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TITLE: The Role of hCDC4 as a Tumor Suppressor Gene in Genomic Instability  
Underlying Prostate Cancer

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14. ABSTRACT This study investigates the role of a newly identified gene called hCDC4 in prostate cancer. The hCDC4/FBXW7 gene encodes a protein that functions in a cellular process called proteolysis, or protein degradation. hCdc4 degrades a protein called cyclin E1, which is a central component of the cell division machinery. Cyclin E1 is involved in initiating DNA replication in cells. However, in many types of human tumors cyclin E1 protein level is aberrant and this phenotype has been shown in vitro and in vivo to be oncogenic. Very little is known regarding cyclin E1/hCdc4 in prostate tumors. We are exploring whether hCDC4/FBXW7 functions as a tumor suppressor gene in prostate cancer. We have completed a genetic screen of prostate tumors and found an hCDC4/FBXW7 gene mutation. We have shown that this mutant hCdc4 cannot bind cyclin E1 substrate in vivo and mislocalizes in cells. We are currently exploring the effects of hCDC4/FBXW7 knockdown in prostate cancer progression and identifying substrates of dysregulation of cyclin E1 kinase activity due to hCDC4/FBXW7 inactivation using protein array technology.					
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## Introduction

This study investigated the role of a newly identified gene called *hCDC4/FBXW7* in prostate cancer. The *hCDC4/FBXW7* gene encodes a protein that is a member of the F-box protein family that functions in a cellular process called proteolysis, or protein degradation. hCdc4/Fbxw7 functions in the degradation of several important proteins in cell cycle regulation such as cyclin E1, c-myc, Aurora-A, and Notch 1/4 (Strohmaier, H. *et al.* 2001; Moberg, K.H. *et al.* 2001; Koepp, D.M. *et al.* 2001). Cyclin E1 is involved in initiating DNA replication in mammalian cells and abnormalities in its expression have been reported in many types of human tumors (Sandhu, C. and Slingerland 2000). Evidence implicating a role for deregulated cyclin E1 associated kinase activity in prostate tumorigenesis is suggested through studies of the cyclin E1/Cdk2 inhibitor p27. In prostate tumors, p27 protein levels are low or absent and this phenotype is associated with poor patient prognosis (Macri, E. and Loda, M. 1998). In this proposal we explored whether *hCDC4/FBXW7* functions as a tumor suppressor gene in prostate cancer through its role in regulating cyclin E1 proteolysis.

### **Aim1- Determine the role of *hCDC4/FBXW7* as a tumor suppressor in prostate cancer (Months 1 - 20)**

#### **a. *Identify and isolate fresh-frozen and/or archival prostate tumor specimens from the tissue bank at The Sidney Kimmel Cancer Center (Months 1-2).***

We obtained 40 prostate tumor specimens from the SKCC Tumor Bank. These samples were processed in collaboration with a trained pathologist. Sections were trimmed to include the highest possible tumor purity. Four sections of 10 µm thickness were obtained for each fresh-frozen tumor specimen.

#### **b. *Isolate DNA, RNA and protein from fresh frozen prostate tumor specimens (Months 2-3)***

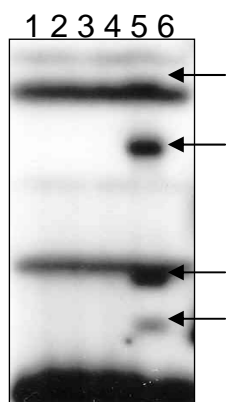
Two 10 µm sections of each tumor specimen were used for DNA isolations using the QiaAmp DNA Isolation Kit (Qiagen). Approximate total yield of DNA for each sample was 20 µg. We diluted each DNA sample to a concentration of 20 µg/ml. To obtain protein, two tumor specimen sections were lysed in mammalian RIPA lysis buffer and cellular proteins extracted. A total yield of approximately 100 µg protein was obtained/sample.

#### **c. *Microdissect matching normal DNA tissue from paraffin-embedded archival tissue specimens (Months 2-3)***

Normal tissue for each tumor specimen was marked by microscopic examination and microdissected under a light microscope (magnification x 40). DNA was isolated by standard proteinase K digestion technique. This DNA was used as control in loss of heterozygosity (LOH) determinations.

