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FOREWORD

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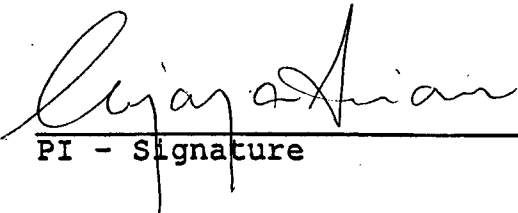
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TABLE OF CONTENTS:

Front Cover	1
SF298 Report Documentation Page	2
Foreword	3
Table of contents	4
Introduction	
Relevance and Nature of Problem	5
Background	5
Hypotheses to be Tested	8
Methods	8
Results	10
Conclusions and Recommended Changes	11
Bibliography	12
Addenda	16

INTRODUCTION

Relevance and Nature of Problem

One in 8 American women will develop breast cancer in her lifetime. Despite the advances in detection and treatment of breast cancer, the mortality from breast cancer has not changed significantly over the last forty years. Breast cancer treatments significantly include radiation and chemotherapy. These regimens are acutely limited by the lack of ability to specifically target tumor cells. The emotional state of the patient which could be a critical factor in combating the disease is gravely debilitated by the psychosomatic trauma of these severe treatment procedures. Even those patients that survive, face the possibility of remission and an uncertain future. Unlike the survival after many other cancers, which tend to level off after 5 years, survival after diagnosis of breast cancer continues to decline. Even women who try to make preventive life-style changes cannot alter the most significant risk factors like age or family history. In this grim scenario, a basic understanding of the cellular processes underlying breast cancer is mandated before effective therapies can be developed or even attempted.

β -catenin is a multifunctional protein that primarily helps link the cadherins (at the adherens junctions) to the cytoskeleton. However, β -catenin is also a crucial signaling molecule that participates in differentiation and proliferation pathways. The *wnt* signaling pathway, known to reverse contact inhibition in mouse mammary cells *in vitro* and to cause mammary cancer in mice (7), results in increased levels of cytoplasmic β -catenin (8). *Wnt*-1 stimulation results in decreased activity of glycogen synthase kinase (GSK)-3 β , that normally phosphorylates the tumor suppressor adenomatous polyposis coli (APC) gene product (8,9). When APC is not phosphorylated, it leads to the stabilization of β -catenin. The stable β -catenin interacts with the transcriptional activators LEF/TCF (10). The β -catenin-TCF/LEF complex translocates to the nucleus and effects gene expression (1,2). The genes activated may include those that stimulate proliferation or antagonize apoptosis (11,12). And finally, stable forms of β -catenin by themselves are oncogenic (3,12,13). These observations strongly point towards the stability of cytoplasmic β -catenin as a “smoking gun” (12) linking cell adhesion and tumorigenesis. Thus, a strategy of down-regulating β -catenin could constitute a potential way of treating breast cancer.

In this study, we investigate the regulation of cytoplasmic β -catenin.

Background

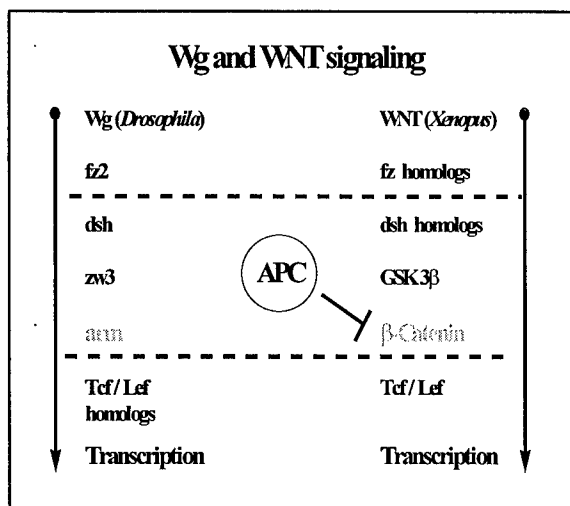
β - Catenin and breast cancer

Cells touch one another through a number of different surface molecules; among the most intriguing are the cadherins and their associated proteins (14). These proteins, in addition to maintaining adhesion of adult tissues, via the adherens junctions, are critical during development and tumorigenesis (15). Cadherin function has been shown to depend on several associated proteins, namely; α , β , and (plakoglobin) γ catenin (16). These molecules, link cadherins to the actin cytoskeleton and are probably involved in relaying cadherin-mediated-contact signals (17).

