REGULATION OF THE TRANSCRIPTION FACTOR p63 BY βTRCP

by

Jayme Renée Gallegos

A DISSERTATION

Presented to the Department of Biochemistry and Molecular Biology

and the Oregon Health & Science University

School of Medicine

in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

January 2008

School of Medicine
Oregon Health & Science University

CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. thesis of

Jayme Renée Gallegos

has been approved

_____

Professor in charge of thesis – Dr. Hua Lu

_____

Member – Dr. Peter Rotwein

_____

Member – Dr. Ujwal Shinde

_____

Member – Dr. Molly Kulesz-Martin

_____

Member – Dr. Xiao-Jing Wang

_____

Member – Dr. Charles Lopez

DEDICATION

This dissertation and my academic career are dedicated to my grandparents, Lloyd Mancebo, Ruby Louise Mancebo, and Frank Riley and Anita Riley.

To Grandpa Mancebo for starting me on science, answering millions of “why”s, and teaching me how a car engine worked when I was six without stopping to question whether I would understand.

To Grandma Mancebo for being the backbone of our family, listening *always*, and still being the wisest woman I know.

To Grandma and Grandpa Riley for making my childhood happy, inspiring me to become a cancer biologist, and constantly enabling me to keep my work in perspective.

TABLE OF CONTENTS

List of Figures and Tables ……. iii

Table of Abbreviations …… v

Acknowledgements …… vii

Abstract ……. x

Chapter 1: Introduction ……. 1-44
- 1.1 A brief history of p63 as a member of the p53 tumor suppressor family
- 1.2 p63's gene and protein architecture
- 1.3 p63's biological functions – roles in development and trends in cancer
- 1.4 p63's transcriptional control - links to p63's stability and post-translational modification
- 1.5 Tales of Two Murine Phenotypes: Potential links between IKK/βTrCP and p63
- 1.6 The Versatile Hierarchy of Ubiquitylation and the SCF complex
- 1.7 βTrCP's gene and protein architecture dictate its substrate recognition
- 1.8 βTrCP's history: the mystery partner to critical cell regulatory proteins
- 1.9 βTrCP1's biological functions

Chapter 2: Materials and Methods ……. 45-58
- 2.1 Cell culture
- 2.2 Antibodies and vectors
- 2.3 Transient transfection/siRNA transfection
- 2.4 Preparation of purified his-TAp63γ, and GST-Flag-βTrCP1
- 2.5 Western blot analysis (WB)
- 2.6 *In vitro* binding
- 2.7 Immunoprecipitation (IP)
- 2.8 Stability assays
- 2.9 Semi-quantitative and quantitative (real-time) PCR
- 2.10 Flow cytometry
- 2.11 Chromatin Immunoprecipitation (ChIP)
- 2.12 Preparation of Roc1-SCF$^{\beta TrCP1}$ Complex
- 2.13 Preparation of Roc2-SCF$^{\beta TrCP1}$ Complex (Appendix A)
- 2.14 *In vitro* ubiquitylation assays
- 2.15 *In vivo* ubiquitylation assays

Chapter 3: Activation of p63 by βTrCP1 ……59-84
- 3.1 βTrCP1 expression stabilizes TAp63γ
- 3.2 p63 binds βTrCP1
- 3.3 βTrCP1 binds TAp63γ's N- and C-termini
- 3.4 TAp63γ upregulation of p21, but not Bax, is amplified by βTrCP1 expression

3.5 TAp63γ's binding at the *p21* promoter increases in the presence of βTrCP1 in cells
3.6 TAp63γ's activation of growth arrest is increased by βTrCP1 expression

Chapter 4: Ubiquitylation of p63 by βTrCP1 activates p63 …….. 85-107
4.1 TAp63γ is ubiquitylated by $SCF^{\beta TrCP1}$
4.2 Characterization of TAp63γ's ubiquitylation by $SCF^{\beta TrCP1}$
4.3 TAp63γ's activation by βTrCP1 is mediated by its ubiquitylation

Chapter 5: Conclusions, Discussion, and Future Directions …… 108-132
5.1 Ubiquitin as an activating signal
5.2 p63 isoform specificity
5.3 The function of a second *p21* promoter binding region
5.4 Potential *in vivo* effects – upstream signals and relationships to other pathways

Summary …….. 133

References …… 135
Appendix A: TAp63γ is also ubiquitylated *in vitro* by a $SCF^{\beta TrCP1}$ complex containing Roc2 …… 148

Appendix B: Hyperacetylation and hyperphosphorylation induced by trichostatin A And okadaic acid inhibits the ubiquitylation of TAp63γ ……. 150

LIST OF FIGURES AND TABLES

Chapter 1: Introduction
1.1 – p63's gene and protein architecture
1.2 – p63's binding proteins and post-translational modifications
1.3 – Ubiquitylation is a hierarchical modification
1.4 – βTrCP1 gene and protein architecture
1.5 –$SCF^{\beta TrCP1}$ architecture

Table 1 – p63 binding proteins and modifications in detail
Table 2 – βTrCP1 interacting proteins

Chapter 2: Materials and Methods
2.1 – Annotated TAp63γ sequence used for cloning
2.2 – $SCF^{\beta TrCP1}$ purification

Chapter 3: Activation of p63 by $SCF^{\beta TrCP1}$
3.1 – The steady-state level of TAp63γ is reduced in *βTrCP1*$^{-/-}$ MEFs
3.2 – The exogenous steady-state level of TAp63γ is increased when co-expressed with βTrCP1
3.3 – βTrCP1 expression stabilizes exogenous TAp63γ
3.4 – βTrCP1 levels are reduced by βTrCP1/2 siRNA
3.5 – Endogenous p63 is destabilized by βTrCP1/2 knockdown
3.6 – TAp63γ and ΔNp63γ bind βTrCP1 in cells
3.7 – TAp63γ binds βTrCP1 *in vitro*
3.8 – TAp63α and ΔNp63α bind βTrCP1 in cells
3.9 – ΔNp63α and TAp63γ, but not TAp63β bind βTrCP1 endogenously
3.10 – Schematic of the TAp63γ truncation mutants
3.11 – βTrCP1 binds the N and C termini of TAp63γ *in vitro*
3.12 – TAp63γ's activation of *p21*, but not *Bax*, is increased by βTrCP1 expression at the mRNA level
3.13 - TAp63γ's activation of p21, but not Bax, is increased by βTrCP1 expression at the protein level
3.14 – βTrCP1 co-expression increases the amount of TAp63γ bound to chromatin *in vivo*
3.15 - Schematic of the *p21* promoter
3.16 - TAp63γ binding at the *p21* promoter increases in the presence of βTrCP1 in cells
3.17 - TAp63γ's activation of growth arrest is increased by βTrCP1 expression

Chapter 4: Ubiquitylation of p63 by βTrCP1 activates p63
4.1 – ΔNp63 is ubiquitylated less than TAp63 in cells
4.2 - $SCF^{\beta TrCP1}$ ubiquitylates TAp63γ *in vitro*
4.3 - $SCF^{\beta TrCP1}$, but not ΔFβTrCP1, ubiquitylates TAp63γ in cells
4.4 - $SCF^{\beta TrCP1}$ ubiquitylates the N-terminal of TAp63γ *in vitro*
4.5 - The ubiquitylation of TAp63γ by $SCF^{\beta TrCP1}$ is through a K48 ubiquitin linkage

4.6 - ΔFβTrCP1 co-expression does not stabilize TAp63γ
4.7 - ΔFβTrCP1 co-expression does not upregulate *p21*
4.8 - TAp63γ binding to the *p21* promoter decreases in the presence of ΔFβTrCP1
4.9 - Schematic of TAp63γ's putative canonical βTrCP1 binding domain and mutations
4.10 - The TAp63γD61R and TAp63γS62A mutants bind βTrCP1 with similar affinity to TAp63γ in cells
4.11 - The TAp63γD61R and TAp63γS62A mutants show striking ubiquitylation differences from TAp63γ in cells
4.12 - The TAp63γD61R and TAp63γS62A mutants show altered stability that is greater than TAp63γ's stability in cells
4.13 - The TAp63γD61R and TAp63γS62A mutants activate *p21* similarly to TAp63γ when co-expressed with βTrCP1

Chapter 5: Conclusions, Discussion and Future Directions
5.1 – Model for the ubiquitylation and activation of TAp63γ by $SCF^{\beta TrCP1}$

Appendix A: TAp63γ is also ubiquitylated *in vitro* by a $SCF^{\beta TrCP1}$ complex containing Roc2

Appendix II: Hyperacetylation and hyperphosphorylation induced by trichostatin A and okadaic acid inhibits the ubiquitylation of TAp63γ

TABLE OF ABBREVIATIONS

General terminology

ADULT	Acro-dermato-ungual-lacrimal-tooth
AEC	Ankyloblepharon ectodermal dysplasia clefting (Hay-Wells)
APC/C	Anaphase-Promoting Complex/Cyclosome
ASPP	Apoptosis stimulating proteins of p53
C-	carboxy-terminus
Cul	cullin
D domain	Dimerization domain
Dlg	Disks large tumor suppressor (hDlg is human variant)
DBD	DNA binding domain
DNA	Deoxyribonucleic Acid
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating/carrier enzyme
E3	Ubiquitin-ligase
EEC	Ectrodactyly ectodermal dysplasia clefting
Emi1	Early mitotic inhibitor 1
HOS	Homolog of Slimb (another name for βTrCP2)
GSK3β	Glycogen Synthase Kinase 3β
IκB	Inhibitor of NF-κB
IKK	IκB kinase
LMS	Limb-mammary syndrome
MDM2 (HDM2)	Mouse Double Minute 2 (HDM2 is human variant)
N-	amino-terminus
NBP	Non-p53RE p53RE-binding protein
NF-κB	Nuclear factor κB
NES	Nuclear export sequence
NLS	Nuclear localization sequence
OD	Oligomerization domain
PBS	Phosphate buffered saline
PP2A	Protein phosphatase 2A
PRD	Proline-rich domain
RE	Response element
RING	Really interesting new gene, class of E3s
Roc1/RBX1	RING-finger protein component of SCF
Rub/NEDD	Related to ubiquitin (yeast/metazoans)
SAM	Sterile alpha motif
SCF	Skp1-Cullin1-F-box complex
SHFM	Split-hand/foot malformations
SSRP1	Structure specific recognition protein 1
SUMO	Small ubiquitin-related modifier
TA	Transactivation domain
TAC	Transient amplifying cells of the epidermis
βTrCP	β-Transducin repeats containing protein
UBL	Ubiquitin-like protein

UTR	Untranslated region
Vpu	Human immunodeficiency virus type-1 protein

Methods

BSA	Bovine serum albumin
ChIP	Chromatin immunoprecipitation
DAI	DNA affinity immunoblotting
DMEM	Dulbecco's modified eagle medium
EMSA	Electrophoretic mobility shift assay
FBS	Fetal bovine serum
GST	Glutathione-S-transferase
His	6x histidine tag
IgG	Immunoglobulin type G
IP	Immunoprecipitation
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PI	Propidium iodide
qPCR	Quantitative (real-time) PCR
qRT-PCR	Quantitative (real-time) reverse transcriptase PCR
RT-PCR	Reverse transcriptase PCR
SDS	Sodium dodecyl sulfate
TNFα	Tumor necrosis factor α
UV	Ultraviolet radiation (UVA, UVB, UVC)
WB	Western blot

ACKNOWLEDGEMENTS

My time as a graduate student would not have come to fruition without the support of several important people. I would like to thank my advisor, Dr. Hua Lu, for allowing me to tackle this project, providing a wonderful laboratory in which to work, and teaching me to appreciate science as a practice whose limits can always be tested. Under his guidance, I have learned an extraordinary amount about myself, both as a scientist and a person (and even a few words in Chinese to boot). I would also like to thank the members of my research advisory committee Drs. Peter Rotwein, Ujwal Shinde, Molly Kulesz-Martin, Xiao-Jing Wang, and Charles Lopez for all of their input and positive encouragement along the way. I would also like to give special thanks to Molly and the Kulesz-Martin laboratory for "adopting me" in my last months and for providing a positive and productive environment in which to finish my experiments and write.

Also, I would like to thank our collaborators, Drs. Keiko and Keiichi Nakayama for kindly giving us the βTrCP1 mouse cell lines, and Dr. Yi Sun for generously providing the βTrCP1 and ΔFβTrCP1 vectors, as well as the SCF baculoviral vectors. I would also like to thank the various members present and past of the Lu lab for their support, reagents, discussion, fun, and other assistance. Thank you to Shelya Zeng for keeping us all in order and bringing good treats to keep us pipetting, to Dave Keller for helping me begin in the laboratory and the invaluable input since (especially with ChIP), to Mushui Dai for teaching me FACS, proofing my paper and hundreds of other questions, to Igor Landais for teaching me how to use the qPCR machine and good coffee, to Anuhudra Kumari for advice and spirited discussion (scientific and not), listening to my practice talks and great Indian food, to Hunjoo Lee for cloning many of the vectors

for this project, Korean barbeque and party coordination, and to Joel Litersky for beginning this project with me. Also, I would like to thank Xiaoxin Sun for being my bay mate and always being good for a bright smile, and Yanping Li for being my BMB classmate, helping me with qPCR, teaching me ChIP, and for a wry sense of humor on tough days. Also, I would like to give special thanks to Mary Mac Partlin and Yetao Jin. Mary has helped me in countless ways, was my p63 compatriot in the lab, my bay-mate, looked at nearly every major assignment both for class and the lab, listened to nearly all of my talks and has been a wonderful friend. Yetao was my bay-mate and lab "big brother" for the better part of five years; he taught me everything I ever wanted to know about ubiquitylation assays, protein purification and a million other tiny things, helped me with my physical chemistry homework, proofread this entire thesis, chose the conge for my tattoo and is one of the most positive people I know.

I would also like to thank my family, especially my mom and dad, and my friends and classmates for their wonderful support and encouragement. If I expounded on all the ways you have all helped me, I would have a document larger than this dissertation. I would like to particularly like to thank my mom for proofreading my primary publication and also portions of my grant applications, and my dad for checking in, even if it meant being woken up when I came home after 11pm. Particularly, I would like to thank my dearest friends George and Jennifer Niemann, Grace Van Ness and Melissa Noeth for venting at the hard times, laughing at the good times, beer and being my Portland family and main support system. Also, I would like to thank Jodi Johnson for proofreading this thesis, being a fellow p53-family enthusiast, my roomy for the p53 Workshop, a writing buddy, and a great example as a senior graduate student, Janna Mundt for quite literally

putting a pipette in my hand for the first time, convincing me to come to Portland, Mountain Dew and the *Dark Tower*, Lara Shamieh for helping me learn the ropes, her old study guides and violets, and all of them for being good friends. I would also like to thank Mihail Iordanov for not only providing the keratinocytes for this project and also being a great scientific sounding board, but being a good friend as well and always being game for Japanese food. I would like to thank Lisa Shu for proofreading this document, wonderful ubiquitin discussions, and all the Taekwon Do and Wii. I would also like to thank my Taekwon Do teachers and friends, John Shu and Amanda Matsuda for serenity and strength. What they have taught me transcends all aspects of my life including my scientific practice.

Lastly and most greatly, I would like to thank my husband, Anthony Gallegos, for looking at me unflappably when I announced to him at 17 that if he wanted to stay with me than he would have not only follow me to Tucson, but also to graduate school. Words can not begin to describe how much he has supported me in this endeavor, or how much cooking and laundry he has done so that I can have more time to write this dissertation. It is insufficient to say that without his love and support these last years would not have happened in quite the same way.

ABSTRACT

p63 is a member of the p53 tumor suppressor family that is critical for epithelial differentiation and cancer progression. Unlike p53, p63 is not a classical tumor suppressor because of the various roles of its isoforms. The balance of these isoforms is critical to cell homeostasis and perturbation of this balance is a hallmark of cancer. Currently, the molecular mechanisms regulating p63's function remain largely unclear. Therefore, studies of p63's signaling pathways and post-translational modifications are important to discerning the mechanisms governing p63's role and that of the larger p53 family in cellular homeostasis. This study is one of a few which examine a specific protein governing p63's post-translational modification, identifying $SCF^{\beta TrCP1}$ as an E3 ubiquitin ligase for p63. My study begins with the striking observation that βTrCP1 raises the steady-state levels of endogenous TAp63γ, but not TAp63β. These data are corroborated by studies demonstrating that the protein half-life of TAp63γ increases with co-expression of βTrCP1 endogenously and exogenously. Then, I determined that this increased stabilization is in fact due to direct interaction of $SCF^{\beta TrCP1}$ with p63, as βTrCP1 binds the TAp63α, ΔNp63α, TAp63γ, and ΔNp63γ isoforms exogenously with a higher affinity for the TAp63γ isoform versus the ΔNp63γ isoform. Further, this interaction occurs endogenously, as βTrCP1 binds TAp63γ and ΔNp63α, but not TAp63β in keratinocytes. Further, βTrCP1 interacts with TAp63γ through regions on TAp63γ's N- and C- termini which may allow for differential regulation of the various isoforms according to our protein binding assays. Then, to study the functional outcome of this direct effect of βTrCP1 which increased p63's stabilization, I performed several assays

on downstream promoter function and also cell cycle regulation. For these functional assays, I chose to focus on TAp63γ, since it is the most transcriptionally active p63 isoform. I found that the stabilization of TAp63γ leads to upregulation of p21 at the mRNA and protein level that is associated with an enrichment of TAp63γ at the *p21* promoter at both canonical and a novel p63 binding sites. This overall increase of p21 causes an increase in G1/S phase cell cycle arrest.

