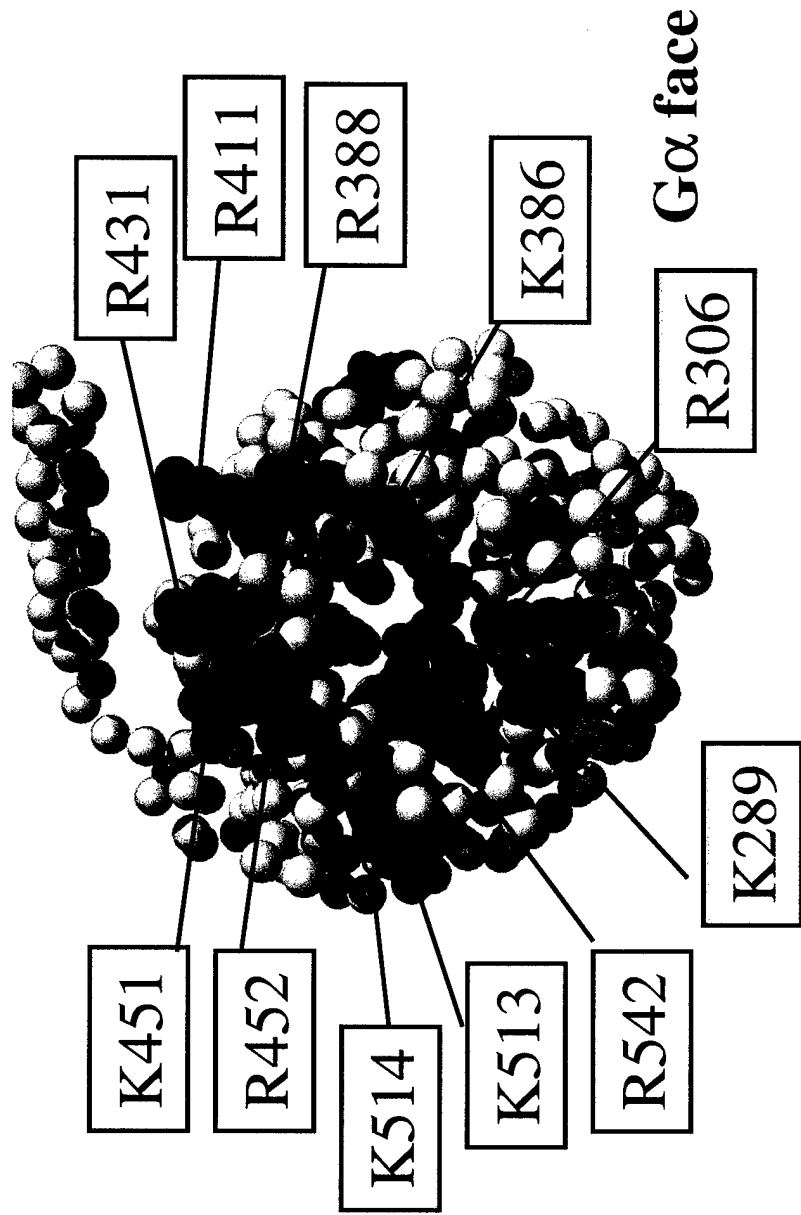


Figure 2. Model of  $\beta$ -TRCP based on the structure of  $\beta$ -transducin. Lysine and arginine residues on the surface of TRCP and corresponding to the face of  $\beta$ -transducin that binds  $\alpha$ -transducin are shown in blue. The residue numbers chosen for mutagenesis are