The β -catenin/cadherin association requires serine phosphorylation of the cadherin molecule (17). β -catenin is itself a substrate for tyrosine phosphorylation and can also act as a link between Growth factor receptors (such as the EGFR) and the adherens junction complex (18,19). Mutation of the β -catenin gene in mice, by homologous recombination, results in embryonal lethality. When the expression of E-cadherin and the catenins was analyzed in human breast carcinomas, lobular breast carcinomas showed disturbances of E-cadherin and catenins in a high frequency of cases (20). In ductal breast carcinomas (where E-cadherin is often unchanged), a high frequency of cases showed disturbance of alpha- and/or gamma-catenin expression. 50 % of cases with defects in E-cadherin and catenins had lymph node metastasis, whereas this number was low in cases with undisturbed cadherin/catenin expression (20).

A truncated stable form of β -catenin itself acts as an oncogene (9). The phosphorylation state of β -catenin can also influence the transformed phenotype (19,21). Further, cytoplasmic β -catenin associates with the tumor suppressor adenomatous polyposis coli (APC) gene product (19). Over-expression of APC results in the cell cycle being blocked at the G1/S boundary (19). Recent evidence indicating that the tumor suppressor effects of APC are dependent upon its ability to destabilize β -catenin, strongly argue the significance of β -catenin in the control of cell proliferation (5,22).

β -catenin is a signaling molecule



β -catenin participates in developmental patterning in *Xenopus* (23). Ectopic expression of β -catenin by mRNA injection into the ventral region of *Xenopus* embryos induces a secondary dorso-anterior body axis, giving rise to two heads, notochords, and neural tubes (24). Wnt-1, the vertebrate homologue of wingless is known to reverse contact inhibition in mouse mammary cells *in vitro* and to cause breast cancer in mice (24). Wnt-1 stimulation results in decreased activity of glycogen synthase kinase (GSK)-3 β , that normally phosphorylates the tumor suppressor adenomatous polyposis coli (APC) gene product (5,8). When APC is not

phosphorylated, it leads to the stabilization of β -catenin through an unknown mechanism. Now, β -catenin interacts with transcriptional activators LEF/TCF, translocates to the nucleus, and effects gene expression (10,25). The genes activated may include those that stimulate proliferation or antagonize apoptosis.

Taken together, these finding strongly argue the significance of β -catenin and its cytoplasmic levels in the integration of adhesion, differentiation and proliferation pathways. A clearer understanding of this crucial signaling pathway holds tremendous potential to offer realistic strategies to combat breast cancer.

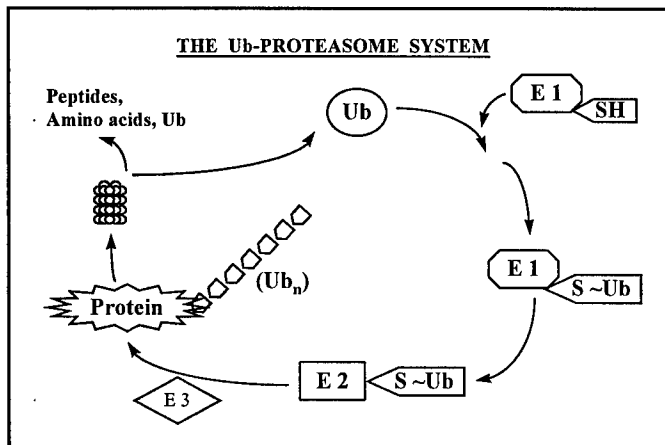
β -catenin stability and APC

The Adenomatous polyposis Coli (APC) gene is a tumor suppressor, found mutated in most human colon cancers. APC directly binds β -catenin (26). APC is a part of the *wnt* signaling pathway, and when phosphorylated by GSK3 β , down-regulates β -catenin levels. Cancer cells with mutant APC contain abnormally high levels of cytoplasmic β -catenin (4,5). Over-expression of APC blocks progression of the cell cycle from G₀ to the S phase (27). This observation suggests that loss APC activity (resulting in β -catenin stabilization) could lead to uncontrolled cellular proliferation. Indeed, the *wnt* signal, thought to inactivate APC, can cause cell proliferation in certain tissues. Although APC has been primarily studied in colon cancer, there is strong evidence that loss of heterozygosity at the APC locus may be involved in mammary tumors in humans. The Multiple intestinal neoplasia (Min) allele is a mutant allele of the murine APC locus. Min-/+ mice are predisposed not only to intestinal but mammary carcinoma as well (7). Among Min-/+ mice exposed to carcinogenic material, over 75% developed mammary tumors, while Min +/+ mice displayed no evidence of mammary tumors (7). These observations suggest that APC, by virtue of its ability to regulate β -catenin, can play an important role in predisposing breast tissue for further hyperplastic events (11).

The ubiquitin-proteasome pathway

Our preliminary evidence demonstrates that the cytoplasmic, “signaling pool” of β -catenin is regulated at the level of stability by the ubiquitin-proteasome pathway.