Next, since $SCF^{\beta TrCP1}$ is an E3 ubiquitin ligase, I characterized the ubiquitylation of p63. My data showed that the TAp63 isoforms had a higher degree of global ubiquitylation than the ΔNp63 isoforms. Further, $SCF^{\beta TrCP1}$ ubiquitylated TAp63γ *in vitro* and in cells. Using *in vitro* ubiquitylation assays, I found that ubiquitylation of p63 occurred on p63's N-terminus region, and extended from TAp63γ through a K48 poly-ubiquitin linkage. This ubiquitylation was lost in the presence of ΔFβTrCP1, a mutant which can bind, but not ubiquitylate p63. In fact the stabilization and activation of TAp63γ by βTrCP1 is due to ubiquitylation, since TAp63γ was not stabilized, could not upregulate *p21*, and had significantly decreased binding at an established *p21* promoter site and a novel-p63 binding region when co-expressed with ΔFβTrCP1. Also supporting the effect of ubiquitylation on the stability of TAp63γ are data showing that two point mutants of a putative canonical βTrCP1 interaction motif in the N-terminal domain, while not displaying any difference in binding, displayed differing ubiquitylation and stabilization patterns compared with TAp63γ. Therefore, my study reveals $SCF^{\beta TrCP1}$ as an E3 ligase which activates TAp63γ through ubiquitylation, providing a new mechanism for differential activation of p63's isoforms in development and cancer.

CHAPTER ONE

INTRODUCTION

According to current statistics, one in every three people will develop cancer in their lifetime (54). Further, cancers account for 25% of all adult deaths, recently overtaking cardiovascular disease as the number one cause of mortality in the United States (54). On a yearly basis, 0.5% of the population is diagnosed with cancer (54). At the heart of this disease are accumulated genetic or genomic mutations which lead to a disruption of tumor suppressor genes and a deregulation of oncogenes. One of the major families of tumor suppressor proteins involved in cancer is the p53 family. p53, p63 and p73 are part of a family of genes known for their roles in cell growth regulation and tumor suppression (101). Each protein has both shared and individual roles in cellular homeostasis. Since they were cloned 10 years ago, research on p63 and p73 has shown that unlike p53, p63 and p73 are not classical Knudson tumor suppressor genes (72). Rather, p63 and p73 may have the capability to be tumor suppressors or oncogenes, depending on the balance of their isoforms which are the products of both alternate coding and splicing regions. Since p63 and p73 are genetically wild type in most cancers, a great portion of this isoform imbalance arises from deregulation of their protein stability (101). At the time I began this project, there were few post-translational modifications known for p73 and though the existence of ubiquitylation was known for p63, an E3 ubiquitin ligase had not been identified that could modify p63. Thus I set out to identify an ubiquitin ligase for p63 and determine its effect on p63's function. A clue for the identity of this ligase came from a comparison of the current literature which

showed that the *IKKα* (Inhibitor of Nuclear Factor-κB, or NF-κB) knockout mouse displayed a very similar phenotype to the *p63* knockout mouse. Thus I wondered whether p63, IKKα and the E3 ligase that often works in parallel with it, $SCF^{\beta TrCP1}$, might interconnect in cells. This dissertation describes a novel ubiquitin-mediated regulatory pathway for p63 showing that p63 is ubiquitylated and activated by $SCF^{\beta TrCP1}$. In order to set the stage for our work I will first describe p63 at the molecular level and its role in development and cancer, detail the similarities in the knockout models of *p63* and *IKKα* which hinted at a link to βTrCP1, and discuss why βTrCP1's role as an important growth regulatory gene makes it an apt candidate for p63's regulation at the molecular and biological level. I will then detail the discovery of the interaction with and the modification of p63 by βTrCP1, the effect of this pathway on cell growth arrest through the cyclin-dependent kinase inhibitor, $p21^{WAF1/Cip1}$ (hereafter called simply p21), and the dependence of this activation on the ubiquitin modification itself. Then, I will discuss the broader implications of this novel signaling pathway on the roles of both p63 and βTrCP1 in cell growth, development and tumorigenesis.

1.1 A brief history of p63 as a member of the p53 tumor suppressor family

p63 (p51A/B, NBP, p40, $p63^{AIS}$) is a member of the p53 tumor suppressor family. This family includes p53, p63, and p73 - homologs characterized for their roles as transcription activators critical for cell growth control. p53 gained historical status as the "guardian of the genome" because of its importance in cancer as illustrated by its transcriptional activation of genes involved in growth arrest, cell senescence, and apoptosis in response to cell stress. It is estimated that 50% of all cancers have a mutation in p53, and the other ~45% have a mutation in a connected regulatory pathway

which compromises the function of p53 (101). Thus the finding of p63 and p73 generated much excitement in the field. p63 and p73 were cloned in 1997 by several groups (61, 112, 133, 148, 167). Our laboratory later demonstrated that p63 was NBP (non-p53RE p53RE-binding protein) (174). Now after a decade of study, it is clear that p63 and p73, while sharing some common functions and targets with p53, also have individual functions in development and cancer. In fact, p63 and p73 are evolutionarily older than p53 and are thought to have evolved from a common p63/p73 archetype (61, 167, 169). Currently, most of what is known about p63 and p73 is based on similarities to p53, such as shared target genes, binding proteins, and hotspot mutations. There is currently a push in p53 family research to determine more of the unique signaling pathways for each family member. When I began this study, little was known about p63's individual role in signaling or its post-translational modification. The purpose of this study was to identify a novel ubiquitin ligase for p63 and to discern what effect this modification has on p63's activity. Since this dissertation focuses on p63, the discussion of p53 and p73 from this point on will be limited largely to their relationship with p63.

1.2 p63's gene and protein architecture

p63 shares a high degree of homology at both the sequence and protein level with the other p53 family members. Evolutionarily, this common gene structure is conserved from mollusk to human (93, 101, 157). A schematic of p63's gene and protein architecture is provided in Figure 1.1. All of the p53 family members have three major domains: an N-terminal transactivation (TA) domain, a central DNA binding domain (DBD) and a C-terminal oligomerization domain (OD) (101). p63 and p73's TA, DBD, and OD domains have approximately 25%, 60% and 35% amino acid identity with p53

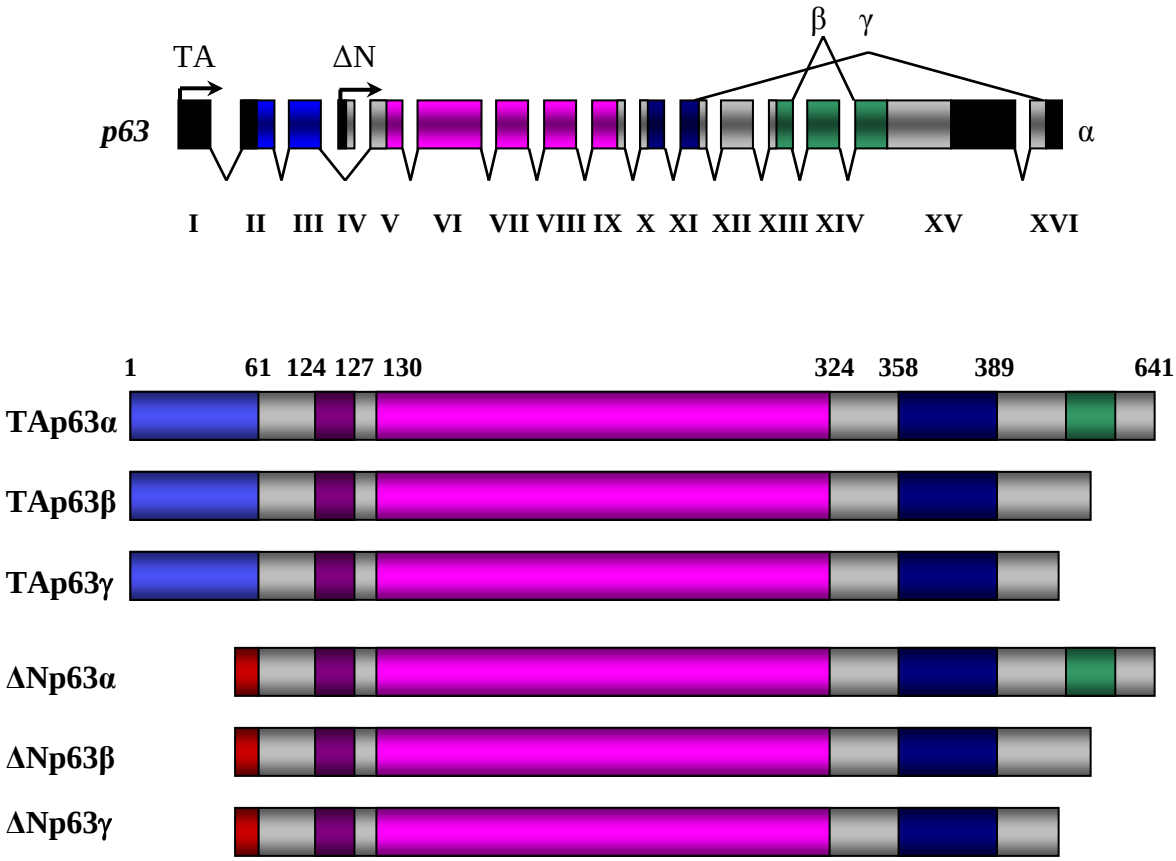


Figure 1.1 – p63's gene and protein architecture. Top – The p63 gene, with alternate promoters and splice variants shown. 5' left, 3' right. Exons are color coded to correspond with protein domains below. Black regions are 5' and 3' UTRs. Bottom – Protein architecture of p63's isoforms. N- terminus left, C- terminus right. Blue is the TA domain, red is the alternate TA domain unique to the ΔNp63 isoforms, purple is the PRD, pink is the DBD, deep blue is the OD and green is the SAM domain.

respectively and are more highly conserved between p63 and p73 (101, 157). Also, all of the known DNA contact and structural residues that are hotspots for p53 mutations in human tumors are also conserved in p63 and p73, even though p63 and p73 are rarely mutated in cancer (101).

The p53 family members encode several isoforms from a combination of varied upstream promoters and splice variants. p63 is the least complex of all the family members in this aspect because it encodes 6 gene products. These isoforms are encoded from 2 N-terminal promoters, the P1 promoter in the 5' untranslated region (UTR) upstream of the noncoding exon 1, and the P2 promoter within the 23 kb spanning intron 3 (101). The P1 and P2 promoters encode two distinct classes of isoforms, the TAp63 isoforms (that have the TA domain), and the ΔNp63 isoforms (that lack the conserved TA domain), respectively. The ΔNp63 isoforms act as potent dominant-negative inhibitors of the TAp63 isoforms *in vivo* (167). In fact, *in vitro* studies determined that a single ΔN isoform is all that is required to inhibit a p53 family tetramer, but this inhibition may vary *in vivo* (18). However, it is worth noting that the ΔNp63 isoforms also have their own unique TA domain and can activate their own sets of target genes (47). This difference in activation and regulation has led to the hypothesis that the TAp63 isoforms act tumor suppressors, but that the ΔNp63 isoforms act as oncogenes (168). This topic will be discussed in greater detail below.

A second degree of versatility is introduced by the three C-terminal splice variants, termed p63α, p63β, and p63γ from longest to shortest. These isotypes are created by alternative splicing of exons 11-15 (see Figure 1.1). The most striking function of these different tails is their variation in transcriptional activity, with p63γ being the most

transcriptionally active isotype, and the p63α and p63β variants much weaker (167). The functional strength of the p63γ isoforms may be due to the fact that the p63γ variants most closely resemble p53, harboring a small C-terminal extension of 30 amino acids past p53's C-terminus. In fact, TAp63γ is as potent as p53 in many transactivation and apoptosis assays (167). This similarity is also why I have chosen TAp63γ as a focus of my study, because the higher transcriptional activity of the TAp63γ isoform makes it a robust and easy to detect readout in functional assays, and the primary focus of our laboratory is transcription. The p63α isoforms contain an additional sterile alpha motif (SAM) domain that is conserved in p63, p73 and forms of p53 in lower organisms. The SAM acts as a protein-protein interaction motif and is found in a wide variety of developmental proteins, such as the ETS transcription factor, the TEL leukemia protein, the polycomb group of homeotic transcription factors, and the ephrin receptors (101). According to its crystal structure, p73's SAM domain features a 5-helix fold that is conserved in other SAM domain containing proteins. This fold is also capable of binding anionic and zwitterionic lipid membranes, and it is thought that p63's SAM may function similarly (7,154). This long C-terminus is one reason for the exceedingly weak transactivational activity of p63α because it contains a 27 kd C-terminal region is able to curl around and bind the proline-rich domain (PRD, see Figure 1.1) in p63's N-terminus that is homologous to the MDM2 binding site (134). This folding pattern also greatly affects the stability and biology of the p63α isoforms, as will be discussed below. *In vivo*, the balance of these isoforms is crucial to homeostasis and each cell type expresses a specific combination of isoforms during and post-development. Generally, the ΔNp63α isoform is the most predominantly expressed isoform in adult epithelial tissue, while the

expression of the p63γ and p63β isoforms is lower. While this study does touch on most of the p63 isoforms, the primary focus of this study is the p63γ isoform because of its potent role in transcription.

1.3 p63's biological functions – roles in development and trends in cancer

As mentioned above, p63 is not a classical Knudson tumor suppressor (72); rather its major functions are epithelial and limb development. *p63*$^{-/-}$ mice show severe limb truncations or the absence of limbs and craniofacial malformations (100, 167). The most striking phenotype is the lack of most epithelial structures, such as teeth, hair, and mammary, prostate, sweat and lacrimal glands (100, 167). Additionally, the epidermis is non-stratified. The lack of upper skin layers is most likely what causes these pups to die within a few days of birth due to dehydration (100, 167). Further, it was found that basal layers of human epithelium, including the epidermis, strongly express p63 proteins, generally at a 100:1 ratio of ΔNp63:TAp63 (167), but lose them when these cells terminally differentiate (118, 161). In support of this observation are the data that keratinocyte differentiation is associated with the downregulation of ΔNp63α, while the expression of the p53 family target genes, *p21* and *14-3-3σ* which are involved with cell cycle control increase, as would be expected with loss of a transcriptionally repressive isoform (161). This release of repression is also found in some of the human syndromes associated with loss of p63, as will be discussed below (161). In addition to its role in epidermal differentiation, p63 is also important for regulating the development of other tissues. p63 is indispensible for the differentiation of transitional urothelium and is also expressed in normal bladder urothelium (149). Later, it was determined that p63 is found in the apical ectodermal ridge of the developing limb bud where the p63 expressing cells

create a signaling center (118). Also, p63 is used as a differentiation marker in human skin, myoepithelium of the human breast, and human prostate (6, 85, 102, 124, 137, 138). Therefore, it is clear that p63 has a critical role in development and cell fate.

These observations have been incorporated into our larger understanding of p63's role in development, which has been both more closely defined and debated, beginning with the generation of two *p63* knockout models and followed by other transgenic studies. Currently, two separate groups have created *p6*3 knockout models and the phenotypic differences between these knockouts have created debate in the field over the mechanisms of how p63 mediates these *in vivo* functions. For ease of discussion, these knockout models will be referred to by their primary publications. These mice have in common the macroscopic phenotype of failed differentiation in the epidermis, craniofacial abnormalities, and limb truncation, as discussed above. However, they vary in the presentation of the skin developmental phenotype, which leads each group to make very different hypotheses about p63's role in development. One group (Yang *et al*.) holds to the hypothesis that p63 is necessary for stem cell self-renewal. This conclusion came from the observation that their mice had clumps of differentiated keratinocytes as well as the undifferentiated layers common to both mouse models. These clumps were interpreted as cells that had stratified but had lost the ability to maintain them. Thus, in analysis of this model, p63 is not required for commitment to the epidermal lineage and functions to maintain the stem cell pool, preventing it from terminal differentiation (167, 168).

In the second model (Mills *et al*.), TAp63 is required first for commitment to the epithelial lineage, and a subsequent downregulation of TAp63 by ΔNp63 is required for

commitment to terminal differentiation of mature epithelium (76, 78, 80). They came to this conclusion after noting that their mice had a single layer of undifferentiated epithelial cells covering the dermis. Thus, they concluded that these epithelial cells were blocked in their transition to become mature keratinocytes (76, 78, 80). The second part of this hypothesis was further supported by elegant experiments using an inducible *TAp63α* mouse model where *p63* was lost, and the TAp63α was added back under the control of a conditional K18 ectodermal-specific promoter (76, 78, 80). They found that TAp63α could initiate a stratification program and inhibited epidermal differentiation. Further, this hyperproliferation must be balanced by the ΔNp63 isoforms which would halt the increased growth and allow for terminal differentiation. Therefore, it is debated whether p63's major role in the epidermis is to maintain the stem cells in the basal layer of the skin, or if it instead works to commit the progenitor cells to a differentiation program and later acts to regulate stratification.

The true function of p63 in development is likely somewhat of a fusion of these two hypotheses, as shown by later experiments. p63 is most likely not simply required for maintenance of a stem-cell pool, since transient amplifying cells (TAC) cells, the immediate progeny of stem cells, are equally proliferative, but have already lost p63 expression (118). Additionally, zebrafish embryos require ΔNp63-mediated inhibition of p53 to allow for epidermal proliferation and limb development, and *xenopus* models show conservation of the regulation of ΔNp63/TAp63 isoform balance during development (80, 89, 93, 147). Therefore this interplay of p53 family isoforms is likely a very ancient mechanism in cell signaling and indicates the importance of understanding p63's independent and p53-family dependent signaling pathways.

Mutations of p63 cause several disorders in humans that have similar developmental phenotypes to those in animals. Heterozygous germ line point mutations of p63 cause six rare autosomal dominant disorders: Ectrodactyly Ectodermal dysplasia Clefting (EEC), Split-Hand/Foot Malformations (SHFM), Acro-Dermato-Ungual-Lacrimal-Tooth (ADULT), Ankyloblepharon Ectodermal dysplasia Clefting (AEC or Hay-Wells), Limb-Mammary Syndrome (LMS) and Rapp-Hodgkin Syndrome. EEC and AEC are somewhat related and were the first discovered. Of 29 mutations discovered in 90 affected families with EEC, 28 were point mutations of the DBD, some of which correspond to p53 hotspot mutations (17). Not surprisingly, these mutations affect all p63 isoforms and inhibit the binding of TAp63 to DNA, while EEC mutations in the ΔN isoforms ablate their dominant-negative activities towards the p53 family members (17). In contrast, AEC mutations are only in the SAM domain and effect only the p63α isoforms. In this case the phenotype is caused by loss of interaction with apobec-1 binding protein-1, which then also causes loss of the K-SAM splice variant of fibroblast growth factor receptor 2 which is essential for epithelial differentiation (31). The other four diseases extend beyond the strict genotype:phenotype correlation and are under current study (101). It should be mentioned that a hallmark of many of these diseases is also the altered stabilization of p63 which will be discussed in greater detail in the section on p63's post-translational modification.