#### **d. *Screen prostate tumors for *hCDC4* gene mutations by SSCP (Months 3-6)***

We screened 40 prostate tumor specimens for *hCDC4/FBXW7* gene mutations by single-stranded conformation polymorphism (SSCP) analysis as developed by us. Eighteen different PCR reactions were used to cover the 13 different exons of the *hCDC4/FBXW7* gene. An aberrant SSCP banding pattern was detected for a single prostate tumor specimen corresponding to the α-exon of *hCDC4* (Fig. 1).

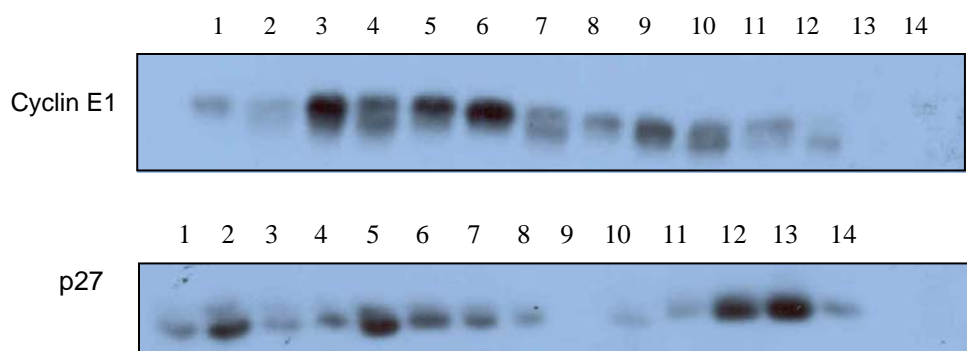


**Figure 1. hCDC4/FBXW7 gene mutation in a prostate tumor detected by SSCP analysis.** SSCP analysis of the  $\alpha$ -exon of *hCDC4/FBXW7* demonstrated an aberrant banding pattern for tumor in lane 5 (arrows). DNA sequencing revealed the mutated allele contains a three base pair insertion (CCG) introducing an in-frame proline residue in the N-terminus of the hCdc4/Fbxw7 protein (see below).

**e. Sequence *hCDC4* gene mutations (Months 5-6)**

We cloned the *hCDC4/FBXW7* alpha-exon for the prostate tumor containing an aberrant SSCP banding pattern into pCRII-TOPO (Invitrogen). DNA sequencing revealed a three base pair insertion in the gene which is predicted to introduce an in-frame proline residue in the hCdc4 protein.

**f. Western blot analysis of cyclin E and hCdc4 protein in prostate tumor specimens (Months 8-10).** We isolated protein from 40 fresh frozen prostate tumor specimens (see above). Approximate yield of protein for each sample was 100  $\mu$ g. We then performed Western blot analysis of all 40 prostate tumor specimens using antibodies specific for cyclin E1 and p27 (Fig. 2). As expected, we found that numerous prostate tumors contained an elevated level of cyclin E1 protein. Additionally, numerous tumors were found to contain a low or absent level of p27.



**Figure 2. Western blot analysis of cyclin E1 and p27 in prostate tumors specimens.** 40 prostate tumor specimens were obtained from the SKCC Tumor Bank and the proteins extracted in RIPA buffer. 50 micrograms of protein were separated on SDS-PAGE gels and Western blotted using antibodies to cyclin E1 and p27. Several tumors show evidence of cyclin E1 overexpression and low or absent expression of p27.