The ubiquitin-proteasome pathway is involved in the processing and rapid degradation of many short-lived regulatory proteins. Mitotic cyclins, cyclin-dependent kinase inhibitors, the tumor suppressor p53, transcriptional activators NF- κ B, v-jun, and v-fos are examples of proteins that are degraded by this highly specific pathway (28-31).



The ubiquitin pathway effects the degradation of proteins in two steps (28). First, multiple ubiquitin moieties are covalently attached to a target protein. Second, the multi-ubiquitinated protein is degraded by the 26S proteasome complex. Conjugation of ubiquitin moieties, to a substrate, is performed in a three-step process. Following activation of the C-terminal glycine of ubiquitin by enzyme E1, one of several E2 enzymes transfers the activated ubiquitin to the

substrate that is specifically bound to an enzyme E3.

E3 catalyzes the formation of an isopeptide bond between the activated glycine on the ubiquitin and ϵ -NH₂ group of a lysine residue in the substrate (or in the previously conjugated ubiquitin moiety). The E2 and E3 enzymes bind the substrate, and help transfer the ubiquitin moieties. There are dozens of genes, unrelated to each other, that encode E2 and E3 enzymes. The specificity of the ubiquitin pathway is thought to reside in the E3 enzymes (28). Following

targeting (e.g. phosphorylation, as in the case of NF- κ B) and multi-ubiquitination, the substrate protein is rapidly degraded by a large multi-subunit structure called the proteasome.

PURPOSE

The general aim of this investigation is to study the regulation of cytoplasmic β -catenin stability, and the involvement of the tumor suppressor APC in this process.

Our **working model** is that β -catenin is recruited by a cadherin to the plasma membrane where it is phosphorylated on serine residues (analogous to I κ b). This phosphorylated β -catenin can either be recruited to an adhesion complex or can interact with APC via its armadillo domains. In the presence of active GSK3 β , APC is phosphorylated and its E3 activity is activated. β -catenin is also phosphorylated by GSK3 β . In response to these events, β -catenin is ubiquitinated and degraded by the proteasome. When APC or β -catenin or both are mutated, the result is accumulation of cytoplasmic β -catenin. At elevated levels, cytoplasmic β -catenin is oncogenic.

SPECIFIC AIMS (Years 1-2)

Year 1 (presented in annual report - 1996):

Aim 1. To test the hypothesis that cytoplasmic β -catenin is regulated at the level of stability by the ubiquitin-proteasome pathway.

Aim 2. To establish an *in vitro* cell-free model to study β -catenin ubiquitination and degradation

Year 2:

Aim 3. To test the hypothesis that the tumor suppressor adenomatous polyposis coli (APC) is involved in the ubiquitin-proteasome pathway that regulates β -catenin stability (Collaborator: Dr. Paul Polakis)

Aim 4. To test the hypothesis that the APC gene product is an ubiquitin-ligase (E3) that ubiquitinates β -catenin and targets it for degradation by the proteasome (Collaborators: Dr. Aaron Ciechanover, Dr. Allan Weissman, Dr. Richter King)

METHODS

Aim 1. To test the hypothesis that cytoplasmic β -catenin is regulated at the level of stability by the ubiquitin-proteasome pathway.

- The effect of proteasomal inhibitors on cytoplasmic β -catenin stability was tested. The breast cancer cell line SKBR3 (APC+/+) and the colon cancer cell line SW480 (APC-/-) were used in this experiment. Cells were treated with the proteasomal inhibitors (peptidyl aldehydes) ALLN and Lactacystin (32) for 12 hr. Cells were lysed in a hypotonic lysis buffer and dounce homogenized, clarified in a ultracentrifuge (100,000g for 1 hr) to yield the S100 cytoplasmic fraction (free of membranous components). To obtain cytoplasmic fractions including membrane vesicles, the dounced lysate were clarified in a table-top microfuge (10,000g for 10 min).
- The half-life of β -catenin in E36ts20 cells (33), that harbor a temperature sensitive E1 enzyme, was monitored at permissive and non-permissive temperatures

- SKBR3 cells were transiently transfected with His₆-tagged ubiquitin (34) and HA-tagged β -catenin. Cells were treated with/without proteasomal inhibitors, ubiquitinated proteins were purified with Ni-NTA columns (34), and Western blotted with anti-HA antibody (helps distinguish from native β -catenin).

Aim 2. To establish an *in vitro* cell-free model to study β -catenin ubiquitination and degradation.

In vitro ubiquitination and degradation assays will be established according to published protocols (29,35,36).