Along with the description of whole physiological phenotypes of p63's deregulation in the murine models and human disease, p63 expression has also been described at the molecular level from animal and human tissue samples; though for the purposes of this study, I will focus on mammalian expression in order to provide a basis

for the following discussion of p63's deregulation in cancer. As mentioned previously, in human adult epithelia, the ΔNp63 isoforms are often readily detectable, though in other tissues the balance of isoforms can widely differ, such as the tissues of the eye (117). In adult epidermis, p63 is largely restricted to the nuclei of basal cells of normal epithelia (skin, esophagus, tonsil, prostate, urothelium, ectocervix, and vagina) and specific populations of basal cells in glandular structures of the breast, prostate and bronchi (149). In the case of this study, I detected endogenous p63 mRNA and protein levels in primary mouse embryonic fibroblasts (MEF). Even though most of the dermis shows lack of p63 immunolabeling, this result is most likely due to the low population of fibroblasts in the dermis, since other groups have shown p63 protein expression in MEFs which is increased upon typical activating stimuli (30). p63 is a specific marker for the myoepithelial cells of the breast (6). It is also a marker for the basal cells of the prostate, making it an excellent cancer marker, as a large majority of prostate cancers and pre-invasive prostate intraepithelial lesions have lost p63 expression (138). Also, the TAp63 isoforms are highly expressed in the tissues of the eye, while the ΔNp63 isoforms may be involved in cell migration during corneal wound healing (25, 117). Thus a proper balance of p63 isoform expression must be maintained for normal tissue homeostasis.

p63 is not a classical tumor suppressor but is often deregulated in cancer. The analysis of p63 in cancers of patients with germ line mutations or somatic mutations in p63 reveals a lack of genetic mutation, but an upregulation in p63 expression. For instance, no p63 mutations were found in 47 bladder cancers or 68 head and neck squamous cell carcinoma samples (115, 158). Also, only 1 missense mutation (Ala148Pro) was found in 66 human tumor samples and 2 missense mutations in 35

human cells lines (112). However, despite the lack of mutation, p63 is located on chromosome 3q27-28, a region that is frequently amplified in squamous cell, cervical, and prostate carcinomas (101). These data suggest a role for p63's deregulation, rather than loss, in cancer.

A portion of p63's deregulation in cancer likely stems from a perturbation of the wild type p63 isoform balance, as shown by several studies using both tumor tissue and cancer cell lines. In less differentiated gastric carcinoma, both the TAp63 and ΔNp63 isoforms are highly expressed, suggesting the overexpression of p63 can promote growth in this tissue (146). Some lung cancers and head and neck squamous cell carcinomas show an increase of p63 increase correlated with an increase in copy number of the *p63* gene (48). These increased copy numbers have been shown in squamous cell carcinomas to translate to not only an overall increase in transcript, but a particular stabilization of the ΔN isoforms, predominantly p40AIS (48). p40AIS has also been shown to act as an oncogene in nude mice and in Rat1a focus formation assays (48). Similarly nasopharyngeal carcinoma shows an upregulation of the ΔN isoforms, even though most of these tumors have wild type p53 (20). In 25 nasopharyngeal carcinoma samples, nearly all tumor cells showed positive staining for ΔNp63 isoforms, even though the normal tissue had expression restricted to the basal layer (20). Further, in esophageal squamous cell carcinoma the predominantly expressed isoforms are the ΔNp63 isoforms (20). Thus the ΔNp63 isoforms may help the cells maintain a proliferative phenotype for some cancers. However, tumors are heterogeneous and there are data which also present the other side of the proverbial coin in p63's role in cancer.

For all the cases in which p63 is amplified in some cancers, its downregulation is characteristic of specific types and stages of other cancers. For example, ΔNp63α is frequently undetectable in cutaneous lesions, like basal cell carcinoma, even though it is strongly expressed in the basal layers of normal skin (22, 23). Adenocarcinoma of the prostate also shows a loss of p63 staining, as it is devoid of the basal cells which usually stain positive for p63 (24, 159). Also interesting are cases, where one set of isoforms is preferentially lost. For example, in 30 of 47 bladder cancers ΔNp63 was upregulated (115). However, TAp63 was concomitantly downregulated in 25 of the 47 tumors (115). Another study of 160 bladder transitional cell carcinomas examined this phenomenon more closely (74, 149). Where 93% of low-grade papillary superficial tumors expressed p63, only 68% of the intermediate- and high-grade superficial tumors were positive (74, 149). Thus the loss of p63 in transitional cell carcinoma is correlated with the progressive stage and grade of the tumor (74, 149). Yet another example is cancers of the breast, where p63 expression is rarely found in carcinoma *in situ* and is never expressed in invasive carcinoma, even though it is a marker for the normal myoepithelium (6, 24, 101, 127). Therefore, the role of p63 in cancer is complex and most likely is rooted in the specific balance of each isoform within a particular cell and tissue type which is deregulated by one or more mutations during tumorigenesis. As shown by the studies above, the most commonly increased isoforms in tumors are the ΔNp63 isoforms. The function of this increase will be discussed further in the next section on signaling and post-translational control, continuing the emphasis of p63 isoform deregulation and disease. Adding to this complexity is the interplay of p63's isoforms with each other, the

other p53 family members, and the latticework of their shared and individual signaling pathways.

1.4 p63's transcriptional control - links to p63's stability and post-translational modification

The literature on the mechanisms of p63's activity is still in an immature state due to the complex nature of p63 signaling and the changes that distinguish p63 from p53 and p73 and impact p63's transcription, stability and signaling. These differences were found through analysis of p63's activity in animal models and the description of how the genetic differences in the animal models translated to differences in binding and activity on common p53 family target promoters. The differential regulation of the p53 family members on chromatin leads to varied regulation at the transcriptional level and is closely tied with p63's stability and post-translational modification which are often altered in disease.

As mentioned in the previous sections, p63 shares common and individual transactivation targets with p53 and p73. These common targets include p21, GADD45, 14-3-3σ, BTG2, PIG3, Bax, ribonucleotide reductase p53bp2, and IGFBP3 (101). Since my study focuses on p21, the literature on the direct regulation of p21 transcription by p63 will be addressed in detail in the Discussion. This functional overlap between the p53 family members suggests that a certain family member would predominate in certain situations - specifically a group of isoforms would control a given signaling outcome after induction with a specific stimulus. The data which demonstrate that p63 is rarely mutated in most cancers and is only lost in advanced state and that the ΔNp63α isoform is frequently overexpressed in tumors supports this concept. The body of data now suggests

that the TAp63 isoforms most likely act as tumor suppressors because of their high transcriptional activity and role in apoptosis, and that the ΔNp63 isoforms are largely oncogenic because of their dominant-negative activity and overexpression in tumor tissue. However, the mechanisms which drive the competition between isoforms of the p53 family and their target promoters are largely unknown.

To begin to tease apart separate roles for the three p53 family members and their contributions to tumor development, there have been several elegant studies using mouse knockout models. The first of these studies showed in both MEFs and mice knocked out for each of the family members, that loss of *p63*, *p73* or both, ablated p53's apoptotic response, but not its growth arrest response (30). This result was first demonstrated by RT-PCR showing that induction of p21 occurred normally in the $p63^{-/-}$, $p73^{-/-}$ and $p63^{-/-}/p73^{-/-}$ animals, but that the induction of the apoptotic targets Bax, Noxa, and PERP was compromised (30). This loss of apoptotic gene expression was demonstrated by chromatin immunoprecipitation (ChIP) to originate from loss of p53 binding to the *Bax, PERP, and Noxa* promoters in the absence of either p63 or p73, but only partial loss of binding on the *p21* promoter (30). Later studies showed that even though they are not classical tumor suppressors, heterozygosity of p63 or p73 crossed with a p53 heterozygous background exacerbates the tumorigenic phenotype of any of the single p53 heterozygous mice. Also the $p63^{+/-}/p73^{+/-}$ mice developed tumors, and loss of the second copy of p63 or p73 was a hallmark of more aggressive, advanced tumors as in the human cancers discussed above (29). These studies used $p63^{-/-}$ knockout mice from the Yang *et al.* model (which supports the hypothesis that p63 is required for stem-cell maintenance). Later studies with the Mills *et al.* knockout model (which supports the hypothesis that

p63 is required for commitment to the epithelial lineage and the terminal differentiation program) agreed with these data and showed the major difference that their *p63*$^{+/-}$ mice did not develop spontaneous tumors (77). The conclusion from the Yang *et al*. model argues that p63 has a larger role as a tumor suppressor and is supported by the data showing loss of p63 in a subset of advanced tumors. In the Mills *et al.* model, p63 is interpreted as an oncogene, since their *p63*$^{+/-}$ mice do not develop spontaneous tumors and reactivated TAp63α in these mice activates tumor development and progression. Further, this model is supported by the data that genomic mutations are rare and that increased p63 expression is common in most epithelial tumors (77). Again there is no clear-cut rule as to whether p63 is an oncogene or a tumor suppressor and the two knockout models provide a different interpretation of p63's activity as they did in the developmental papers. The difference in these models and in p63's signaling is postulated to result from a difference in p63 regulation, which is an integrated network of p63 control of promoter binding and protein stability.

Currently, a number of studies on p63's transcriptional regulation have focused largely on the p63α and p63γ isoforms, with the weight of the literature describing p63α. As mentioned above, the TAp63α isoform is a weak transcriptional activator, most likely due to the folding of its C-terminal tail, while the TAp63γ isoform is considered to be as potent as p53. Similarly, TAp63α lacks significant apoptotic inducing activity while TAp63γ is very potent in induction of both transcription activation and apoptosis (167). However, TAp63γ's apoptotic activity is controversial, as one study showed that cells expressing TAp63α, ΔNp63α, TAp63γ or ΔNp63γ showed poor or undetectable apoptosis, while TAp63γ was clearly able to activate p21 (26). In fact, TAp63γ's activity on the

p21 promoter is clearly under tight and subtle regulation. One group demonstrated that ectopic TAp63γ expression induced p21 in an erythroleukemia cell line to induce differentiation, rather than apoptosis (64). Similarly, our own laboratory's work on p300's activation of TAp63γ showed an upregulation of p21, but no distinct apoptosis, similar to the increase of p21 by TAp63γ and βTrCP1 seen in this study which also does not elicit an apoptotic response (35, 95). Yet overexpression of TAp63γ induced apoptosis in a hamster kidney cell line, and recently TAp63γ was shown to be a critical factor in the apoptotic response in developing neurons, arguing that TAp63γ's role in apoptosis is important and its function is most likely regulated in a tissue-specific manner (53, 101). Also, while there is a general balance between the TA and ΔN isoforms generated by their dominant-negative activity, as detailed above, there is also feedback between ΔNp63α and TAp63γ specifically (167). It was found that p53 is a transcriptional regulator of p63 and it was later shown that TAp63γ regulates the expression of ΔNp63 isoforms in a manner that is sensitive to p53 (43, 101). Several recent studies have focused in the description of several p63-specific response elements in an attempt to understand these differences in the current data in terms of the individual transcription response mediated by a particular p63 promoter complex.

p63 has both common and unique DNA response elements (RE) when compared with p53. p53 recognizes a consensus sequence composed of two or more half sites. Each half site consists of a palindromic decamer with the sequence 5'-RRRC(A/T)(A/T)GYYY-3', where R is a purine residue, and Y is a pyrimidine residue (120). Several lines of evidence suggest that p63 would also recognize a pattern of half sites common or similar to p53's favored half sites. First, the DBD is the most conserved

within the family between p53 and p63 and displays similar global fold (120). Second, p63's DBD can be modeled based on the resolved p53 structure (120). Third, p63 can bind known p53 REs in both reporter assays and endogenous chromatin contexts (120). However it was also shown that the DBDs of p53 and p63 display different biophysical properties that may underlie differences in the DNA sequences optimally recognized by each protein (120). There have been several studies characterizing the p63 consensus motif. The core domain has been characterized to differ from p53's slightly, either CGTG, or C(A/G)(T/A)G (112, 119, 120). My study shows these sequences at a higher frequency near the transcription start site of *p21*, leading to a second unique recognition site for p63 at the *p21* promoter. Another group suggests that the core domain does not differ from the p53's, but that the purine and pyrimidine rich flanking sequences change to A/T rich sequences at p63-prefered binding sites (111). What is common about these studies is that p63 displays a more degenerate binding site than p53, perhaps enabling it to rescue p53 function under some circumstances. Also, a recent report showed during p63's activation of PKCΔ that p63 and p53 seem to differentially recognize REs with the same core consensus sequence (122). This differential recruitment to binding sites has been shown to translate to varied recruitment of protein machinery to chromatin, leading to differences in transcriptional regulation, and it has been further suggested that each p63 isoform would have the innate ability to preferentially recruit transcriptional proteins to target promoters *in vivo* (120). Therefore, it is highly likely that the affinity of separate family members and isoforms for an individual RE is one of the many factors contributing to p63 regulation.

The differences in the p63 isoforms' functions results from the interplay of p63 with its own isoforms and that of the other p53 family members to recruit specific complexes to promoters; the mechanisms for this interchange are deeply intertwined with p63's folding and stability both on and off the promoter. It is well-established that p53's stability is directly tied to its folding and transactivation activity; further, many of the modifications important for regulation of p53's stability occur on p53's N- and C- termini (101, 157). This control is unsurprising as many regulatory proteins, such as MDM2 (mouse double minute 2), the major E3 ligase for p53, binds to the N-terminus in the transactivation domain, the three nuclear localization signals (NLS) reside in the C-terminus, the two nuclear export signal (NES) are in the N- and the C-termini, and the repressive domains are in the C-terminus (10). Also, through analysis of the regulation of p53 through these domains and the data on the hotspot mutations of p53 (largely in the DBD) in cancer it has been demonstrated that p53's transactivation and DNA binding capability of are linked directly to p53's stability. The N- and C-terminal domains are also important for p63 regulation. The variable C-termini for p63, while being associated with differences in transactivational activity, are also closely tied to p63's stability. In general, the TAp63 isoforms (about a 6 minute *in vitro* half-life) are much less stable than the ΔNp63 isoforms (a greater than 5 hour *in vitro* half-life). Also, the p63α isoforms are more stable than the p63γ isoforms, most likely due to the inhibitory effect of the longer C-terminal domain, particularly the SAM domain which allows for a more stable folding pattern with the N-terminal domain, as mentioned previously. Also, DNA binding activity does play some role in stability because disease-related DNA binding mutants of p63 are very stable. However, one study found that the correlation between DNA

binding activity and stability may not be as straightforward for p63 as with p53, as p63 DBD mutants were able to be degraded efficiently upon co-expression of a functional TA isoform in cells (96). Along these lines, the unique transactivation domains in the ΔNp63 mutants, and the presence of the SAM domain in the p63α isoforms may be two of the many sources of the differential control of p63's stability.

As with the transcriptional data, the information on p63 is in an early state and growing rapidly. Several co-factors have been described for p63 (summarized in Table 1 and Figure 1.2). As displayed by the chart, the isoforms examined in the studies vary, with a heavy focus on ΔNp63α. Also, as one would expect, most of these proteins bind to the N or C-termini (Figure 1.2). Our laboratory found that SSRP1 (Structure Specific Protein 1) bound to TAp63γ and activated its expression of genes such as *p21* and *Bax* which I also tested in our current study (173). Other co-factors include the ASPP (apoptosis stimulating proteins of p53) family of apoptotic co-factors and the transcriptional repressor hDaxx which modulates p63's apoptotic response (9, 39, 141). Also, ΔNp63α has been shown to be in a complex with the B56α subunit of Protein Phosphatase 2A (PP2A) in a complex with Glycogen Synthase Kinase 3β (GSK3β) and the β-catenin oncogene (116). The implications of this activation pathway will be discussed in more detail in the Discussion, since β-catenin is also a substrate of βTrCP1, as described below. All of these proteins bind p53 and/or p73 except for βTrCP1; our study is the first showing a unique co-activator for p63, but with the numerous array studies currently being conducted it is only a matter of time before others are found.

Further, other upstream signals are directed and altered by the pattern of post-translational modifications on p63 at any specific time, and therefore competition

modifer	type of modification	which isoforms	interacting regions	modified residues	function
ASPP 1/2	none - cofactor	γ	DBD - 7 conserved residues	N/A	activation
Arf	none - cofactor	all	PRD residues 109-120	N/A	inhibition
hDaxx	none - cofactor	α	OD (used p53's OD)	N/A	inhibition
MDM2/MDMX	uncertain	all (largely α and γ)	TA domain	?	inhibition (cytoplasmic loc.)
PP2A (B56α)/GSK3β/β-catenin	none - regulated by p63	ΔNp63α	N-terminus, 1-20 of ΔNp63α	N/A	activates β-catenin
SSRP1	none - cofactor	γ	N-terminal (TA)	N/A	activation
p300/PCAF	acetylation	α, γ	N-terminus, 1-131	?	activation
unknown kinase	phosphorylation	α	?	S66, S68	activation leads to rapid deg.
RANBP2	sumoylation ??	ΔNp63α	?	K637, K549 ??	?
UBC 9	sumoylation	α	C-terminus	K637, K549	inhibition (26S deg. ΔN only)
Itch	ubiquitylation	α/all	PY domain, K449/109-120	K193,K194	inhibition (26 S deg.)
14-3-3σ/ RACK1	ubiquitylation	all (ΔNp63α)	553-559 a.a./5' to SAM domain	?	inhibition (26 S deg.)
βTrCP1	ubiquitylation	α (not modified), γ	N- and C- (1-131, 389-448)	N terminal and ?	activation
NEDD 4	ubiquitylation	ΔNp63α	C-terminal PY motif	K637 and others?	inactivation (deg.)

Table 1 – p63 binding proteins and modifications in detail. A summary of the current literature, see text for discussion and references.