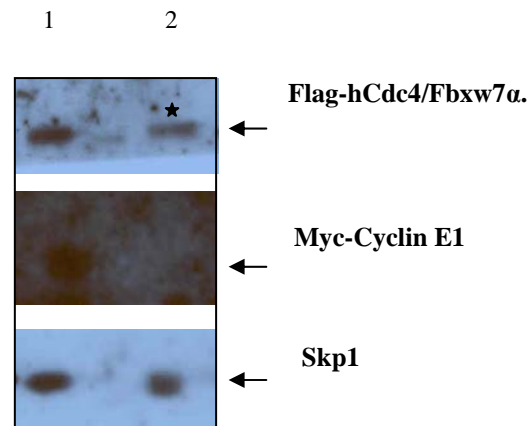
We encountered several problems in our attempts to determine the level of hCdc4/Fbxw7 in prostate tumor specimens by Western blot analysis. We tried numerous commercially available antibodies to no

avail, likely do to the extremely low level of the protein in cells. We also developed a human antibody in collaboration with researchers at the Karolinska Institute in Sweden, which could detect overexpressed protein in 293T cells but was unable to detect hCdc4/Fbxw7 in tumor specimens (data not shown). These results have necessitated our undertaking of LOH and Real-time PCR analysis to substitute for hCdc4 western blot analysis in hCdc4 expression determinations.

**g. Immunohistochemical staining of archival prostate tumor specimens for cyclins E, A and B1 (Months 9-12).** We immuno-histochemically analyzed prostate tumor specimens containing the *hCDC4/Fbxw7* mutation or wild-type alleles. Archival paraffin-embedded specimens were analyzed for cyclin E1 and cyclin A expression. Slides were analyzed microscopically for the percentage of positive staining nuclei. Determinations for cyclin E1 were found to be difficult to interpret due to high background, but a determination was made. No correlation was observed between the level of cyclin E1 and hCdc4/Fbxw7 mutation status in the single tumor (data not shown).

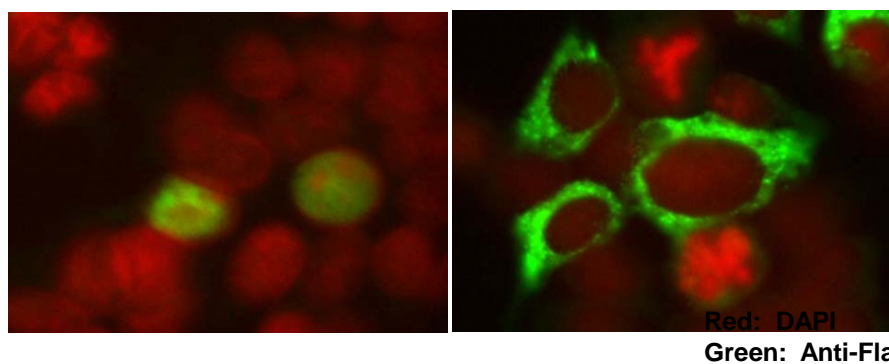
**h. Functional analysis of the mutant hCDC4/FBXW7 allele (Months 9-12).** We cloned the mutant *hCDC4/FBXW7*  $\alpha$ -exon for the prostate tumor containing an aberrant SSCP banding pattern into pCRII-TOPO (Invitrogen). DNA sequencing revealed a three base pair insertion in the gene. This sequence is predicted to introduce an in-frame proline residue in the hCdc4 protein. To test whether this mutation caused an alteration in hCdc4/Fbxw7 function, we tested the ability of the mutant protein to complex with cyclin E1 *in vivo*. We constructed mammalian expression vectors that express wild-type or mutant hCdc4/Fbxw7 tagged with the Flag epitope. Human embryonic kidney 293T cells were co-transfected with vectors that express wild-type or mutant hCdc4/Fbxw7, together with myc-tagged cyclin E1. To determine whether the mutant hCdc4/Fbxw7 protein can complex with SCF core components, we also co-transfected 293T cells with a vector that expresses Skp1. Protein complexes were isolated using anti-Flag agarose and proteins separated by SDS-PAGE. As shown in Fig. 3, the wild-type hCdc4/Fbxw7 protein was shown to bind cyclin E1 and the Skp1 component. In contrast, although the mutant hCdc4/Fbxw7 could bind Skp1, it was incapable of binding cyclin E1 *in vivo*. These results show that the *hCDC4/FBXW7* mutation found in the prostate tumor destroys the substrate recognition function of hCdc4/Fbxw7.