- Recombinant β -catenin was generated in a combined *in vitro* transcription-translation system (Promega). mRNA synthesized from 2 μ g of template DNA was used in a 100 μ l translation reaction mixture containing 50 μ l of rabbit reticulocyte lysate (RRL).
- Conjugation assays (29,35) are performed essentially as described by Dr. Ciechanover (29,30,32). Briefly, the reaction mixture (30 μ l) consisted of 1 μ l of either programmed RRL containing the HA-tagged β catenin or unprogrammed RRL, 10 μ l SKBR3 hypotonic lysate (5-6 mg/ml) or, 20 ng E1, 20 ng E2 (UbcH5b) and, either 40 ng E3 (E6-AP)(recombinant enzymes were kindly provided by Dr. Allan Weissman) or 40 ng of various APC deletion constructs. 5 μ g ubiquitin, 40 mM Tris HCl (pH 7.6), 5 mM MgCl₂, 2mM DTT, 0.5 μ g of ubiquitin aldehyde (kindly provided by Dr. Keith Wilkinson), 5mM ATP γ S, 10mM Phosphocreatine, and 5 units Phosphocreatine Kinase, were also included in the reaction mixture. In Assays without ATP γ S, 20 mM EDTA, 0.5 μ g of hexokinase and 10 mM 2-deoxyglucose substituted for ATP γ S. Ubiquitination assays were performed for a 1 hr period at 25°C. Degradation assays included ATP (instead of ATP γ S, which cannot be used by the proteasome but can be utilized by the ubiquitination enzymes), and were performed at 37°C for 2 hr. Following incubation, reaction mixtures are resolved by Tris-glycine SDS-PAGE (4-12%).
- Western blotting was performed using anti-HA (BabCo) and anti- β -catenin antibodies (Transduction labs).

Aim 3. To test the hypothesis that the tumor suppressor adenomatous polyposis coli (APC) gene product is involved in the ubiquitin-proteasome pathway that regulates β -catenin.

- *In vitro* ubiquitination assay with SW480 (APC^{-/-}) cytoplasmic fraction and recombinant APC constructs with/without the HECT consensus.
- Test the effects of alkylating agents (alkylating agents like N-ethyl maleimide (NEM) block the free cysteine residues necessary for the thiol-ester transfer of ubiquitin) (36) on the ligase activity of recombinant APC
- Transient transfection of SW480 (APC^{-/-}) cells with APC (4), monitor cytoplasmic β -catenin levels in response to proteasomal inhibitors.

SW480 cells (APC^{-/-}) were co-transfected with a vector encoding His₆-tagged ubiquitin (34) and vector encoding APC 25 (4) (the smallest construct including the free cysteine consensus) or vector alone, using Lipofectamine™ (GIBCO) according to manufacturer's instructions.. 24 hr after transfection, the cells were treated with the proteasome-specific inhibitor, Lactacystin for

6hr. Ubiquitinated proteins were purified by Ni-NTA chromatography, and Western blotted with anti- β -catenin antibody

Aim 4. To test the hypothesis that the APC gene product is an ubiquitin-ligase (E3)

- *In vitro* reconstitution experiments (35-37) with recombinant β -catenin, E1, E2, and various APC constructs with/without the HECT consensus.

RESULTS

Aim 1. To test the hypothesis that cytoplasmic β -catenin is regulated at the level of stability by the ubiquitin-proteasome pathway.

1. Results indicate that treatment of SKBR3 cells with proteasomal inhibitors ALLN and Lactacystin (32) result in the accumulation of high-molecular weight, β -catenin-ubiquitin conjugates in the cytoplasm (Addenda; Fig. 1).
2. E36ts20 cells harboring a thermolabile Ubiquitin activating (E1) enzyme (33), when grown at the non-permissive temperature (39.5°C) accumulate β -catenin (half-life is extended, compared to cells grown at the permissive temperature; 30°C) (Fig. 2).
3. In a more direct approach, SKBR3 cells were co-transfected with a vector encoding His₆-tagged ubiquitin (34) and a vector encoding HA-tagged β -catenin (Fig. 3). 48 hr after transfection, the cells were treated with the proteasome specific inhibitor, Lactacystin, for 6 hr. Ubiquitinated proteins were purified by Ni-NTA chromatography (34) and Western blotted with anti-HA antibody (Fig. 4). β -catenin was found to accumulate as high- molecular weight ubiquitinated conjugates, in response to the proteasome-specific inhibitor Lactacystin. These observations demonstrate that cytoplasmic β -catenin is regulated at the level of stability by the ubiquitin-proteasome pathway.

Aim 2. To establish an *in vitro* cell-free model to study β -catenin ubiquitination and degradation.

In vitro ubiquitination and degradation assays were established according to published protocols (29,35,36) and with the help of our collaborators Drs. Aaron Ciechanover and Allan Weissman.