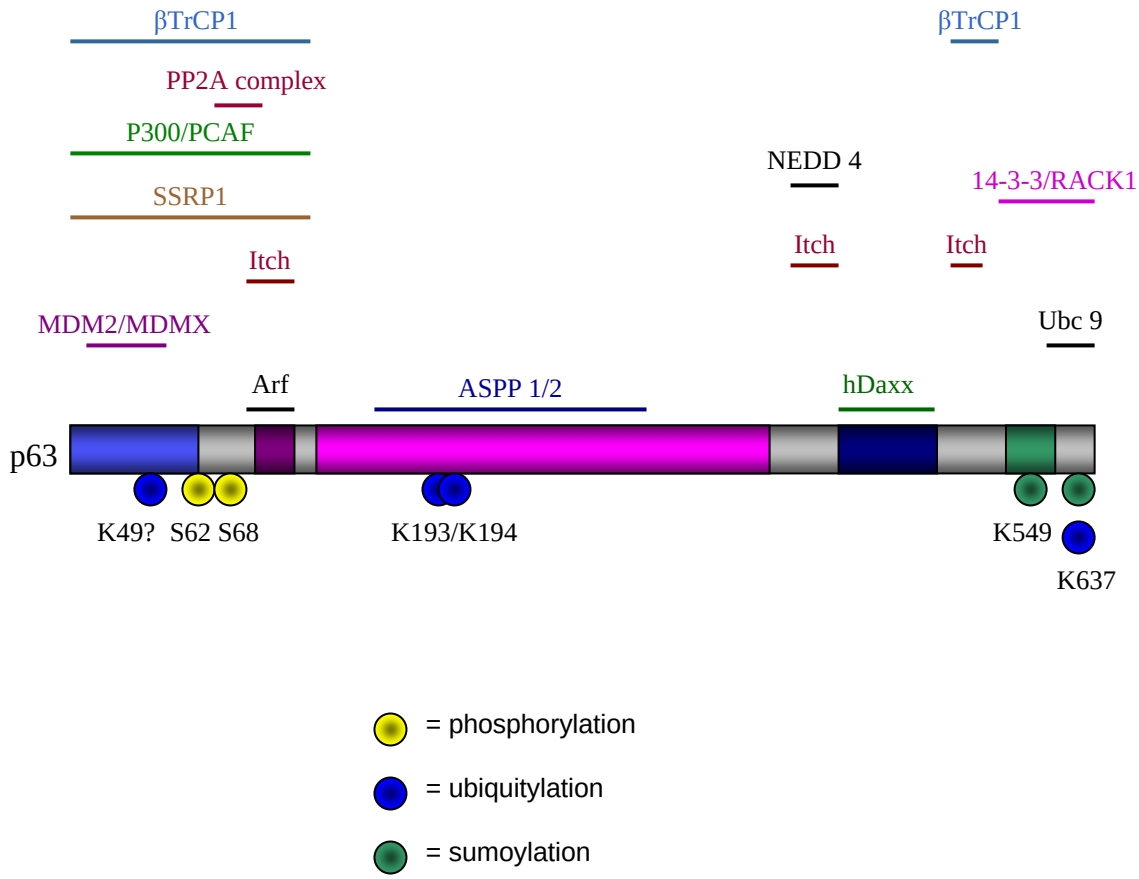


Figure 1.2 - p63 binding proteins and post-translational modifications. Post-translational modifications are shown on the bottom of TAp63α according to the key with residues shown. Known protein binding sites are shown above. Blue is the TA domain, purple is the PRD, pink is the DBD, deep blue is the OD and green is the SAM domain. For details see Table 1; references are in the text.

between modifying proteins which determines the ultimate modification that persists on the molecule and the consequent functional outcome. These modifying proteins are grouped into the bottom section of the chart, with the exception of MDM2/MDMX and Arf which have a debated role in p63 regulation. To date, only a few post-translational modifications have been found on p63. Where a full gamut of acetylases, deacetylases, kinases, ubiquitin ligases, deubiquitylases, nedd-ligases, SUMO-ligases, isomerizes, etc are known for p53, within the past five years p63 has only been shown to be acetylated, phosphorylated, sumoylated and ubiquitylated (10, 101, 157). A summary of known modifications and associated enzymes is provided in Table 1 and Figure 1.2. Our laboratory showed that p300 acetylates p63's N-terminus, increases TAp63γ's upregulation of p21, and that this increase was inhibited by ΔNp63γ (95). Also, a recent study showed that ΔNp63α is phosphorylated on S66 and S68 as part of a pathway upstream that activates Akt to inhibit UVB-mediated apoptosis, though a kinase for p63 has yet to be published (107). As a parallel project to the ubiquitylation of p63 by βTrCP1 our laboratory is currently examining IKK as a potential kinase of p63.

Sumoylation and ubiquitylation are the post-translational modifications of p63 and p73 which have received major focus due to the role of their stability in disease. The mechanistic details of these modifications will be described in a later section. Sumoylation mediated through the Ubc9 E2 SUMO enzyme and the RanBP2 SUMO-ligase has been characterized for the p63α isoforms (37, 51). Sumoylation occurs primarily at K637, with K539 being a secondary affinity site for both enzymes and leads

to transcriptional inactivation and 26S proteasomal degradation of the ΔNp63α isoform (37, 51). This modification was directly related to the SHFM disease phenotype (51). No other sumoylation sites were found on the shorter isoforms. Ubiquitylation is thought to be the major modification regulating stability of the p63 isoforms, since all the p63 isoforms are ubiquitylated. The MDM2/MDMX (also known as MDM4) regulation of p53 is a major feedback loop in its regulation, but the results of several studies with p63 have been controversial. The data shows that p63 can bind MDM2 and MDMX, and that this binding causes inactivation (14, 60, 155). However, the mechanism for this inactivation is debated, since MDM2 and MDMX do not seem to ubiquitylate p63 (14, 60, 155). However, MDM2 and MDMX can mediate the nuclear export of p63 and have been shown in most cases to show a downregulation of function (14, 60, 155). Likewise, reports on Arf have been controversial. With p53, Arf regulates MDM2/MDMX leading to the downstream stabilization and activation of p53 (101). Arf directly binds p63's N-terminus and inactivates it, though the link between Arf and p63's ubiquitylation has not been firmly established (15, 155). The ΔN isoforms are preferentially degraded through a mechanism by which 14-3-3σ drives p63 nuclear export in response to genotoxic stress, followed by RACK1 (a scaffolding protein and receptor for protein kinase C)-mediated proteasomal degradation (114). 14-3-3σ is regulated by all three p53 family members. RACK1 itself does not contain the typical ubiquitin ligase domains (HECT and RING) and is thought to recruit an E3 ligase or to have an uncharacterized ubiquitin ligase activity (114). The first definite demonstration of a p63 ubiquitin ligase was NEDD4. NEDD4 ubiquitylates and degrades ΔNp63α and is involved with dorso-ventral patterning in zebrafish (3). Interestingly, the targeted lysine for NEDD4 ubiquitylation is

also one of the C-terminal lysines used for sumoylation. Recent work has also shown the epidermal protein Itch as a p63 ubiquitin ligase. This work was shown by two different groups, one of which showed that Itch binds the C-terminus of p63 and only ubiquitylates the p63α isoforms, and the second which showed that it recognizes a region in the DBD and is able to modify all of p63's isoforms (99, 128, 130). In both cases, the ubiquitylation led to increased turnover of p63 (99, 128, 130). Future work will certainly define more of p63's post-translational control network.

Therefore, since the signaling pathways governing p63 expression are largely unknown and the weight of data suggests that p63's regulation of cell growth is largely mediated by a mis-regulation of isoform stability, I wished to look for specific factors that could mediate p63 stability in cells. Thus I began to search for an E3 ubiquitin ligase that could regulate p63's stability and activity, since at the time there were no ubiquitin or ubiquitin-like modifiers clearly shown for the p63 isoforms. While screening potential ligases, I found a paper in the literature which provided a tantalizing potential lead. Therefore, I began my project examining the regulation of p63 by βTrCP1.

1.5 Tales of Two Murine Phenotypes: Potential links between IKK/βTrCP and p63

It is through comparison of the literature on the striking epidermal phenotype of the *p63*$^{-/-}$ mouse with another knockout model that I first postulated that there could be a link between the post-translational modifiers associated with the NF-κB (nuclear factor κ-B) pathway and p63. The *IKKα* knockout also has a striking epidermal phenotype, characterized by a lack of terminal differentiation that is found in the p63 knockout (100, 139). However, where the *p63*$^{-/-}$ skin displays a single basal layer of undifferentiated tissue and no hair follicles, the skin of an *IKKα*$^{-/-}$ mouse does stratify to a few layers thick,

but does not complete the differentiation program (100, 139). As far as the limb phenotype, among the important developmental regulators lost as a result of impaired IKKα signaling is FGF8, which was also shown to be lost in the *p63*$^{-/-}$ phenotype (100, 139). The mesodermal phenotype of the *IKKα*$^{-/-}$ mouse is shown by the authors to be caused by an alteration of the epithelial-mesenchymal transition due to the loss of terminal epithelial differentiation, rather than the direct loss of IKKα (139). Thus, even though there are some differences in phenotype, the activation of IKKα, like p63, is critical for part of the skin developmental program, implying that these pathways may be interrelated.

IKKα is an important kinase in the NF-κB pathway. NF-κB is well-characterized because of its important role in pro-apoptotic signaling, and because it is often deregulated in cancer. In brief, NF-κB is a transcription factor that is controlled largely through sub-cellular localization. When the pathway is inactive, NF-κB resides in the cytoplasm bound by its inhibitor IκB (Inhibitor of NF-κB) (63). Upon an activating stimulus, the IKK kinase complex, consisting of a trimer of IKKα, IKKβ, and IKKγ (NEMO) phosphorylates IκB (63). This phosphorylation creates a charged binding site for recognition by a βTrCP (β-Transducin Repeats Containing Protein)-containing ubiquitin ligase complex. This complex ubiquitylates IκB and marks it for degradation, allowing NF-κB to traffic to the nucleus and act on DNA (63). Therefore, I wanted to examine whether IKK and βTrCP might post-translationally modify p63. It is worth noting that IKK phosphorylation of p63 is an ongoing project in the laboratory and, since this study, other genomic work on p63 has demonstrated that *IKK* is a downstream target for p63 activation (16). Meanwhile, I have demonstrated that βTrCP1 works in a unique

ubiquitin-mediated activation pathway for p63. Not only does my study find the first substrate to be directly activated by βTrCP1, but it also has striking implications in the greater understanding of the developmental and cell stress pathways mediated by both p63 and βTrCP1, as suggested by comparison of the *p63*$^{-/-}$ and *IKKα*$^{-/-}$ mouse models and the endogenous functions of both proteins.

1.6 The Versatile Hierarchy of Ubiquitylation and the SCF complex

As mentioned above, βTrCP proteins are substrate recognition components of an E3 ubiquitin ligase complex. However, to understand βTrCP's overall importance in post-translational modification and signaling, it is first necessary to understand ubiquitin's role as a post-translational modification.

Ubiquitin is a 76 amino acid polypeptide that is highly conserved among eukaryotes, having only 3 amino acid changes from yeast to human (121, 160). It has a globular hydrophobic core comprised by folding of its three β-sheets around a central α-helix (160). It is critical for a myriad of cellular functions, regulating processes such as cell cycle progression, in which it was first studied, to organelle biogenesis, apoptosis, cell proliferation, cell differentiation, protein quality control in the endoplasmic reticulum, protein transport, inflammation, antigen processing, DNA repair, and the cell stress response. In these functions it closely resembles another post-translational modification: phosphorylation; thus it is unsurprising that these modifications are often intimately associated (160). Further, ubiquitin can be grouped broadly under modifications known as ubiquitin-like proteins (UBLs). All UBLs, including ubiquitin, are characterized by the inclusion of a glycine residue at their activated C-terminus that forms an isopeptide bond with ε-amino groups on their substrate (121, 160). These modifications also include

neddylation, for modification by NEDD8 (or rubylation, for Rub-1, or Related to Ubiquitin, in *S. Cerevesiae*), sumoylation, for modification by SUMO-1 (Small ubiquitin-related modifier) (160). However, ubiquitin is unique from the other UBLs in its ability to form multi-chain linkages through its seven lysine residues (K6, K11, K27, K29, K33, K48, K63) (160). It is this chain formation which gives ubiquitin its amazing versatility as a post-translational modification, allowing it to be involved in both the activation and proteasomal degradation of proteins. While the known outcome of the type of modification will be discussed more thoroughly below, it is important to note that there is constant opposition to the covalent ubiquitin chain attachment to the substrate by de-ubiquitylating enzymes (121, 160). These enzymes are a subject unto themselves and so will not be discussed further except to note that in the case of every ubiquitylation modification there is constantly a tightly regulated balance between the kinetics of the initial ubiquitin addition on open substrate residues, the ubiquitin chain growth from lysines in ubiquitin, and the action of de-ubiquitylating enzymes.

Ubiquitin's ability to form chains is only one of many features that mark this modification as being extremely hierarchical in nature. The control of every aspect of the modification, from the initial activation of the ubiquitin peptide to its fate on the substrate and in signaling, is inherent in the design of the reaction itself. As illustrated in Figure 1.3, free ubiquitin is first activated on its C-terminus in an ATP-dependent fashion by the formation of a high-energy thiol-ester bond with the ubiquitin-activating enzyme (E1) (121, 160). The activation kinetics consist of a sequential binding between Mg^{++}ATP followed by ubiquitin that forms a ubiquitin adenylate intermediate (121, 160). Each E1 carries 2 activated ubiquitin molecules – one in the thiol ester form, the other as the

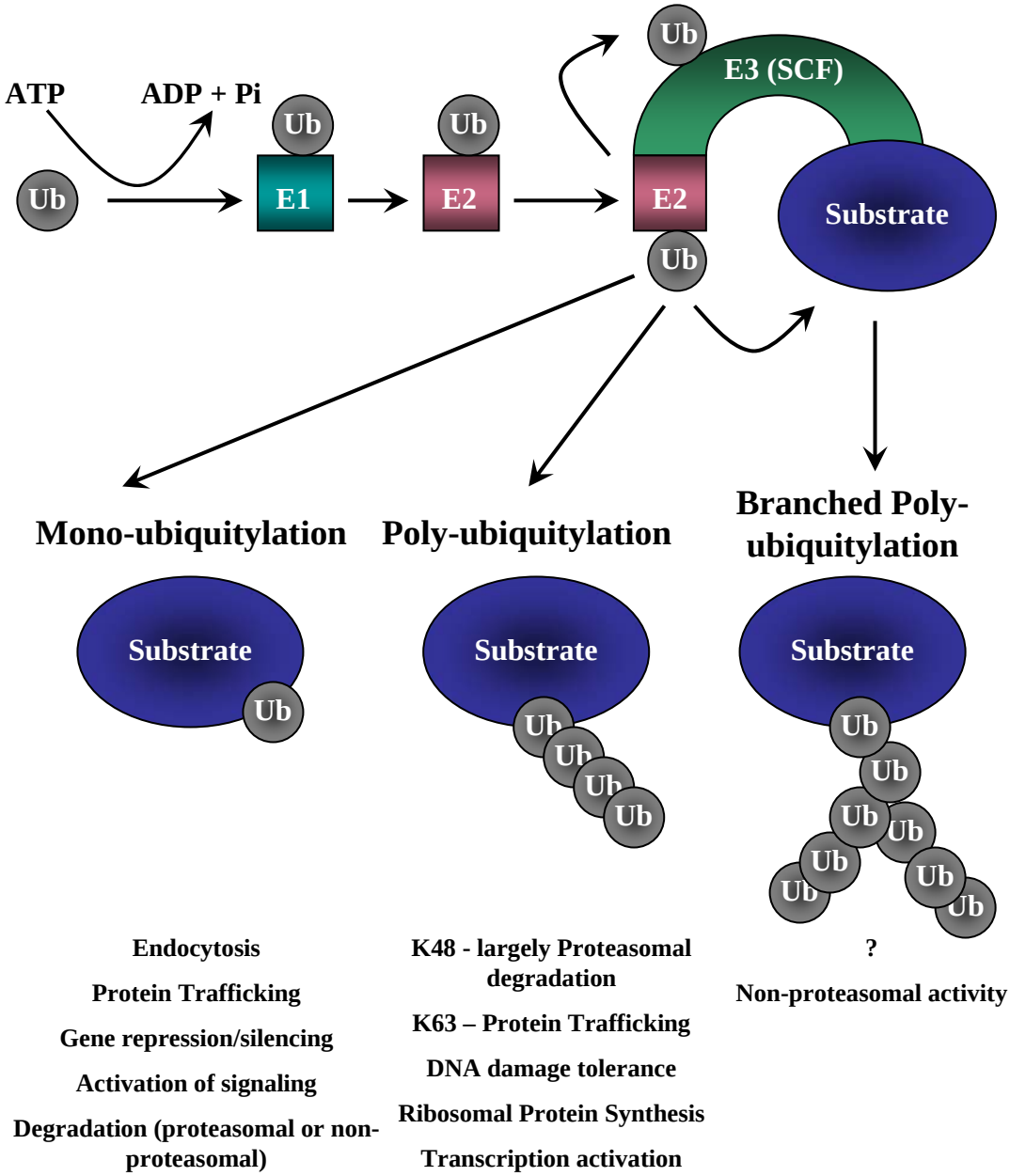


Figure 1.3 - Ubiquitylation is a hierarchical modification. The ubiquitylation reaction and possible linkages. Ubiquitin is recruited to the E1 through an ATP-dependent reaction. The E1 then transfers the ubiquitin to the E2 which is recruited to the E3 (in this case the SCF complex). The ubiquitin is then transferred either directly from the E2 to substrate or from the E2 to the E3 where it is then transferred to the substrate. Ubiquitylation can occur in mono-, poly- or branched poly-ubiquitin structures. Known functions for each linkage are given.