**Figure 3. A mutant hCdc4/Fbxw7 protein detected in a prostate tumor specimen cannot bind cyclin E1 substrate *in vivo*.** 293T cells were co-transfected with expression plasmids for Skp1 and myc-cyclin E1, together with either wild-type hCdc4/Fbxw7 (lane 1) or mutant hCdc4/Fbxw7 (lane 2). Extracts were then immunoprecipitated using anti-Flag agarose and immunocomplexes resolved on SDS-PAGE gels. Note that the mutant hCdc4/Fbxw7 binds Skp1 but not cyclin E1.



**i. Functional analysis of an N-terminal mutant of Fbxw7/hCdc4 reveals the location of a nuclear localization signal in hCdc4/Fbxw7.** Substrate recognition by Fbxw7/hCdc4 is believed to be

mediated through the interaction of key residues on the  $\beta$ -propeller surface formed by the 7 WD40 repeats of Fbxw7/hCdc4 and phosphodegron motif(s) contained in the various substrates. To further test the significance of the prostate tumor mutation on hCdc4/Fbxw7 function, we next determined whether the mutation had any effect on protein localization. To determine this, we transfected 293T cells with expression vectors that express the mutant or wild-type hCdc4/Fbxw7 protein and determined cellular localization using anti-flag antibodies. Immunofluorescence analysis showed that wild-type  $\alpha$ -Fbxw7/hCdc4 was found almost exclusively in the nucleus, whereas  $\alpha$ -Fbxw7/hCdc4<sup>+p16</sup> exclusively localized to the cytoplasm (Fig. 4). These results show that the insertion of proline at position 16 abolished the putative NLS of the protein, preventing interaction of hCdc4/Fbxw7 with cyclin E1 substrate in the nucleus.



**Figure 4. N-terminal mutation in hCdc4 in a prostate tumor causes a mislocalization of the  $\alpha$ -isoform to the cytoplasm.** Immunofluorescence analysis of  $\alpha$ -Fbxw7/hCdc4<sup>+p16</sup>. Cells were transfected with expression plasmids for Flag- $\alpha$ -Fbxw7/hCdc4 (left) or Flag- $\alpha$ -Fbxw7/hCdc4<sup>+p16</sup> (right) and protein location determined using anti-Flag FITC antibodies.

### **Accomplishment of goals-Aim1**

We have accomplished all of the points listed in Aim1 of the SOW. We are currently evaluating the data for publication purposes.

### **Aim2- Determine if hCdc4/Fbxw7/cyclin E1 abnormalities are involved in prostate tumor progression (Months 1-24).**

**a. Design of siRNAs.** We successfully designed siRNAs to silence *hCDC4/FBXW7* in mouse cells. These siRNAs were designed based on our preliminary data obtained in *hCDC4/FBXW7* silencing in 293T human cells. The ability to down-regulate expression was evaluated in immortalized mouse embryonic fibroblasts (MEFs) created by us.

**b-c. Construction of siRNA vector.** A *hCDC4/FBXW7* shRNA vector was created which contains the siRNA with upstream transcriptional stop codon flanked by 2 loxP recombination sites. We obtained mice that express the Cre-recombinase under control of the probasin promoter, which is prostate-specific for expression.

**d-f. Construction of hCdc4/Fbxw7 siRNA expressing mice.** We outsourced the transfection of the hCdc4/Fbxw7 siRNA expression plasmid into embryonic stem cells to the Transgenic Core Facility at the University of California at San Diego. Blastocyst injection was also performed by this facility. We screened approximately 10 mice for germline transmission of the siRNA construct and identified several progeny mice. These mice have been crossed with normal C57BL/6 mice to start a founder population.

### **Accomplishments of goals-Aim2**

We have succeeded at constructing an siRNA targeting vector for *hCDC4/FBXW7* down-regulation based on our preliminary data and tested its effectiveness in vivo. We have also created transgenic mice containing the siRNA vector. We have not, however, been able to cross these mice with the Cre-recombinase expressing mice and tested the effectiveness of down-regulation of *mCDC4/FBXW7* in animals. This has been due primarily to time constraints and unforeseen circumstances involving the PI of this project (Audrey van Drogen, see below).