1. Cytosol that included membrane and/or particulate material was able to ubiquitinate β -catenin more efficiently than a S100 preparation that lacked them (Fig. 5).
2. Cytosol extracted from cells in different phases of the cell cycle, strikingly varied in their ability to ubiquitinate β -catenin (Fig. 6).
3. The most efficient ubiquitination activity was observed in the extracts from cells in M-phase (Fig. 6).

Aim 3. To test the hypothesis that the tumor suppressor adenomatous polyposis coli (APC) gene product is involved in the ubiquitin-proteasome pathway that regulates β -catenin.

1. Recombinant APC constructs help degrade β -catenin in SW480 (APC^{-/-}) cell lysate, *in vitro*. APC constructs 4 and 25 (Fig.8) include a free-cysteine-HECT consensus of ubiquitin ligases, whereas APC 3 does not. Lane 6; In the presence of a proteasomal inhibitor, a high molecular weight band is observed, which probably is a mono-ubiquitinated β -catenin (Fig.10).

2. Anti-APC antibodies and N-ethyl Maleimide (NEM) (4) inhibit the ubiquitination of β -catenin, *in vitro* (Fig. 9).

3. In SW480 cells transfected with APC (Fig. 11), β -catenin was found to be stabilized as ubiquitinated conjugates, in response to lactacystin, a specific proteasomal inhibitor. In cells transfected with vector alone, the proteasomal inhibitor had no effect. However, in Lane 6, the APC 3 transfection did not seem to have worked well in this particular experiment. Further experiments monitoring the effect of vectors including APC constructs that lack the HECT domain are currently in progress.

Aim 4. To test the hypothesis that the APC gene product is an ubiquitin-ligase (E3)

In vitro reconstitution experiments with recombinant β -catenin, E1, E2, and various recombinant APC fragments (with/without the HECT consensus) have proved to be elusive and efforts to standardize reaction conditions continue with the help of Dr. Allan Weissman (NIICB, NIH).

Conclusions

1. Cytoplasmic β -catenin is regulated at the level of stability by the ubiquitin-proteasome pathway.
2. *In vitro*, cytosol that included membrane and/or particulate material ubiquitinated β -catenin more efficiently than a S100 preparation that lacked them.
3. Cytoplasmic extracts from M-phase cells displayed maximal ability to ubiquitinate β -catenin, *in vitro*.
4. Anti-APC antibodies and N-ethyl Maleimide (NEM) inhibit the ubiquitination of β -catenin, *in vitro*
5. Recombinant APC constructs help degrade β -catenin in SW480 (APC^{-/-}) cell lysate, *in vitro*.
6. In SW480 cells transfected with APC, β -catenin is stabilized as ubiquitinated conjugates, in response to a proteasome specific inhibitor.

Recommended Changes

The above observations lead to the question, "Do changes in physiologically relevant signaling, i.e. GSK3 β activity, lead to changes in the ubiquitin-proteasomal turnover of β -catenin?"

We hope to partially answer this question *in vivo*, by studying changes in β -catenin turnover in cells treated with inhibitors of GSK3 β activity, like Rapamycin and Wortmannin. Further analysis of GSK3 β activity, its effect on APC phosphorylation, and subsequent effects on β -catenin ubiquitination and stability will be addressed using the *in vitro* assays described below. For these experiments, pre-phosphorylated APC, de-phosphorylated APC, with/without purified GSK3 β , and GSK3 β inhibitory peptides will be used (these reagents were kindly provided by our collaborator Dr. Paul Polakis).

Preliminary studies on the cell cycle dependent fluctuations of β -catenin, APC, and GSK3 β are currently in progress.

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ADDENDA

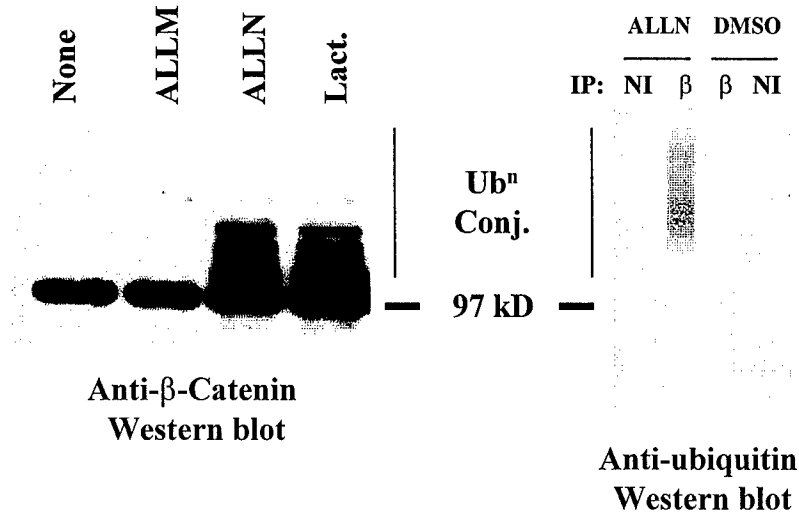
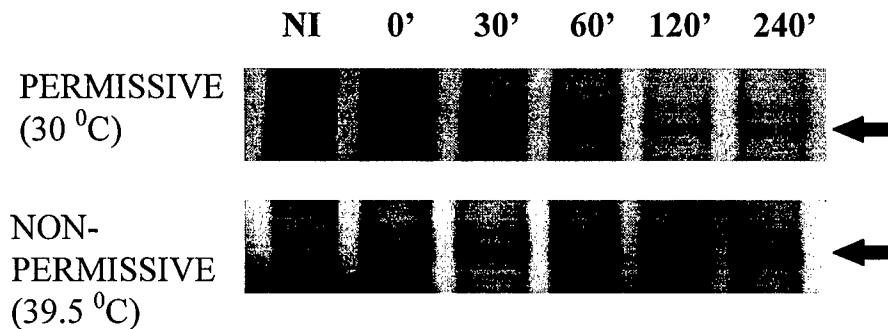