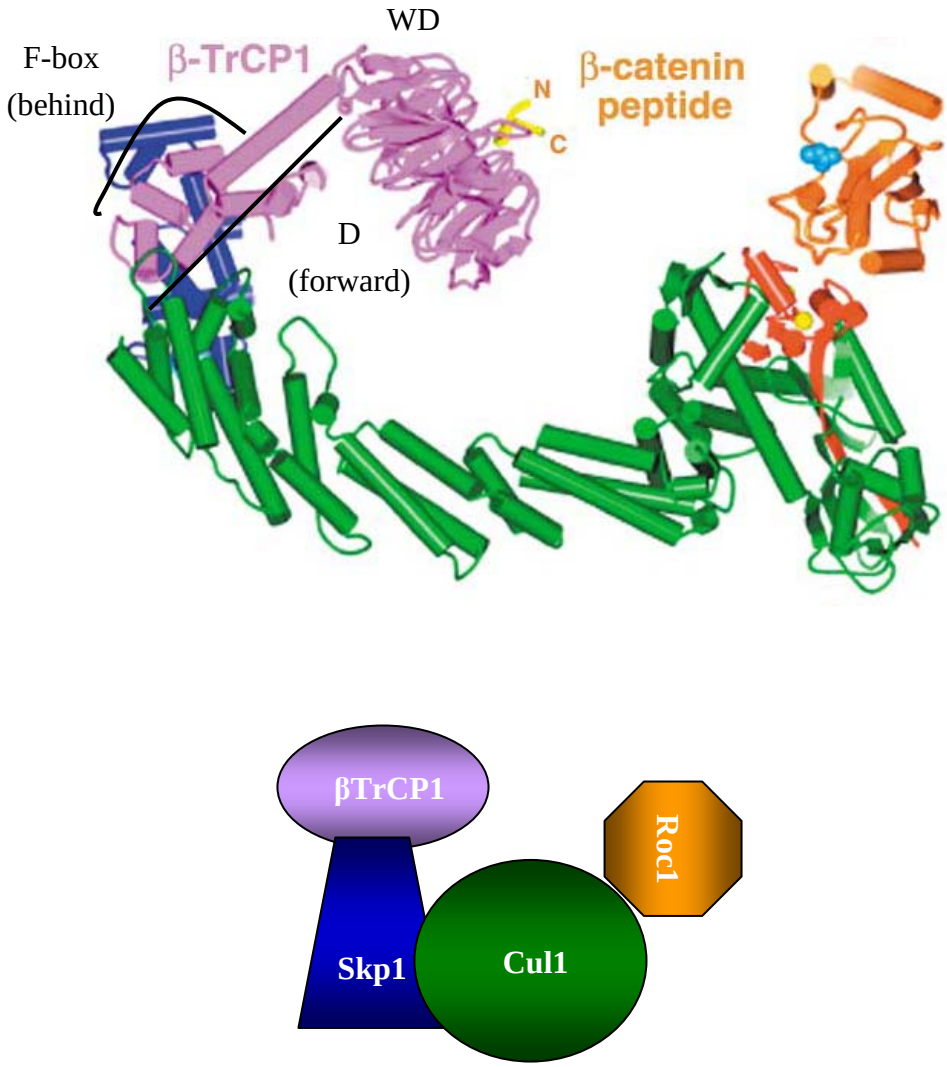


Figure 1.4 – SCF$^{\beta TrCP1}$ architecture. The SCF complex components and their crystal structure as modeled bound to β-catenin peptide. Roc1 is in orange, Cullin 1 is in green, Skp1 is in blue, and βTrCP1 is in purple. F-box, D, and WD domains are labeled. F-box helices are the three in back, the D domain helices are the three in front. Though it is shown as a monomer here, note that βTrCP1 binds as a dimer *in vivo*. The crystal structure is solved to 2.95 Å and taken from Wu G *et al*, 2003.

adenylate (121, 160). There are a handful of E1 enzymes in mammals; the E1 used in these studies is UBA1. The E1 then passes the ubiquitin to the ubiquitin-conjugating/carrier enzyme (E2) through a *trans*-thiolation reaction involving the C-terminus of ubiquitin (121, 160). There are a significant, but limited number of E2s, all of which share an approximately 150 amino acid conserved core domain (121, 160). Even though the core domains are highly conserved, the E2s work in conjunction with the E3s to specify the function of the ubiquitylation. This specification can be achieved through both direct binding and selection of the ubiquitin ligase (E3), and also the E2's ability to pass the activated ubiquitin to the E3, or directly onto the substrate (121, 160). In the case of this study, the E2 enzyme UbcH5b, recognizes both of the two major families of E3s (HECT and RING-finger, see below) and also may pass the ubiquitin directly onto the substrate while docked on the E3, depending on the class of E3 (67).

There are 500-1,000 different E3 ligases in mammals (121, 160). These enzymes not only recruit the substrate for modification, but they also have a very important role in specifying the outcome of the ubiquitylation reaction through their localization and linkage, as discussed below. These E3s divide into four classes according to their structural motifs: U-box, PHD finger, HECT and RING-finger (106, 121, 160). HECTs were the first characterized E3 family, and were considered the largest family until they were recently eclipsed by the RING-finger (Really Interesting New Gene) family. Now the largest family of E3s, the RING-family can be split into several sub-families, one of which is the cullin-based (Cul) family of E3s. The cullin-based family includes seven multi-protein complexes, all of which are assembled around a cullin protein backbone (106, 121, 160). The cullin proteins are distinguished by their N-terminal repeats of a 5

helix bundle (cullin repeats) and their distinct, globular C-terminal domains (cullin homology domain) (106, 121, 160). The cullin family is organized into two classes of ligases which often antagonize one another, especially in cell cycle regulation: APC/C (anaphase-promoting complex/cyclosome) and SCF (Skp1-Cul1-F-box) (106, 121, 160).

The SCF complex, is aptly named because it contains a cullin backbone linked on its C-terminus to a Skp1 adapter protein which, in turn, is linked to a substrate-recognition component containing an F-box motif. The F-box motif binds directly to Skp1 provides some of the fine control of the affinity of the ubiquitylation reaction when bound to substrate (for greater detail, see section 1.7 and the Discussion) (106, 165). F-box proteins are important regulators of cell-cycle components. For example, Skp2 is an E3 ligase to p27 and p21, important for cell cycle arrest, largely in the G1/S phase of the cell cycle, and c-myc, a well-characterized oncogene, Fbw7 is also an E3 ligase for c-myc, while the βTrCP proteins regulate many substrates, including β-catenin, and IκB, as mentioned above (106). The SCF is also attached through the N-terminus of Cullin1 to RBX1 (RING-finger protein, hereafter referred to as Roc1) which recruits the E2 to the SCF and also facilitates the linkage of activated ubiquitin to the substrate (see Figures 1.3 and 1.4) (106, 121, 160). The SCF complex which is relevant to these studies consists of a Roc1:Cul1:Skp1:βTrCP1 complex, denoted $SCF^{\beta TrCP1}$; but I have also shown that a Roc2:Cul1:Skp1:βTrCP1 complex could ubiquitylate p63 *in vitro* (see Appendix A). This SCF backbone works in conjunction with βTrCP1 to control the ubiquitylation of βTrCP1's substrates, as discussed below.

Now that the E1, E2, E3 cascade has been discussed in detail, it is important to note the variety of outcomes of this reaction. As mentioned above, what makes

ubiquitylation an important post-translational modification its incredible degree of versatility. Not only is there enormous potential for substrate specificity conferred by the mechanism of ubiquitylation, but there is much specificity conferred by the ubiquitin linkage itself. While most post-translational modifications involve the attachment of a single chemical group or peptide to the substrate, ubiquitin can also form a single modification (mono-ubiquitylation) or can form chains (poly-ubiquitylation) through linkage to one of its lysines, as mentioned above (see Figure 1.3) (106, 121, 160). Mono-ubiquitylation has several functions from degradation to protein silencing, while multi-monoubiquitylation (such as the mono-ubiquitylation on multiple sites that occurs on proteins such as p53) may have another range of consequences. Poly-ubiquitylation, where trimeric chains of ubiquitin are linked covalently to the first mono-ubiquitin on the substrate, is the most common form of ubiquitin, producing the characteristic laddering of ubiquitylated moieties seen in most assays. The mechanism of this poly-ubiquitylation is still largely unknown. However, it is known that the kinetics of the poly-ubiquitin attachment is much faster than the initial mono-ubiquitylation of the substrate, and that these triplicates of ubiquitin may be recycled in their covalent, trimeric form for subsequent poly-ubiquitylations (106, 121, 160). Further, the linkage of these chains through ubiquitin is being intensively studied as a potential mechanism for substrate fate. For example, most K48 poly-ubiquitylated proteins are marked for degradation by the 26S proteasome, while K63-linked molecules often are used in some signaling process (81). However, chain linkage is not a concrete predictor of substrate fate. In fact, while the field has long been operating on the assumption that there is only one type of linkage per ubiquitin chain, one group has demonstrated that branched chains are also possible.

A recent study showed that some E2s, such as UbcH5, have the ability of forming not only chains consisting of a single ubiquitin linkage but also branched multi-ubiquitin chains *in vitro* (67). Theoretically, these chains could be ablated by ubiquitin lysine mutants typically thought to recognize the linkage of an unbranched poly-ubiquitin chain, such as those I use in my study, but rather forming the "smooth" or "kinked" chains in the case of a K63, or K48 poly ubiquitin chain, these branched chains contained several lysine linkages and were not marked for 26S proteasomal degradation (67). Another consideration in the versatility of the ubiquitylation reaction is the sub-cellular localization of the E2 and E3 and how their roles may effect the outcome of ubiquitylation (81, 121, 160). For instance, E3s, such as MDM2, are also involved in subcellular shuttling of their substrates (101). As noted above, in the case of p53 this re-localization promotes its degradation, but in the case of p73 and p63 their relocalization by MDM2 disables their transactivation activity and does not affect their protein stability (101). Lastly, another complication in deciphering the purpose of an ubiquitylation event is that the modification itself does not always rely on the site specificity characteristic of other post-translational modifications (121, 160). In other words, ubiquitylation can occur with similar kinetics on any open lysine residue depending on the substrate. One of the exceptions to this trend is MDM2, which ubiquitylates itself on a very specific residue (101, 160). However, the site of the modification in most cases is more a function of the local environment, including other modifications on nearby residues (121, 160). Thus the outcome of ubiquitylation as a modification is exceedingly complex and depends not only on the E2:E3 exchange, but the open lysine residues at a specific time,

the sub-cellular localization of the substrate, and the ubiquitin linkage that exists at a given time under the ratio of E3 bound to substrate and competing DUBs.

1.7 βTrCP's gene and protein architecture dictate its substrate recognition

βTrCP proteins are a subfamily of F-box ubiquitin ligases that are highly conserved throughout evolution. The family includes Slimb in *Drosophila melanogaster*, a *Xenopus laevis* βTrCP, and includes both βTrCP1 (also E3RS/Fwd1/Fbw1a) (98) and βTrCP2 (also HOS, "Homolog of Slimb"/ Fbw1b) (33) in mammals. In human, βTrCP1 and βTrCP2 are encoded from distinct genes on 10q24, and 5q25 respectively, each comprised of 15 exons (34). *βTrCP1* and *βTrCP2* display an overall identity of 86%. A large part of this conservation is concentrated in the F-box domain and the WD domain, with the WD domain nearly having perfect sequence conservation (34). X-βTrCP and human βTrCP1 and βTrCP2 have multiple splice variants, all of which conserve both the F-box and WD domain (4, 5, 34, 36, 172). Human βTrCP1 has two variants while βTrCP2 has three, both differing by N-terminal exon splicing (4, 36, 75). βTrCP1 and βTrCP2 largely differ due to their patterns of subcellular localization. When exogenously expressed, βTrCP1 is largely nuclear while βTrCP2 is largely cytoplasmic. It is βTrCP1's nuclear localization which made it a prime candidate for interaction with p63 in this study. A summary of βTrCP1 gene and protein architecture is provided in Figure 1.5 (4, 5, 34, 36, 172).

βTrCP proteins have three distinct domains: an F-box domain, a D domain, and a WD domain. The N-terminal F-box domain allows βTrCP to link to Skp1 in the SCF complex, as noted previously, and consists of a 3 α-helical bundle characteristic of all F-box proteins (Figure 1.4) (165). A deletion mutant of the F-box domain (see Figure 1.5)

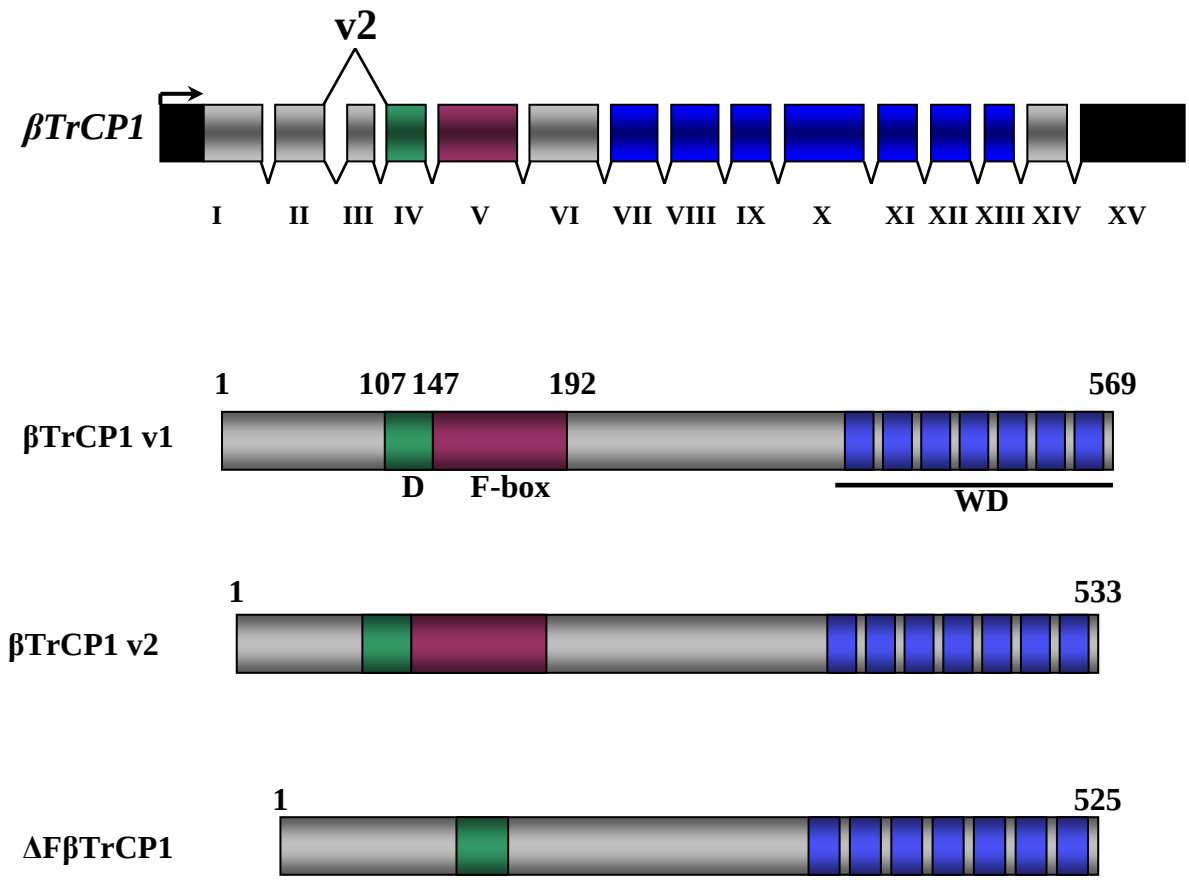


Figure 1.5 - βTrCP1 gene and protein architecture. Top – βTrCP1 gene structure. 5' left, 3' right. Each exon is shown by roman numeral and the v2 splice variant is indicated. Exons are color coded according to protein domain below. Black regions are 5' and 3' UTRs, respectively. The arrow is the transcriptional start site. Bottom – βTrCP1 protein structure. N- terminus left, C- terminus right. βTrCP1 endogenous isoforms v1 and v2 shown with D, F-box and WD domains in green, purple, and blue respectively. ΔFβTrCP1 deletion mutant also shown.

substrate	binding motif	binding sites	βTrCP dimerization	function
ATF4/CREB2	DSGICMS	1	(homodimer)	transcription factor
β-Catenin	DSGIHS	1	homodimer	trancription factor/oncogene
hDlg	DSGLPS	1	(heterodimer)	cell adhesion/tumor suppressor
Fibronectin	DSGVVYS/ DSGSIVVS	2	(heterodimer)	cell adhesion
IkBα	DSGLDS	1	homodimer	inhibitor of NF-κB
IkBβ	DSGLGS	1	homodimer	inhibitor of NF-κB
IkBε	DSGIES	1	homodimer	inhibitor of NF-κB
p105	DSGVETS	1	homo/heterodimer	precursor/inhibitor of NF-κB
p100	DSAYGS	1	homo/heterodimer	precursor/inhibitor of NF-κB
Snail	DSGKGS	1	?	cell adhesion
Vpu	DSGNES	1	homo/heterodimer	viral coat protein/pseudosubstrate
Cdc25a	DDGFLG(x)	2	(heterodimer)	cell cycle promoter
Cdc25b	non-phosphorylated/ charged	1	(heterodimer)	cell cycle promoter (major isoform)
Emi1/2	DSGYSS and Cdk phospho-sites	2+	heterodimer	cell cycle inhibitor/inhibitor of APC
hnRNPu	N- and C-terminal acidic regions	2	homodimer	pseudosubstrate
PER1/2	TSGCSS	1	(heterodimer)	mammalian circadian regulation
p63α and γ	DSDLSD(?) and C-terminal	2	(heterodimer)	trancription factor
Pro-caspase 3	non-phosphorylated N-terminal 38 aa	1	(heterodimer)	apoptotic signalling cascade
Smad4	DLSGLTLQS	1	(heterodimer)	TGF-β transciption activation
Wee1A	non-canonical/S53, S123	11+	(heterodimer)	cell cycle inhibitor

Table 2 - βTrCP1 interacting proteins. A summary of the current literature. Parenthesis for dimerization indicates that heterodimerization shown by knockdown of both βTrCP1/2, versus direct binding or ubiquitylation experiments. References are noted in the text.

was also created to study βTrCP's function in development and cancer; I will use this deletion mutant in my later studies (also see section 1.9). The D domain, or dimerization domain, of βTrCP is part of the central region of the protein which forms a 4 α-helical linker to the WD domain. The D domain is important because the homo- or hetero-dimerization of βTrCP1 and βTrCP2 is controlled through this region. The WD region on the C-terminus is so named for its 7 WD40 repeats which form a propeller which binds the substrate. This propeller forms a cup-like structure, with the center of the propeller sinking in towards the F-box domain with the blades forming a highly charged binding surface to present the substrate to the E2 and Roc1 (Figure 1.4) (165). Structural analysis has demonstrated that substrate binding opens a channel within the cup of the WD domain which greatly influences the affinity and rate of ubiquitylation (34, 165)

The interaction between TrCP1 and its substrates relies heavily on charge, and substrates are often previously phosphorylated on their binding sites or rely on a cluster of highly-charged residues. The canonical βTrCP1 binding motif is a $DSG(X)_{2-3}S$ motif where the aspartic acid can be critical for binding and/or ubiquitylation (depending on the substrate) and the two serine residues are phosphorylated. A list of βTrCP1's many substrates and their known recognition motifs is provided in Table 2. Canonical domain-containing substrates are many of the first substrates identified for βTrCP1 and include ATF4/CREB2 (86), β-catenin (33, 44, 71, 87, 162), the human disks large tumor suppressor (hDlg) (97), fibronectin (125), IκBα (33, 45, 82, 135, 140, 142, 172), β (135, 164), and ε (135), NF-κB p105 (19, 110) and p100 (32), Snail (140), and Vpu (98). Non-canonical substrates are Cdc25a and b (13, 57, 62), Emi1 and 2 (40), hnRNPu (21), PER1 and 2 (136), pro-caspase 3 (145), Wee1A (156), SMAD 4 (170), and p63 (35). In the

case of canonical binding sites, the preferred lysine residue for ubiquitylation is typically 9-13 residues upstream of this site (34, 165). Further, proteins such as Emi1, Cdc25, and p63, as shown in this study, also harbor regions where binding seems to be dictated by a highly-charged cluster of residues, in addition to or independently of a canonical domain. Further, some substrates, as in the case with Cdc25, may also have their binding affinity regulated by a phosphorylation site upstream or downstream of their βTrCP1 binding site, while others, such as pro-caspase 3 and p63 may not require phosphorylation for function. Therefore phosphorylation and the presence of a canonical domain varies depending on substrate, but in all cases interaction with βTrCP1 is dictated largely by clusters of highly charged residues due to the nature of the WD domain.