indicated in boxes. The boxes in blue represent residues that are required for interaction of TRCP with I $\kappa$ B and  $\beta$ -catenin

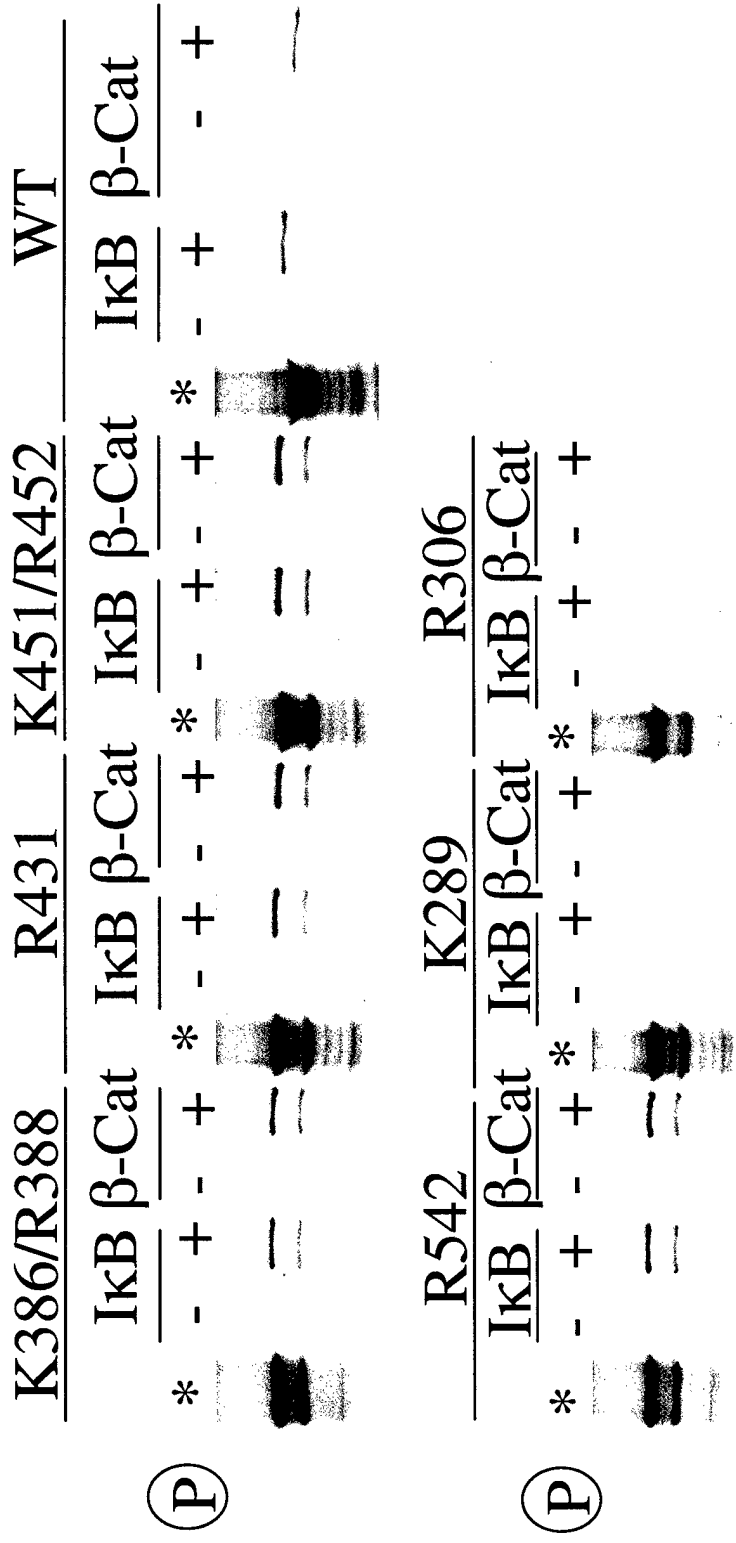
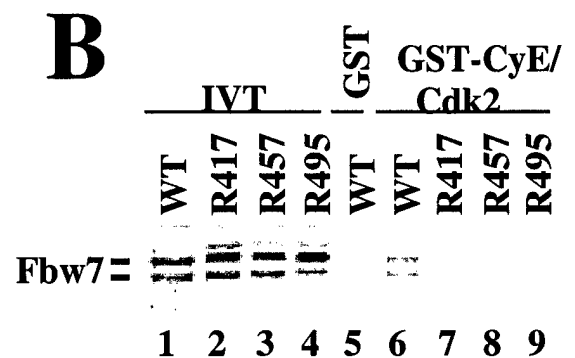
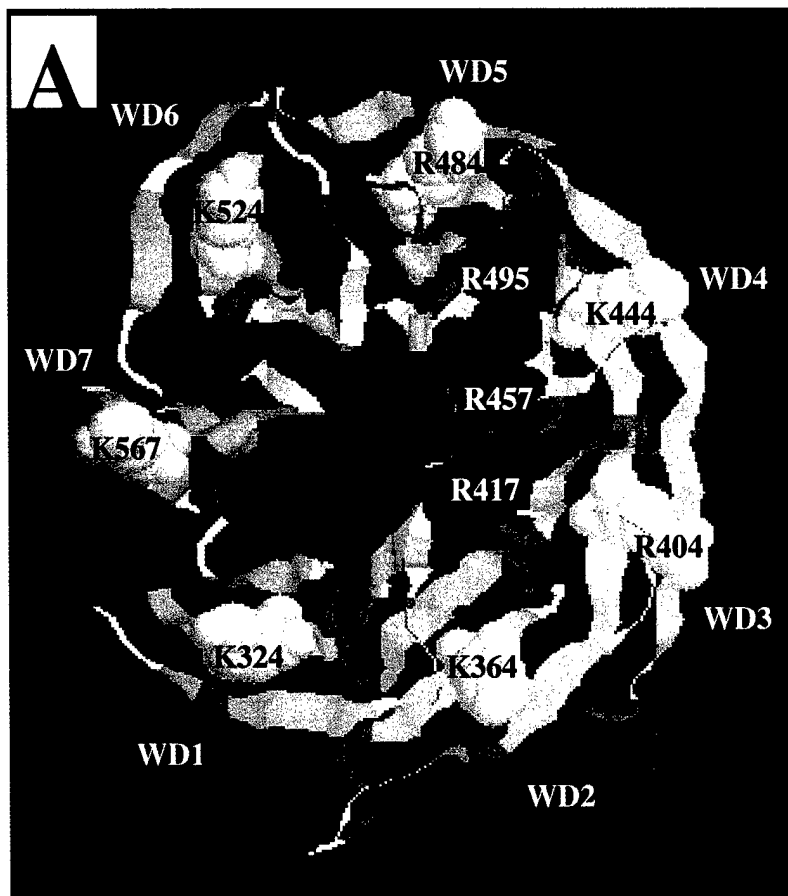


Figure 3. Binding of  $\beta$ -TRCP mutants to I $\kappa$ B and  $\beta$ -catenin phosphopeptides. In vitro translated  $\beta$ -TRCP and mutants (\*) were used for binding reactions with beads containing either unphosphorylated or phosphorylated destruction motifs from I $\kappa$ B and  $\beta$ -catenin. After binding, proteins were separated by SDS-PAGE and detected by autoradiography.