Figure 1. β-catenin accumulates as high- molecular weight ubiquitinated conjugates, in response to the proteasomal inhibitor ALLN and Lactacystin. ALLM is the negative control.

In the right panel, β-catenin was immunoprecipitated with a C-terminal monoclonal antibody from a NP-40 lysate, and Western blotted with anti-ubiquitin antibody. NI: Non-Immune, β: β-catenin.



AUTORADIOGRAPH OF IMMUNOPRECIPITATED [³⁵S] β CATENIN

Figure 2. Pulse chase in E36ts20 cells harboring a thermolabile Ubiquitin activating (E1) enzyme. When grown at the non-permissive temperature, the half-life of β-catenin is extended.

EXPERIMENTAL DESIGN :

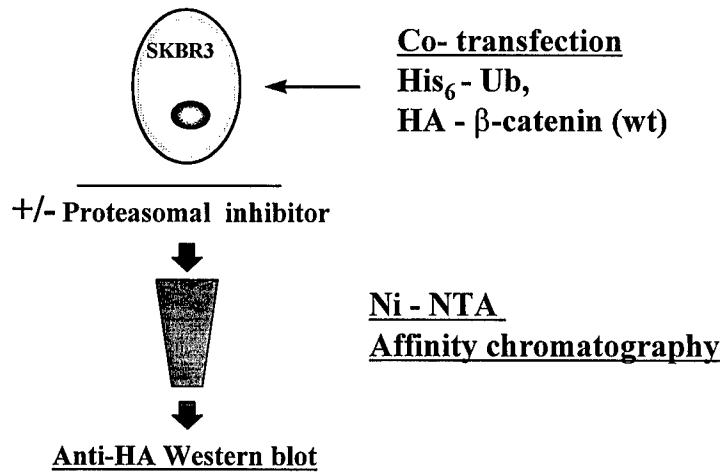


Figure 3. Experimental design to test the hypothesis that cytoplasmic β -catenin is regulated at the level of stability by the ubiquitin-proteasome pathway.

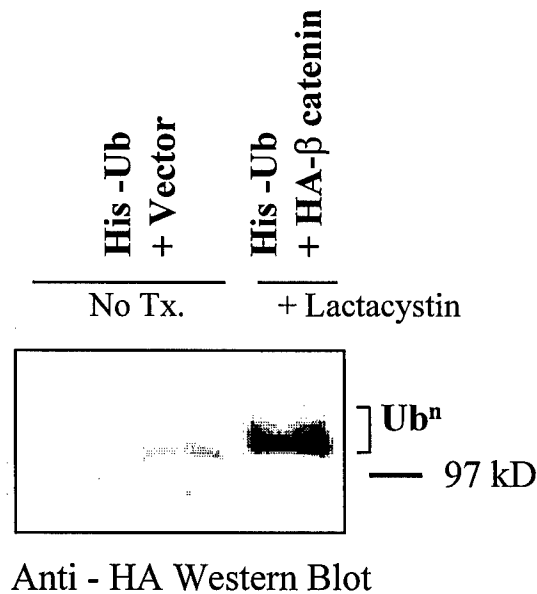


Figure 4. β -catenin accumulates as high- molecular weight ubiquitinated conjugates, in response to the proteasomal inhibitor Lactacystin.