1.8 βTrCP's history: the mystery partner to critical cell regulatory proteins

The importance of the canonical motif and understanding of its function is what originally led to the identification and cloning of βTrCP. Early reports identified the canonical motif of IκB (DpSGXXpS) as essential for its ubiquitylation and degradation (171). Further, it was noted that there was a distinct similarity between this "destruction motif" in IκB to a similar motif identified in β-catenin, a potent oncogene involved in the Wnt signaling pathway, implying that the ubiquitylation and degradation of both of these important cell fate regulators was mediated by the same E3 ligase (1, 109). The next year, it was shown that deletion of the Slimb gene in *Drosophila*, which encoded an F-box/WD repeats-containing protein, led to the accumulation of the β-catenin ortholog, Armadillo; these data implied that a mammalian homolog of Slimb might regulate the ubiquitylation and degradation of both IκB and β-catenin (55). Concomitantly, another group found human βTrCP1 as a protein pulled down during a yeast-2-hybrid screen for E3 ligases

interacting with the HIV-1 Vpu (Human immunodeficiency virus type-1) protein (98). Vpu also harbored a similar canonical interaction motif (98). Later, βTrCP was found to be the E3 ligase involved in IκB and β-catenin degradation as well (33, 172).

Since then, the list of βTrCP1 regulated substrates has grown to include many proteins critical for regulation of the cell cycle, as well as growth and death pathways (see Table 2 and reviewed in (34, 106)). Other than several isoforms of IκB, βTrCP1 has been shown to target the NF-κB subunits p105 and p100 (2, 19, 32, 110). It also targets Cdc25a/b, Emi1 and Wee1, important cell cycle regulators. The degradation of Cdc25a/b, cell cycle promoters, is triggered by a Chk1/2 phosphorylation cascade that marks Cdc25a/b for degradation (13, 57, 62). This degradation promotes G1/S phase cell cycle arrest. Emi1 and Wee1 are cell cycle inhibitors which need to be degraded in G2/M for mitosis to progress (40, 156). Other substrates include the (hDlg), the ATF4/CREB2 transcription factor, Snail, and PER1/2 (additional information in Table 2). Overall, it is clear that βTrCP1, as with other F-box proteins, is highly involved in the regulation of cell division and fate; therefore it is more than reasonable to suggest that p63 might also grace this list.

1.9 βTrCP1's biological functions

Since βTrCP1 touches so many important cell regulatory pathways, including those controlling both cell growth promotion (ie. Wnt pathway inactivation) and arrest (ie. NF-κB activation) it is unsurprising that, like p63, βTrCP1's specific role in cell fate and cancer development is under debate. *Slimb* mutants in *Drosophila* exhibit centrosome overduplication and mitotic defects (163). A murine knockout model for *βTrCP1* showed defects in male fertility most likely due to the accumulation of spermatocytes in

metaphase I (40). Additionally, *βTrCP1*$^{-/-}$ MEFs have an impaired growth rate, most likely due to a high rate of polyploidy, centrosome overduplication, and impaired mitotic progression (40, 104). These defects are largely due to stabilization of Emi1 (40, 104). Therefore depletion of βTrCP1 greatly impairs cell division and growth.

βTrCP1's role in cancer development is murky at best, as both mouse models and human tumors demonstrate that deregulation of TrCP1, either by up- or downregulating its function, is detrimental. Mice transgenic for βTrCP1 in the mammary epithelium have increased ductal branching and increased development of alveolar structures. This increased proliferation is associated with higher NF-κB DNA binding and nuclear localization, and an increase in tumorigenesis over a usual lifespan (83). In contrast, *βTrCP*$^{-/-}$ mice do not have a tumorigenic phenotype, though in the case of this model, there may be some compensation from wild-type *βTrCP2* (40). Further, a recent study showed that mice transgenic for expression of either transgenic human *βTrCP1* or *ΔFβTrCP1* showed an increased incidence of tumors (8). This study not only demonstrates that either up or downregulation of βTrCP1 may be tumorigenic, but also that dosage of βTrCP1 is important. This model echoes previous results from cell culture assays which show that an increase in βTrCP1 itself may act as a dominant-negative in cases where exogenous expression outpaces the upregulation of SCF components (8). These mouse studies also corroborate the data from human samples.

Human tumors also show increased tumorigenesis with down- and upregulation of βTrCP1. One gastric cancer study found a mutation in βTrCP2 which led to an amino acid substitution in the 7th WD40 repeat domain – a domain that is conserved in βTrCP1 (132). Further, a study of lung cancer cell lines demonstrated that βTrCP1 is often lost

and that this loss correlated with the promotion of cell growth and motility through Cdc25 and MMP11 regulation, respectively (46). This study is in contrast to the murine models, and demonstrates that βTrCP1's role in cell growth may differ depending on tissue type and the pathways which are involved, as Cdc25 is largely relevant in human lung cancer, versus the NF-κB activation seen in the mouse studies. Also, in human prostate cancers, alterations in βTrCP1 were found in about 10% (2 out of 22 samples) (36). An intriguing finding in this study is that one of the alterations mimics the βTrCP1v2 splice variant found in the initial cloning study, generating a deletion of amino acids 17-73 in the N-terminal region, which functioned similarly to the ΔFβTrCP1 truncation mutant as far as its dominant-negative role in cell growth (36). Further, all of the alterations in this case were heterozygous, implying that they are either dominant-negative or haploinsufficient (36). Therefore, this study emphasizes the importance of studying the dominant-negative forms of βTrCP1 and also that some of the phenotypic complexity may arise from a yet-undiscovered imbalance with the v2 isoform. Our study provides further evidence of the differences in βTrCP1 versus ΔFβTrCP1 ubiquitylation and control of p63.

However, other tumor types have increased levels of βTrCP1. In a study of hepatoblastoma, 100% of the 23 samples examined showed increased expression of βTrCP1, with the βTrCP1 protein significantly increased in both the nucleus and cytoplasm, that was independent of the β-catenin mutational status (73). Further, 25 out of 45 (56%) of colorectal cancer samples showed an increase of βTrCP1 in conjunction with increases in both the NF-κB and β-catenin levels; these increases also correlated with poor prognosis (113). In this case, βTrCP1 overexpression may in part be due to a

stabilization of βTrCP1 mRNA caused by an increase in Wnt signaling (106). Further, chemoresistant pancreatic cell lines displaying constitutively active NF-κB activity also show significantly increased βTrCP1 levels (103). When treated with RNAi for βTrCP, the NF-κB levels drop, and the cells become more sensitive to chemotherapeutic agents. Therefore, the biological role of βTrCP1 is very complex and encompasses issues such as the balance of gene dosage through mRNA and protein expression of wild type βTrCP1, its subsequent sub-cellular localization, expression of mutant forms of the protein, including dominant-negative forms, potential isoform regulation, and the feedback regulation from various signaling pathways. As most of the βTrCP1 literature is largely confined to the β-catenin and NF-κB signaling pathways, my study showing a novel interaction of βTrCP1 with p63 is an important contribution to the field and provides another lens through which the current tumor data might be interpreted.

In summary, p63 is a transcription factor important for the control of individual and shared p53 family target genes. The alteration of its six isoforms is a hallmark of genetic disease and cancer and its various isoforms have been associated with both oncogenic and tumor suppressive phenotypes *in vivo*. Yet it remains a mystery why p63 is rarely mutated in cancer, and its levels are often upregulated. One mechanism for this upregulation could be the alteration of its stability by post-translational modifications, such as ubiquitylation. The purpose of this study is to discover and define a novel p63 ubiquitin ligase and describe its effect on p63 activity. I found a candidate ubiquitin ligase, $SCF^{\beta TrCP1}$, through an examination of the literature on the *p63* and *IKKα* knockout models. $SCF^{\beta TrCP1}$ was characterized previously for its importance in the regulation of

several growth regulatory pathways. βTrCP1 works in parallel with the IKK kinase complex to regulate the NF-κB pathway and other common targets. While genomic studies and concurrent studies by our laboratory have shown that *IKK* is a downstream target gene of p63 and that IKKα and IKKβ can phosphorylate TAp63γ, respectively, this project focuses on the regulation of p63 by βTrCP1. I hypothesized that βTrCP1 could regulate p63's ubiquitylation and activation. The information provided by this study will be important not only in the elucidation of the mechanisms governing the fine regulation of the p63 isoform balance in disease, since p63's isoform balance is critical to cell fate and often controlled by its protein stability, but also to understand more about how βTrCP1 might link p63 to other pathways critical for development and tumorigenesis.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell culture

Hi-5 insect cells were maintained in HfQ SFX-insect cell media (Perbio) with 1x penicillin-streptomycin (Cellgro) at 25°C. Human embryonic kidney epithelial cells (HEK293) and human non-small cell lung carcinoma cells (H1299) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1x penicillin:streptomycin (Cellgro), in an 37ºC incubator with 5% CO_2 (173). Cells were passed by rinsing once in about 5 mls 1x phosphate buffered saline (PBS), treating with 1 ml 0.5% trypsin:EDTA for 30 sec (Invitrogen), removing the trypsin and allowing the digest to occur for approximately 5 min. Then the cells were brought up in 5-10 mls of media and distributed to plates at an appropriate experimental dilution (1:7 for appropriate transfection confluence). Embryonic MEFs were maintained similarly to above in our DMEM with antibiotic also containing 20% FBS, 1x non-essential amino acids, and 50 mM 2-mercaptetoethanol, and followed the same passage procedure as above with no more than a 1:3 dilution (105). HaCaT and HEKn-E6/E7 cells were obtained from Dr. Mihail Iordanov, OHSU (11, 52). They were cultured as described in his publication, using complete (low calcium) keratinocyte medium (Gibco), in a 37ºC, 5% CO_2 incubator. Cells were passed by rinsing with a PBS rinse, followed by treatment with 2 mls of 2.5% trypsin:EDTA for 5 minutes. The digestion was stopped using 8+ mls of a 4% FBS in PBS solution. The cells were then centrifuged at 1.5 K for

5 mins, the supernatant was removed and the cells were plated at no more than a 1:5 dilution for experiments.

2.2 Antibodies and vectors

p63 was detected using Pab4A4 antibody (Santa Cruz) or p63-NTA antibody (generated by our laboratory). Polyclonal α-GFP antibody (Santa Cruz) was used for exogenous GFP-p63 IPs, and α-his antibody (Qiagen) was used to detect his-tagged p63, as indicated. βTrCP1 was detected using α-FlagM2 antibody (Sigma), or endogenous βTrCP1 antibodies, N-15 or H-300 (Santa Cruz). Tubulin was detected using antibody from Sigma. p21 was detected using p21 polyclonal #sc-H164 (Santa Cruz). Bax was detected using Bax Ab5 (Neomarkers). pCDNA3.1 empty vector, pEGFP-TAp63γ (and related mutants F1-F4), pEGFP-ΔNp63γ, pCDNA3his-ubiquitin were generated as described previously (95, 173). pEGFP-TAp63γ multiple K mutants were generated by J.R.G. and Hunjoo Lee, and pEGFP-TAp63γD61R and pEGFP- TAp63γS62A were generated by Mary MacPartlin. The p63 sequence used for cloning and a summary of mutations is provided in Figure 2.1. pCDNA3Flag-βTrCP1, pCDNA3Flag-ΔFβTrCP1, and baculoviral expression constructs of Roc1. HA-Cul1, his-Skp1, and Flag-βTrCP1 were kindly provided by Dr. Yi Sun, University of Michigan, as was the bacterial GST-Roc2 (144).

2.3 Transient transfection/siRNA transfection

Transient transfection used Transfectin (Biorad) reagent according to the manufacturer's directions at approximately 70% confluence for all cell lines. For each experiment, a 1:2 μg ratio of pEGFP-TAp63γ to pCDNA3-FlagβTrCP1 vector, or a 1:2.5 μg ratio of pEGFP-TAp63γ to pCDNA3-FlagΔFβTrCP1 vector was used and scaled to

1 {msqstqtnef lspevfqhiw dfleqpicsv qpidlnfvde psedgatn*k*i

50 eismdcirmq d}sdlsdpmwp qytnlgllns mdqqiqngss stspyntdha

100 qnsvtapspy aqpsstfdal spspaipsnt {dypgphsfdv sfqqsstaks

150 atwtystelk klycqiaktc piqikvmtpp pqgaviramp vykkaehvte

200 vvkrcpnhel srefnegqia ppshlirveg nshaqyvedp itgrqsvlvp

250 yeppqvgtef ttvlynfmcn sscvggmnrr piliivtlet rdgqvlgrrc

300 fearicacpg rdr*k*adedsi r*k*qq}vsdst*k* ngdgt*k*rpfr qnthgiqmts

350 i*kk*rrspd{de llylpvrgre tyemll*k*i*k*e slelmqylp}q htietyrqqq

400 qqqhqhllqk hllsacfrne lveprretpk qsdvffrhsk ppnrsvyp

Figure 2.1 – Annotated TAp63γ sequence used for cloning. The amino acid sequence of TAp63γ, NCI accession number AAC62633, with amino acid residue number shown. TA, DBD, and OD are bracketed, respectively, N- to C- terminus. Purple is F1 fragment, blue is F2 fragment, all green F3 and green underline is F4. Orange are putative βTrCP1 binding regions. Red are K residues, italicized K residues are point mutants screened for ubiquitylation sites. Point mutants in N-terminal canonical binding site are in gray. K mutants were generated by Hunjoo Lee and JRG, D61R and S62A mutants were generated by Mary Mac Partlin.

the appropriate size of the plate (for ChIP, 0.2 μg of pEGFP-TAp63γ: 2 μg of pCDNA3-FlagβTrCP1 or 2.5 μg of pCDNA3-FlagΔFβTrCP1 was used per 10 cm plate). siRNA oligo infection was performed using cells at approximately 50% confluence. For each experiment a βTrCP1/2 siRNA oligo pool (Santa Cruz) or scramble siRNA oligo mix was added to each plate at a final concentration of 100 nM. The cells were harvested 48 hrs post-transfection.

2.4 Preparation of purified his-TAp63γ, and GST-Flag-βTrCP1

All of the his-TAp63γ, GST-Flag-βTrCP1, and his-TAp63γ fragments (F1-F4) were purified from bacterial cell lysates as follows (95, 173): bacteria expressing each of the his-p63 vectors were grown to log phase in 500 ml LB broth (Sigma) at 37ºC and induced with 1 mM IPTG (Fisher) at 37ºC for 6 hrs, the bacteria were then centrifuged at 3.5 K for 20 min at 4ºC, the supernatant was decanted and the pellet was resuspended in 20 mls nickel lysis buffer (50mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0) with protease inhibitors. After the resuspension, the bacterial solution was lysed 3x using a French press. The lysate was then centrifuged at 10 K for 20 min at 4ºC to remove the debris. 1 ml pre-washed Ni-NTA agarose bead slurry (Qiagen) resuspended in nickel lysis buffer was added to the clarified lysate and incubated at 4ºC for 1 hr. The bead was then precipitated using a 1 min spin at 6K and washed three times in 10 mls nickel wash buffer (50mM NaH_2PO_4, 300 mM NaCl, 60 mM imidazole, pH 8.0) for each wash using the same centrifugation conditions. At the last wash, the beads were loaded onto a polystyrene elution column where six 200 μl fractions were collected using nickel elution buffer (50mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). GST purification was by a similar protocol, but the bacteria were resuspended in PBS with protease

inhibitors before French press, the GSH bead was pre-washed in PBS and 1 ml of 50% slurry was added to the clarified lysate at room temperature for 3-5 minutes. The beads were then washed using PBS. In the case of unbound protein, the eluted fractions were pooled according to concentration and dialyzed in 4 L of Buffer C-100 (20 mM Tris-Cl pH 7.5, 100 mM KCl, 0.2 mM EDTA, and 20% glycerol) for at least 4 hrs. Purification of his-TAp63γ by Poros affinity column was performed by Yetao Jin and J.R.G. His-TAp63γ was purified using Ni-NTA purification, followed by purification on a Poros HQ affinity column (PerSeptive Biosystems). All bead and protein preparations were analyzed by running a fraction of purified sample on and SDS gel followed by staining with Coomassie.