**Figure 4. Analysis of the  $\beta$ -TRCP homolog Fbw7 and its motifs involved in interaction with cyclin E. A model of Fbw7 was generated and conserved arginine and lysine residues not present in  $\beta$ -TRCP found (red). These residues were mutated to alanine and in vitro translation products used for binding to GST-cyclin E/Cdk2. The R417 and R457 mutants displayed no detectable binding while the R495 mutant displayed reduced binding.**

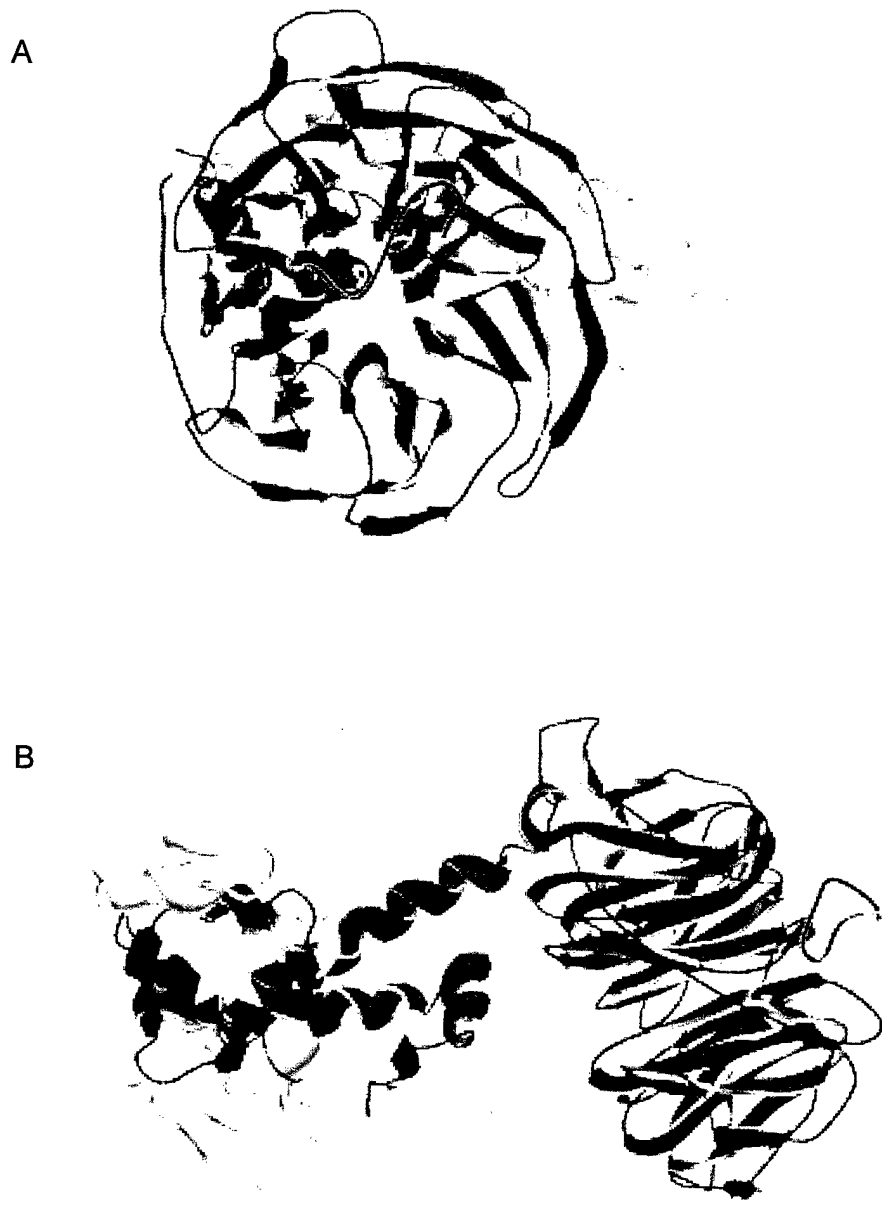
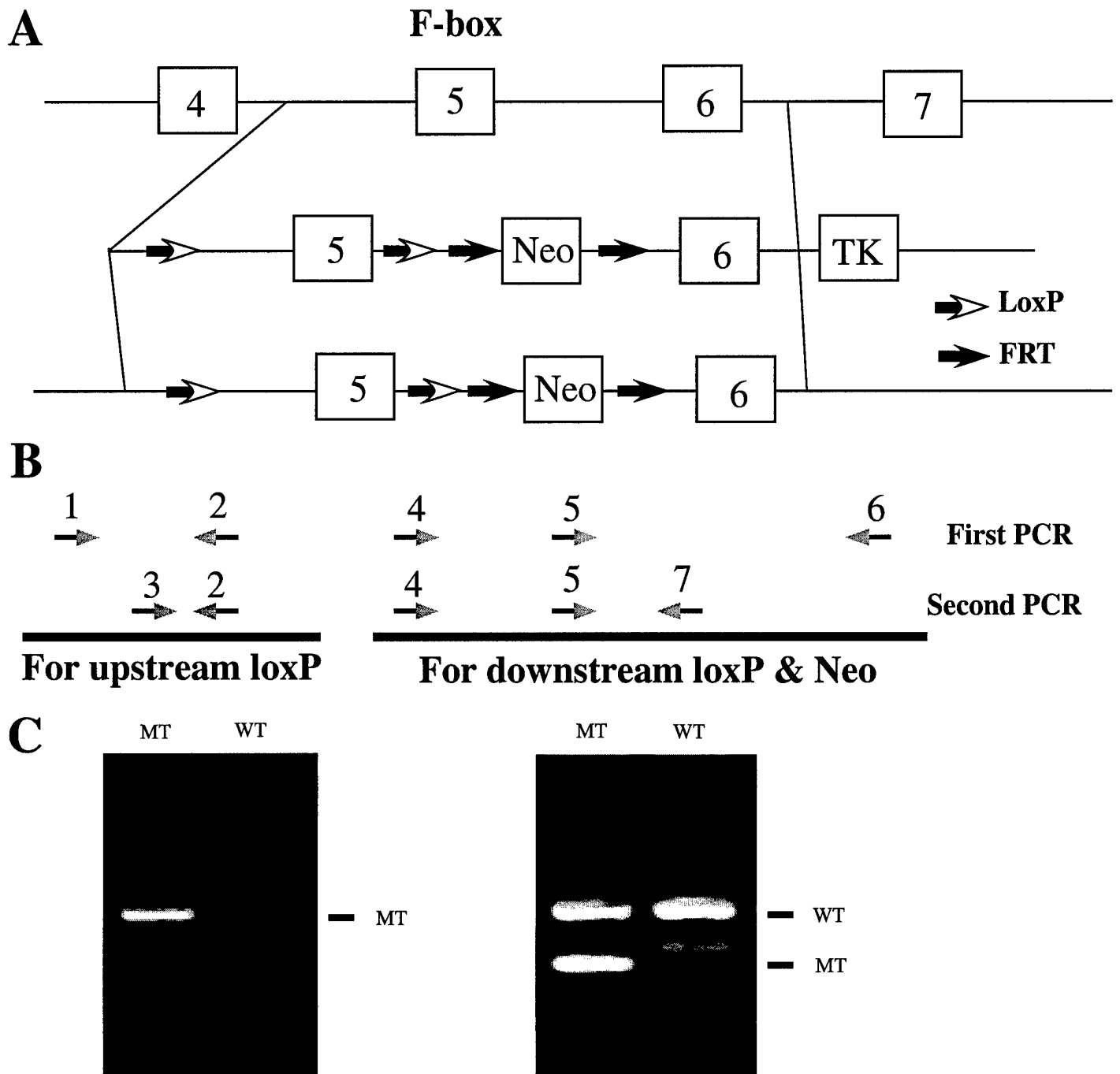


Figure 5. Structure of a Skp1/ $\beta$ -TRCP/destruction motif complex. Skp1 is shown in gray,  $\beta$ -TRCP in green, and a phosphopeptide from  $\beta$ -catenin in red.



**Figure 6.** Strategy for generation of a conditional allele for TRCP2. A loxP site and a loxP-FRT-Neo-FRT cassette were inserted into intron 5 and intron 6, respectively (panel A) Homologous recombination into the TRCP2 locus yields the product shown and these were confirmed by two rounds PCR analysis shown in panel B. For upstream loxP, the first PCR was based on an mutant specific (MT) oligo 2 and an wild-type (WT) oligo 1. The products were reamplified during the second PCR by oligo 2 and another WT oligo 3. For downstream Neo cassette, the long products were amplified by the first PCR with WT oligoes 4, 6 and MT oligo 5. Then, these products were reamplified during the second PCR by oligoes 4, 5 and another WT oligo 7. The results were shown in panel C. The heterozygous mutant ES clones will be reconfirmed by Southern analysis.