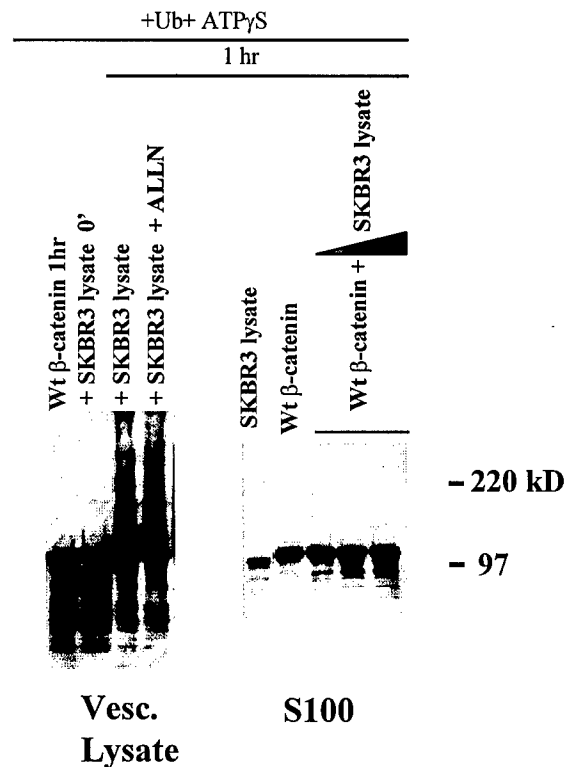


Figure 5. *In vitro* β -catenin ubiquitination assay. Cytosol that included membrane material (vesicular lysate) ubiquitinated β -catenin more efficiently than a S100 preparation that lacked them.

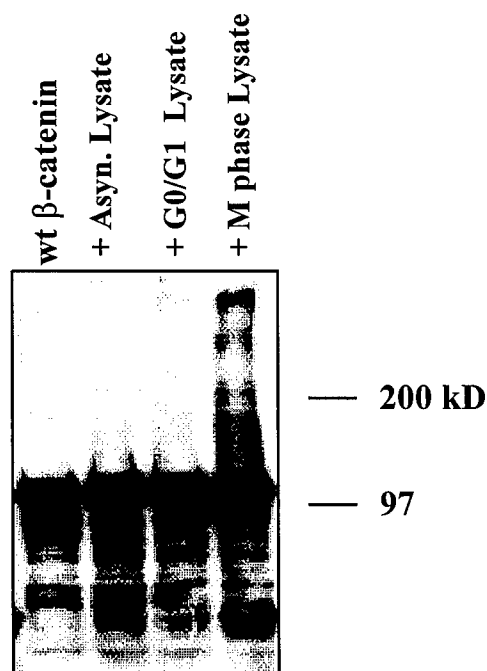


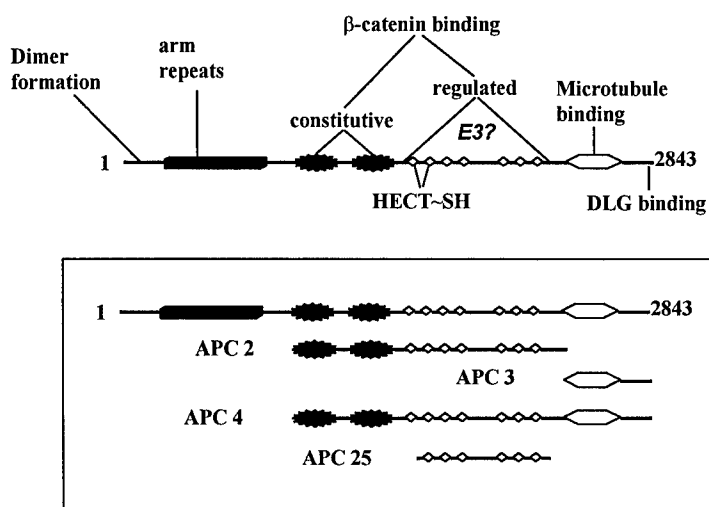
Figure 6. *In vitro* β -catenin ubiquitination assay. Cytosol extracted from cells in different phases of the cell cycle, strikingly varied in their ability to ubiquitinate β -catenin. The most efficient ubiquitination activity was observed in the extracts from cells in M-phase.

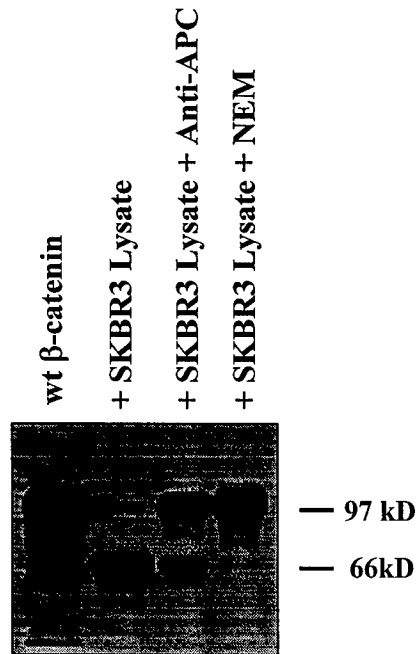
The second β -catenin binding repeat of APC contains a free cysteine consensus region present in the HECT family ubiquitin ligases

	~ SH
	L P L ▼ V V Y
HECT domain	P L X X X X C X X X S X L X X F
Human APC	P L X X X X C X X X S X L X X F
	1387
Mouse APC	P L X X X X C X X X S X L X X F
Xenopus APC	P L X X X X C X X X S X L X X F

Figure 8.