2.5 Western blot analysis (WB)

Clarified whole cell lysates were loaded directly onto an SDS gel and probed with antibodies, as noted in the Figure Legends (95, 173). The standard protocol was as follows: samples were lysed using a standard WB lysis buffer (50mM Tris-HCl, pH 7.5, 5mM EDTA, 150mM NaCl, 0.5% NP-40) for 10 min at 4ºC, the lysates were then clarified using centrifugation at high speed for 10 min at 4ºC and the protein concentrations were read using the Biorad protein assay reagent according to the manufacturer's directions. Once the samples were standardized, they were suspended in 1x SDS sample buffer (50 mM Tris-Cl, pH 6.8, 100mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and boiled for 7 minutes on a heat block. The moisture was brought to the bottom of the tube with a 10 sec centrifugation at high speed before loading onto an 8-10% SDS-PAGE polyacrylamide gel. The gels were then transferred to activated PVDF membrane (Poros) using a semi-dry transfer apparatus (Biorad) for 52

min at 15 v. After transfer the membrane was blocked in a 5% milk solution for 1 hr, before a 5 min rinse and treatment with primary antibody for one hour at room temperature with rocking, 3 washes for 10 mins each, incubation in secondary antibody for 30 mins, and 4 washes for 10 mins each. The washing and blocking buffers were 1x TTBS (20 mM Tris, 137 mM NaCl, 0.05% Tween 20). Blots were probed using Pierce chemiluminescence reagent. Band density was calculated using Adobe Photoshop and Optiquant software and normalized to tubulin. For WB indicating protein levels after co-expression, cells were harvested 48 hrs post-transfection.

2.6 *In vitro* binding

Protein-protein association assays were conducted using the beads and fractions were prepared according to the protocol above (95, 173). GST0 or GST-βTrCP1 bead was mixed with 200-600 ng of each his-TAp63γ preparation and incubated with constant agitation for 30 mins at room temperature. The samples were then washed with 1 ml volumes of the following buffers: 1x WB lysis buffer, 2x SNNTE buffer (50mM Tris-HCl, pH 7.4, 5mM EDTA, 500mM NaCl, 1% NP-40, 50% sucrose), 1x WB lysis buffer. Between each wash, the samples were centrifuged for 30 sec at 6K. The final samples were approximately 30 μl containing 1x SDS loading buffer.

2.7 Immunoprecipitation (IP)

GFP-TAp63γ, GFP-ΔNp63γ, and Flag-βTrCP1 vectors were co-expressed in HEK293 or H1299 cells, according to protocols above and the Figure Legends. Clarified whole cell lysate (concentration indicated per figure) was pre-bound for 2 hrs using 2 μg antibody, monoclonal, or polyclonal IgG, at 4°C, then 30 μL protein G agarose bead slurry (Santa Cruz) was added and the samples continued to rotate at 4°C overnight. The

samples were then washed 1x with WB lysis buffer, 1x with SNNTE buffer, and a second time with lysis buffer. The samples were then prepared using SDS sample buffer and analyzed by WB according to the above protocols. For endogenous assays, the co-IP was performed using the noted endogenous antibodies under the same conditions as above with 800 μg protein lysate harvested from keratinocytes.

2.8 Stability assays

Cells were plated onto 60 mm culture dishes and transfected, as noted above, for regular transfection or siRNA depletion. 48 hrs post transfection, cells were treated with 100 μg/ml cycloheximide and harvested at the indicated time points before preparation for WB.

2.9 Semi-quantitative and quantitative (real-time) PCR

For RT-PCR, cells were plated according to the protocols above. If transfection was used, cells were harvested 24-48 hrs post-transfection. The RNA was isolated using TRIZOL reagent (Invitrogen), according to the manufacturer's directions. cDNA was produced and PCR was performed using our previous protocol and the same primers for *p63*, *p21*, *β-actin* and *Bax*, as noted previously (95, 173). *βTrCP1* primers were as follows: 5'-ATTGTGCCCAAGCAGCGGAAAC-3'and 3'-TGTTCTCAGCGATGTGGTCCAG-5'. Endogenous mouse TAp63 primers were kindly provided by Dr. M. Koster in Dr. D. Roop's laboratory. Image J software was used to estimate band density. Quantitative (qPCR) and qRT-PCR was performed with 2 μL cDNA, as described previously, using the listed primers against *p21* and *GAPDH* (91). Levels were normalized to input and *GAPDH*, then graphed using Microsoft Excel. Data was analyzed for statistical significance using the Student's-T test.

2.10 Flow cytometry

H1299 cells were transfected with vectors as indicated. After 1.5 days, the cells were treated with 150 ng/mL nocodazole to arrest them in G2. The cells were then harvested according to the following protocol: the cells were washed 1x with PBS, trypsinized as for passage and the original media was added back to neutralize the reaction and also for a record of any apoptotic cells, the samples were then washed once in PBS and fixed with 4% paraformaldehyde in PBS for 15 mins, washed 1x with PBS, permeabilized in 0.1% triton X-100 in PBS 2x for 5 mins, washed 2x with PBS, and stained with 300 μl of staining buffer containing 0.1 mg/ml propiduim iodide (PI), 0.2 mg/ml RNase A, and 0.1% triton X-100, for at least 60 mins at 4ºC (95, 173). Cells were sorted for the GFP tag as a positive control for transfection and then analyzed for DNA content with a Becton Dickinson FACScan flow cytometer. Data were analyzed using a multicycle software program (FloJo) using a polynomial S-phase algorithm.

2.11 Chromatin immunoprecipitation (ChIP)

H1299 cells were transfected according to the protocol above. 24 hrs post-transfection, the cells were fixed and processed according to the following protocol (91): the cells were fixed with a final concentration of 1% formaldehyde added directly to the media and were rocked gently at room temperature for 10 mins, then 0.125 M final concentration of glycine was added to the plates and they were rocked for an additional 5 mins at room temperature. The cells were then washed twice with PBS, scraped into 1 ml of PBS with protease inhibitor and centrifuged for 10 mins at 3 K to pellet the cells. For these assays, two 10 cm plates of cells were collected and fixed individually for each assay before being combined for lysis and the following IPs. After the spin, the

supernatant was removed and the pellet was reusupended in 1 ml RIPA lysis buffer (150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, and 5 mM EDTA) containing protease inhibitors and sonicated 10x for 10 sec each with a 1-2 minute rest on ice between each cycle to yield chromatin fragments of approximately 600-1000 bp. The debris was then removed by centrifugation for 10 min at high speed. The supernatant was retained and precleared with a 1:1 50 % protein A agarose: protien G agarose bead slurry for 30 mins at 4ºC. The bead was removed with a centrifugation of 6 K for 30 secs, and the supernatant was removed. 50 µl of this supernatant was kept and the rest was used for IP. The IP was carried out with 2 µg ChIP-grade Pab4A4 (Abcam), or 2 µg monoclonal IgG (Sigma) with rotation overnight at 4ºC, with the 50 µl of 50 % protein G agarose slurry added 2 hrs from the initiation of the IP step. The following day, the beads were washed as follows, using 1 ml solution each time and centrifuge steps of 30 sec at 6 K between each wash: 2x RIPA buffer, 4x ChIP wash buffer (100 mM Tris, pH 8.0, 500 mM LiCl, 1% NP-40, and 1% deoxycholic acid), and 2x RIPA buffer. After all of the RIPA from the final washed was removed, 150 µl of elution buffer (50 mM $NaHCO_3$, and 1% SDS) was added to each sample, it was rocked for 15 minutes at room temperature, and this elution was repeated. The elutants were combined and spun quickly to remove any residual bead. The input samples, which were stored at -80ºC, each received 250 µl of the elution buffer as well. All samples were treated with 1.5 µg/ml final concentration of RNase A before being incubated at 65ºC overnight. 2 µL of the purification elutant was loaded onto a 96 well plate and qPCR was performed as noted above using several previously published primer sets against regions of the *p21* promoter or gene (38). Each sample was normalized to input using Microsoft Excel.

2.12 Preparation of Roc1-SCF$^{\beta TrCP1}$ Complex

Hi-5 cells were maintained in HfQ SFX insect cell medium (Perbio, Belgium) with 1x penicillin-streptomycin (Cellgro, Herndon, VA) at 25°C. Baculoviral expression constructs of Roc-1, HA-Cul1, his-Skp1, and Flag-βTrCP1 were titered for optimal expression in Hi-5 cells. Hi-5 cells were grown to approximately 50% confluence on 100 mm plates before infection. The constructs were infected separately for maximum expression. After 4 days, the cells were then harvested and centrifuged at 3000 rpm for 10 mins at 4°C. The medium was removed, and the cells were washed once in 20 ml of PBS and centrifuged a second time. The pellets were lysed by vigorous pipetting and repetitive freeze-thaw in nickel lysis buffer (50 mM NaH_2PO_4 pH 8, 300 mM NaCl, and 10 mM imidazole) with phenylmethylsulfonyl fluoride and pepstatin A. Lysates were spun at high speed for 10 mins to remove debris, and the supernatants from each construct were combined. The combined lysis was rotated at 4°C for 2 hrs, and then 1 ml of clean 50% Ni-NTA agarose bead slurry (Qiagen, Valencia, CA) was added. The bead was rotated for an additional 2 hrs. The lysate was then centrifuged at 6000 rpm for 3 mins, and the supernatant was removed. Bead pellet was resuspended in Ni wash buffer (50 mM NaH_2PO_4 pH 8, 300 mM NaCl, and 20 mM imidazole) and loaded onto an elution column where it was washed with 1 ml of Ni wash buffer for six times. Proteins were then eluted in 200 µl of a Ni elution buffer (50 mM NaH_2PO_4 pH 8, 300 mM NaCl, and 250 mM imidazole) for six fractions. The fractions were pooled into two groups, according to concentration, and dialyzed in 4 L of Buffer C-100 (20 mM Tris-Cl pH 7.5, 100 mM KCl, 0.2 mM EDTA, and 20% glycerol) for at least 4 hrs. The fractions were checked by WB using polyclonal anti-Roc1 (Neomarkers, Fremont, CA), monoclonal

12CA5 α-HA (generated in Lu Laboratory), monoclonal α-Flag (Sigma), and monoclonal α-his (Covance, Princeton, NJ) antibodies to detect separate complex components. A typical Coomassie for this complex and the accompanying WB are shown in Figure 2.2.

2.13 Preparation of Roc2-SCF$^{\beta TrCP1}$ Complex (Appendix A)

The Roc2-SCF$^{\beta TrCP1}$ complex was prepared as above, except for the Roc2, which was purified separately before complex reconstitution. GST-Roc2 was grown in LB broth to log phase and induced using 0.4 mM IPTG (Fisher, Chino, CA). Bacteria were harvested after 6 hrs at 37°C, centrifuged at 35,000 for 20 mins, and lysed in 20 ml of bacterial lysis buffer (1x PBS, 10% glycerol, and 0.1% NP-40) by French press. The lysate was spun at high speed for 10 mins to remove cell debris and then incubated for 5 mins at room temperature with 500 µl of GSH slurry. After binding, GST-Roc2 beads were washed three times in GST wash buffer (1x PBS, 10% glycerol, and 500 mM NaCl) and eluted using 5 mM reduced glutathione (Sigma). The fractions were dialyzed using the same conditions as above, and the protein was examined for concentration and purity using SDS-PAGE followed by Coomassie staining. The fraction with the highest purity was combined with insect lysates obtained by the same procedure as above. After the steps noted above, purified complex fractions were rebound to GSH beads, washed twice in lysis buffer (50 mM Tris pH 7.5, 5 mM EDTA, 150 mM NaCl, and 0.5% NP-40), and diluted to 50% slurry. *In vitro* ubiquitylation was performed on the same day to maintain complex activity.

2.14 *In vitro* ubiquitylation assays

The SCF$^{\beta TrCP1}$-mediated *in vitro* ubiquitylation assay was performed similarly to previous assays (50), with some modifications. The p63 *in vitro* ubiquitylation assay was

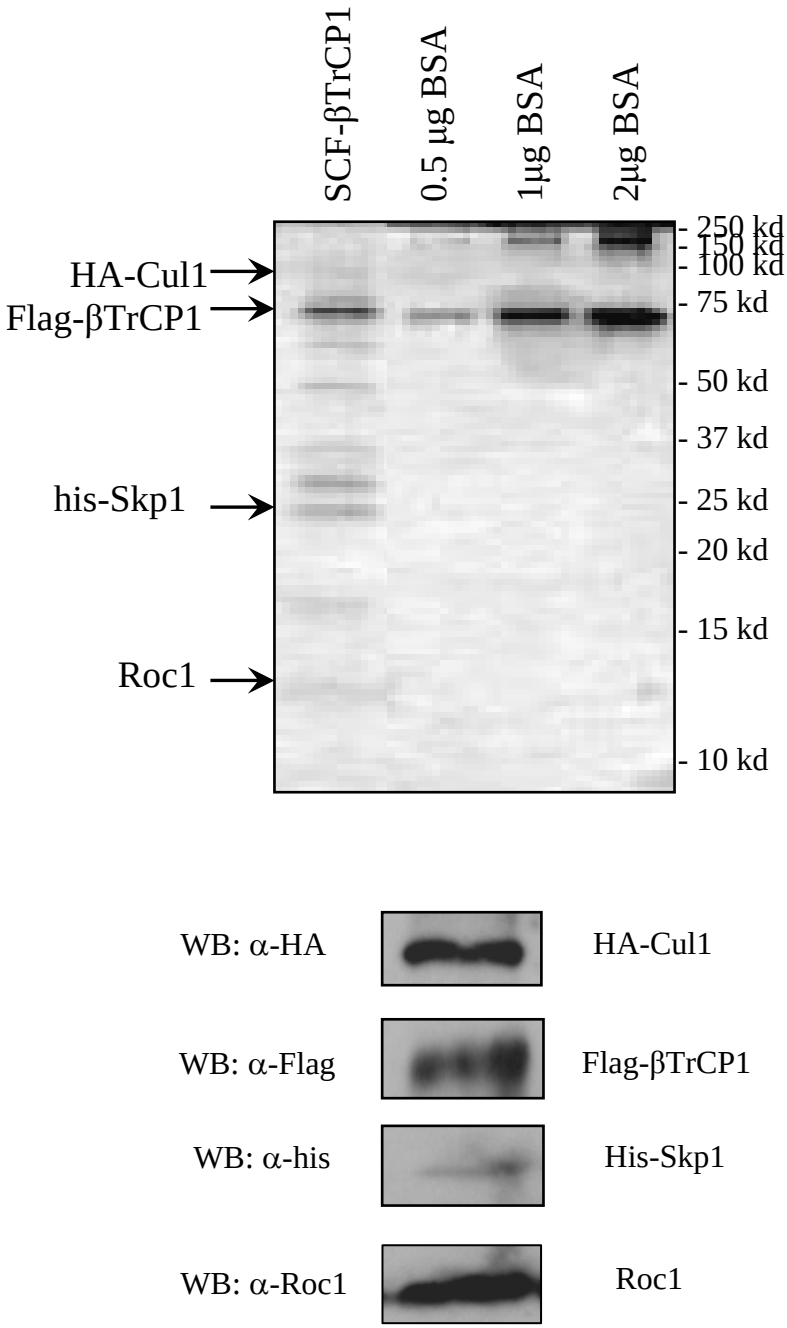


Figure 2.2 – SCF$^{\beta TrCP1}$ purification. SCF$^{\beta TrCP1}$ was purified as noted, and 30 μl complex was run on a 8%-15% gradient SDS gel and stained with Coomassie (top). Bands for each protein are noted. Below, WB for 10 μl complex stained with noted antibodies.

carried out in 40 μl of a reaction mixture containing 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 2 mM NaF, 3 μM okadaic acid, and noted concentrations of his-TAp63γ, 10 μL $SCF^{\beta TrCP1}$, 400 ng of UBA-1 (Boston Biochem), 400 ng of UbcH3 (Boston Biochem), 1000 ng of HA-ubiquitin, 5 mM ATP (Amersham Biosciences), and 1.5 mM ATPS (Fisher ICN). The mixture was incubated at 37°C for 1.5 hrs and analyzed afterward by SDS-PAGE electrophoresis followed by WB, as noted above.

2.15 *In vivo* ubiquitylation assays

10 cm plates were transfected as noted above. 24 hrs post-transfection, the cells were treated with 10 μM MG132 (a protease inhibitor) for 6 hrs. The cells were harvested and the pellet was split in half. Half underwent standard WB sample preparation (above), and half was prepared using our denaturing protocol (59) as follows. The cell pellet was harvested in 250 μl fresh buffer A (6M guanidine-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, pH 8.0, 0.01 M Tris, pH 8.0, 5 mM imidazole, and 10 mM β-mercaptoethanol) and thoroughly dissolved in the lysis buffer with vigorous pipetting. 50 μl of a 50 % Ni-NTA agarose slurry washed and resuspended as noted above in Ni-NTA wash buffer was added and the samples were rotated at room temperature for 4 hrs. Then the beads were washed three times with 1 ml of each of the following buffers with centrifugation steps of 30 sec at 6 K between each buffer change to sediment the bead: buffer A, followed by buffer B (8M urea, 0.1 M Na_2HPO_4/NaH_2PO_4, pH 8.0, 0.01 M Tris, pH 8.0, and 10 mM β-mercaptoethanol), followed by buffer C (8M urea, 0.1 M Na_2HPO_4/NaH_2PO_4, pH 6.3, 0.01 M Tris, pH 8.0, and 10 mM β-mercaptoethanol). On the last wash with buffer C, 900 μl of the wash buffer was removed, and 100 μl of the wash buffer was used to push the bead down the sides of the tube for complete collection before precipitating the bead

with another spin and removing all of buffer C. 20 μl of elution buffer D, was then added (200 mM imidazole, 0.15 M Tris, pH 6.7, 30% glycerol, 0.72 M β-mercaptoethanol, and 5% SDS) and the samples were then agitated at room temperature for 20 mins, before SDS loading buffer was added to 1x final concentration and the samples were boiled as for WB. All the samples were then run on an 8% SDS-PAGE gel, transferred and probed as in the standard WB protocol.