### **Aim3- Identify inappropriately phosphorylated substrates of deregulated cyclin E1 in prostate cells (Months 12-24).**

**a-b. siRNA-mediated knock-down of hCDC4/FBXW7 in LNCaP cells.** We acquired LNCaP cells from the American Tissue Culture Collection (ATCC) and tested our siRNA for its ability to knockdown *hCDC4/FBXW7* level (previously shown in preliminary data). We performed cell cycle analysis of these cells and showed no serious cell cycle effects, suggesting this system was valid for determining cyclin E1 substrates following *hCDC4/FBXW7* down-regulation.

**c-d. SELDI-TOF-MS analysis to identify differentially phosphorylated proteins in hCDC4/FBXW7 knockdown cells.** In our proposal, we proposed to identify differentially phosphorylated substrates deregulated cyclin E1 caused by *hCDC4/FBXW7* inactivation using SELDI-TOF MS analysis. For these studies, phosphorylated substrates were to be isolated by immunoprecipitation of extracts with antibodies specific for phosphothreonine. This approach could be problematic though because our preliminary studies showed that the complexity of was likely too great for this analysis. We have a standing collaboration with Invitrogen (Carlsbad, CA) which is exploring the use of protein arrays for these studies. The protein array we are using has >8000 human proteins spotted onto nitrocellulose slides. We have worked out the conditions for performing these reactions on the array in vitro using cell line extracts and are scheduled to perform the analysis comparing extracts from LNCap cells transfected with *hCDC4/FBXW7* siRNA or control siRNA at different phases of the cell cycle.

### **Accomplishment of goals - Aim3**

We have successfully knocked-down expression of *hCDC4/FBXW7* in LNCaP cells using siRNA and showed that this leads to a deregulation of cyclin E1-associated kinase activity. We have not completed goals c-d of the Aim, but have instead adopted an alternative approach that we feel will likely yield more meaningful data in the long term. This new approach will also integrate state-of-the-art molecular tools into our research.



## Key Research Accomplishments

1. We have detected the first *hCDC4/FBXW7* gene mutation in a prostate cancer.
2. We have analyzed cyclin E1 and p27 in prostate tumor specimens and found abnormalities in each protein.
3. We have found that a mutant hCdc4/Fbxw7 protein found in prostate tumors cannot complex with cyclin E1 substrate in vivo.
4. We have identified the putative nuclear localization signal for hCdc4/Fbxw7.
5. Constructing a siRNA-based vector for *mCDC4/FBXW7* down-regulation in mouse cells.
6. Creating transgenic mice containing the *mCDC4/FBXW7* siRNA construct.
7. Successfully shown siRNA-mediated knock-down of *hCDC4/FBXW7* in LNCaP cells.
8. Explored the use of novel protein array technology to identify substrates of dysregulated cyclin E1 in LNCaP cells.

## Reportable Outcomes

The data on *hCDC4/FBXW7* mutations and the functional characterization of the mutant allele are integrated into a manuscript soon to be submitted to Cancer Research. The protein array experiments and experiments of siRNA-mediated knock-down of *mCDC4/FBXW7* in mouse prostates are still ongoing, but will undoubtedly yield a research publication in the future.

## Conclusions

We have discovered that the *hCDC4/FBXW7* gene functions as a tumor suppressor in prostate cancer. *hCDC4/FBXW7* inactivation/cyclin E1 deregulation may be a major cause of genetic instability and androgen-independent proliferation of prostate tumor cells. We have completed most of the goals outlined in the original SOW and have refined our studies in one case to integrate novel technologies into our research. Some goals in Aim2 have not been completed due to personal reasons of the PI (Audrey van Drogen). During the first year of the fellowship grant, Dr. van Drogen gave birth to a child who was born with a rare genetic defect. The handicapped nature of the child made it difficult to acquire childcare which made it difficult to complete her studies to the fullest degree. Because of the situation, she was forced to return to France prematurely and SKCC returned the funds allocated for salary over this time period. Studies that Dr. van Drogen started will be completed in the coming months by her sponsor, Dr. Charles H. Spruck, and others in the laboratory.

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## Appendices

N/A