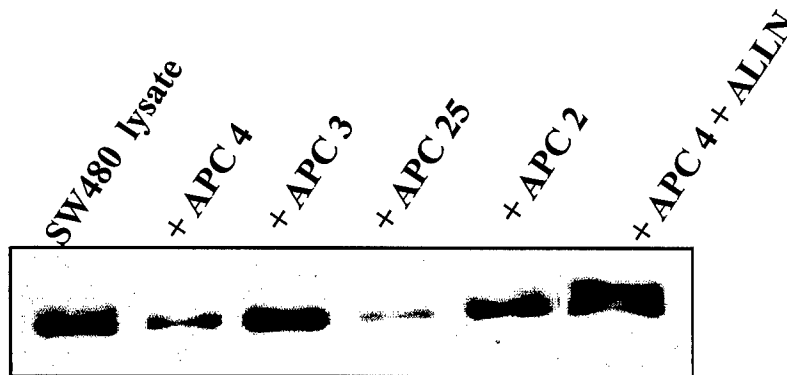
APC Functional Domains and Constructs





Anti- β-Catenin Western blot

Figure 9. *In vitro* degradation assay. Anti-APC antibodies and N-ethyl maleimide (NEM) inhibit the *in vitro* degradation β-catenin



Anti β-catenin Western blot

Figure 10. *In vitro* β-catenin ubiquitination assay. Recombinant APC constructs help degrade β-catenin in SW480 (APC^{-/-}) cell lysate, *in vitro*. Constructs 4, 25, and 2 include a free-cysteine-HECT consensus of ubiquitin ligases. APC 3 does not. Lane 6; In the presence of a proteasomal inhibitor, a high molecular weight band is observed, which probably is a mono-ubiquitinated β-catenin

EXPERIMENTAL DESIGN :

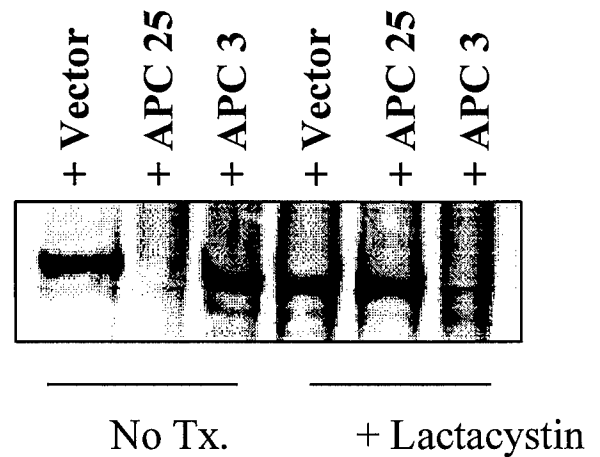
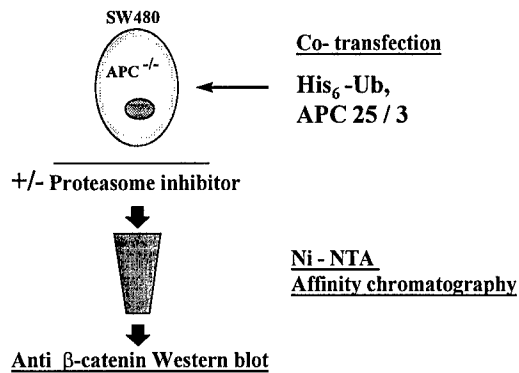


Figure 11. SW480 cells (APC^{-/-}) were co-transfected with a vector encoding His₆-tagged ubiquitin and a vector encoding APC 25 (the smallest construct including the free cysteine consensus) , APC 3, or vector alone. 24 hr after transfection, cells were treated with the proteasome-specific inhibitor, Lactacystin for 6 hr. Ubiquitinated proteins were purified by Ni-NTA chromatography, and Western blotted with anti- β -catenin antibody. In cells transfected with APC 25, β -catenin was found to be stabilized as ubiquitinated conjugates, in response to a specific proteasomal inhibitor. In cells transfected with vector alone, the proteasomal inhibitor had no effect probably because the native ubiquitination machinery is not functional. Lane 6; the APC 3 transfection did not seem to have worked well in this particular experiment. Experiments monitoring the effect of vectors including APC constructs that lack the HECT domain are currently in progress.