CHAPTER 3

ACTIVATION OF p63 BY βTRCP1

A majority of the contents of Chapters 3 and 4 was published in *The Journal of Biological Chemistry* under the title, "$SCF^{\beta TrCP1}$ activates and ubiquitylates TAp63γ" (35).

3.1 βTrCP1 expression stabilizes TAp63γ

To determine if βTrCP1 could act as a regulator for TAp63γ, I wished to determine if βTrCP1 would have an effect on the stability of TAp63γ. To answer this question, I used primary MEFs at passage 2 derived from *βTrCP1*$^{+/-}$ and *βTrCP1*$^{-/-}$ littermates, kindly provided by Drs. Keiko and Keiichi Nakayama (Kyoto, Japan) (104) and examined the endogenous levels of TAp63γ mRNA and protein. Strikingly, there was a significantly lower amount of TAp63γ protein in the *βTrCP1*$^{-/-}$ MEFs compared to the *βTrCP1*$^{+/-}$ MEFs (approximately 30%), though the TAp63β protein level was not affected (Figure 3.1, top). Since the p63 antibody is pan-specific, 03C keratinocytes were used as positive control for the p63 isoforms (70, 84). Further, when the mRNA levels were examined using semi-quantitative RT-PCR with TA-specific p63 primers, little change was observed (less than 0.5 fold) in TAp63 between the *βTrCP1*$^{+/-}$ and the *βTrCP1*$^{-/-}$ MEFs (Figure 3.1, bottom). Since the protein level of TAp63β is minor compared with the level of TAp63γ, by WB, this RT-PCR measurement was interpreted as representative of the TAp63γ RNA levels. It should be noted that while p63β levels are usually low in differentiated tissue, in this case I am confident that the upper band on the WB is in fact TAp63β, versus ΔNp63α, because of the 03C loading control and the

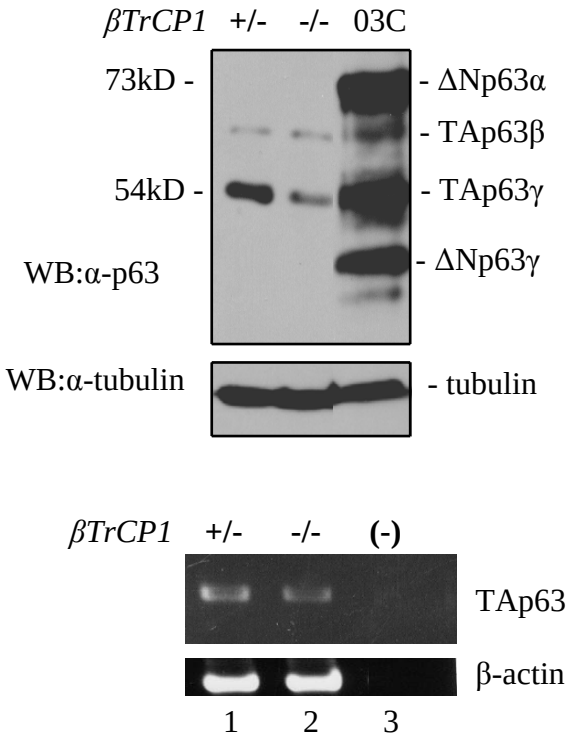


Figure 3.1 – The steady-state level of TAp63γ is reduced in *βTrCP1*$^{-/-}$ MEFs. *βTrCP1*$^{+/-}$ or *βTrCP1*$^{-/-}$ primary MEFs were harvested for either WB (top) or RT-PCR (bottom). In the WB, 150 μg whole cell lysate for MEFs was loaded beside 30 μg whole cell lysate from 03C mouse keratinocytes.

fact that when mRNA levels were examined with ΔN-specific primers there was not a detectible signal after more than 30 cycles (data not shown). Next, to determine if this observation was specific to βTrCP1 expression, I repeated this experiment in H1299 cells, a non-small cell lung cancer line that is p53 null and has undetectable endogenous expression of p63 and p73 by WB. In fact, in the presence of increased βTrCP1, the protein level of TAp63γ increased significantly above the level of TAp63γ alone (Figure 3.2, lane 4 vs. lane 2). This increase was also only at the protein level, as demonstrated by our later control in Figure 3.12. Therefore, TAp63γ protein levels are also increased by a subsequent increase of βTrCP1. These results suggest that βTrCP1 mediates TAp63γ's protein stability though a post-translational mechanism.

In order to verify these intriguing observations, I measured the half-life of exogenous TAp63γ in the presence of βTrCP1 by treating the cells with 100 μg/ml cycloheximide, a commonly used protein translation inhibitor, and looking at the protein half-life over a time course. Again, to my surprise, even though βTrCP1 has been shown to be involved in the degradation of its other substrates, my data demonstrates that βTrCP1 stabilizes TAp63γ. As shown in Figure 3.3, while the exogenous GFP-TAp63γ alone has a half-life of approximately 2-3 hrs, comparable with others' published data (129), the GFP-TAp63γ co-expressed with the Flag-βTrCP1 was highly stabilized over 6 hours. I also confirmed this result using ^{35}S labeled pulse-chase (data not shown).

Further, to examine the effect of βTrCP on endogenous p63 stability, I used a human keratinocyte line with several p63 isoforms and βTrCP1 (HaCaT). The cells were treated with siRNA oligos against βTrCP1/2 (as βTrCP2 often rescues βTrCP1 if knocked down alone) (34). An initial experiment was performed to determine the

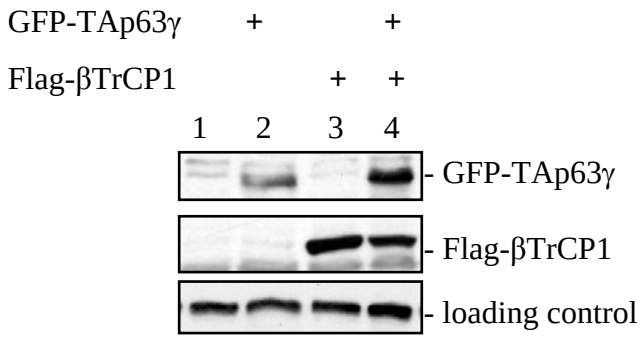
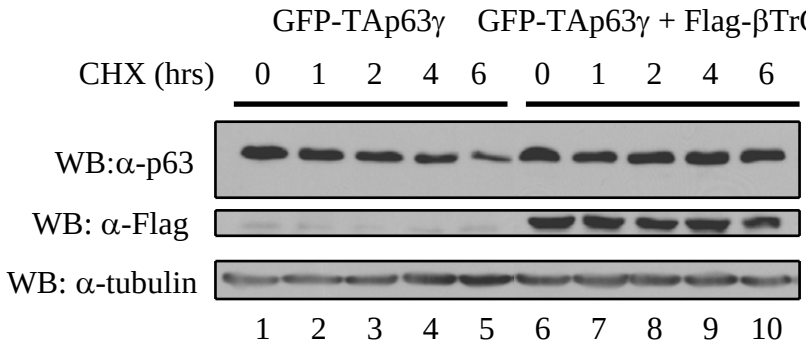
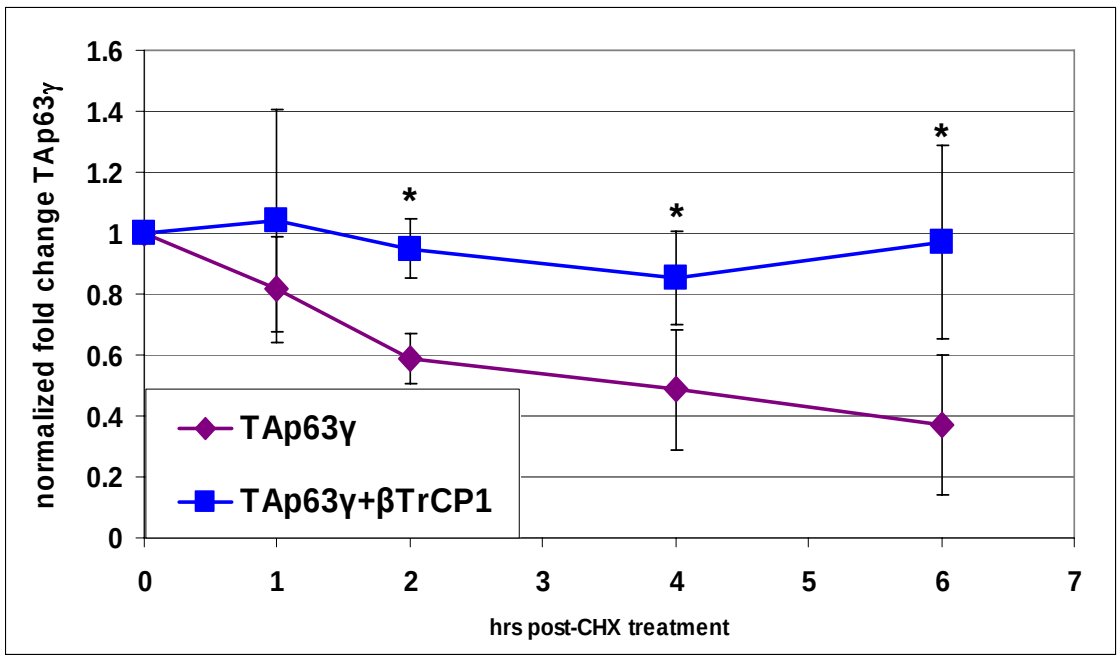


Figure 3.2 – The exogenous steady-state level of TAp63γ is increased when co-expressed with βTrCP1. H1299 cells were transfected for 48 hrs and harvested for WB. 50 μg whole cell lysis was used. Loading control denotes non-specific band.





3.3 - βTrCP1 expression stabilizes exogenous TAp63γ. HEK293 cells were transfected with GFP-TAp63γ and Flag-βTrCP1. 24 hrs later they were treated with cycloheximide and harvested over the indicated time course. Representative WB with quantification of p63 signal intensity/tubulin signal intensity of three experiments graphed below. * = $P \leq 0.03$

efficiency of knockdown at both 24 and 48 hrs post-knockdown to find the optimal time to start the degradation assay. Knockdown was confirmed, at 0.47 fold versus the scramble control at 48 hours (Figure 3.4). This degree of reduction is biologically significant, since several groups have shown that βTrCP1 is effected by both haploinsufficancy and dominant-negative mutants *in vivo* (34, 36). The degree of knockdown could not be maximized further owing to the typical low transfection efficiency of the keratinocytes (about 30%) coupled with their sensitivity to the transfection reagent. Therefore, the half-life time 0 for the cycloheximide exposure in Figure 3.5 is 48 hrs after oligo transfection. As predicted from the data above, the half-life of TAp63γ in the βTrCP1/2 knockdown cells was shorter (about 1.5 hrs), versus the control (about 3.5 hrs) (Figures 3.5A and B). The half-life for the ΔNp63α also showed a significant decrease in the presence of βTrCP1/2 siRNA. However, for reasons discussed in Chapter 5, I believe the control of p63α happens through a different mechanism; therefore I did not show or further pursue this result. The βTrCP1/2 siRNA treatment also reduced the level of endogenous steady-state p63 at low exposure (data not shown), supporting the result in Figure 3.1. Therefore, βTrCP1 co-expression stabilizes TAp63γ exogenously and endogenously.

3.2 p63 binds βTrCP1

In order to determine how βTrCP1 regulates TAp63γ's stability, I next asked whether βTrCP1 could bind TAp63γ in cells. HEK293 cells, which express an undetectable endogenous level of p63, were transfected with GFP-TAp63γ, GFP-ΔNp63γ or Flag-βTrCP1, followed by co-IP and WB. As shown in Figure 3.6, both TAp63γ and ΔNp63γ immunoprecipitated using Flag antibody for βTrCP1 (lanes 11 and 12). Also,

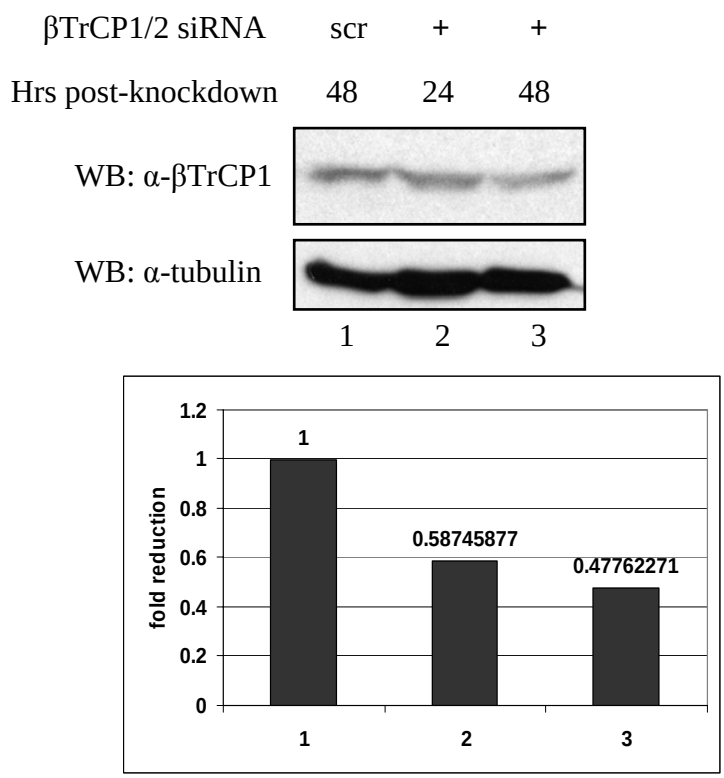


Figure 3.4 – βTrCP1 levels are reduced by βTrCP1/2 siRNA. HaCaT cells were treated with control scramble siRNA oligos or βTrCP1/2 siRNA oligos and harvested after 24 or 48 hrs. 80 μg of whole cell lysate was used for WB analysis. The protein levels were quantified and normalized to tubulin. Numbers shown above are calculated fold reduction. Lane numbers are denoted below.

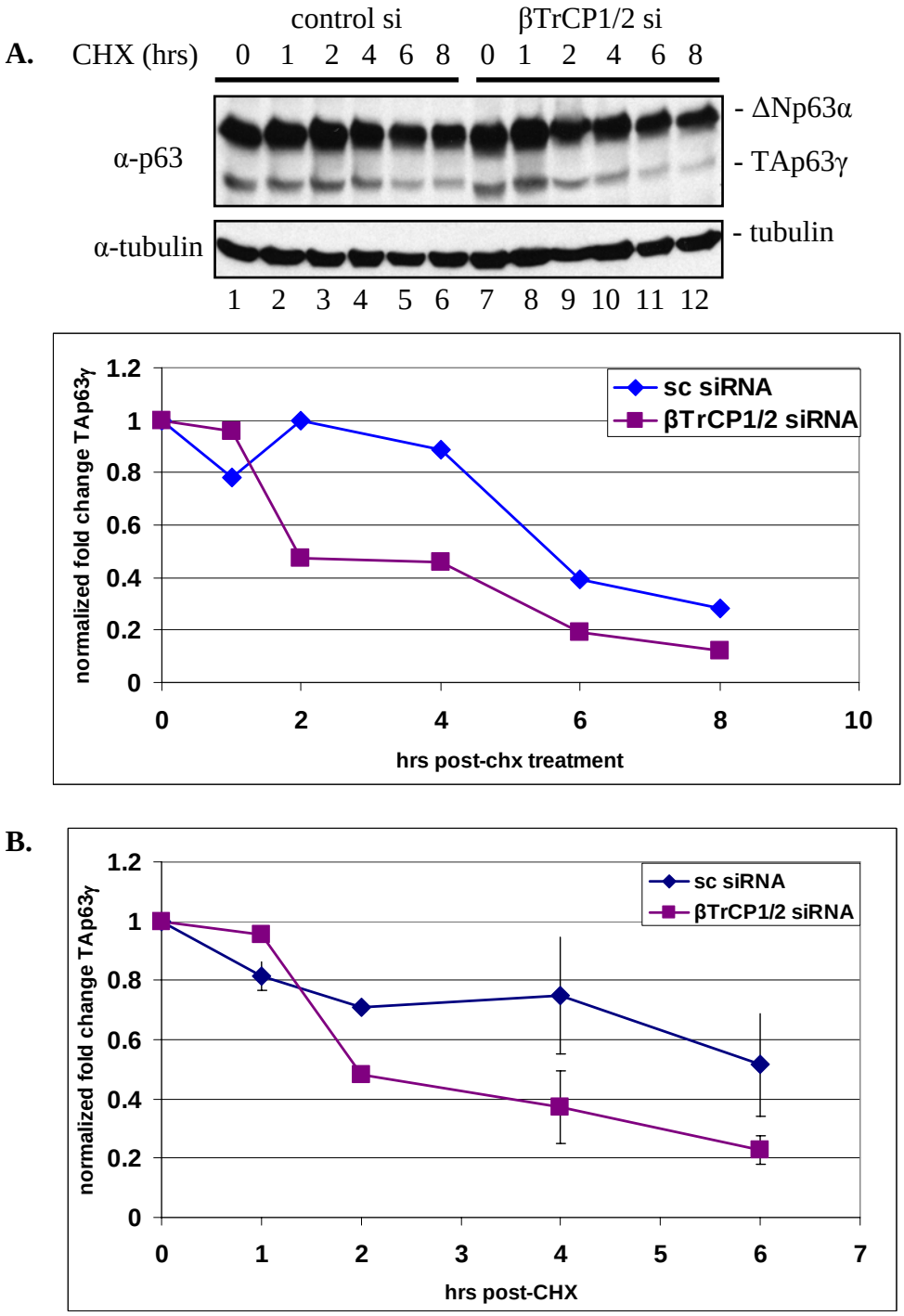


Figure 3.5 - Endogenous p63 is destabilized by βTrCP1/2 knockdown. A. HaCaT cells were transfected with TrCP1/2 siRNA oligos, or scramble control oligos. 48 hrs later they were treated with cycloheximide and harvested over the indicated time course. B. Compiled duplicate experiments. The three points with no discernable error bars have error less than 0.01. In this case statistical significance was not determined because n = 2.

Flag-βTrCP1 immunoprecipitated with GFP antibody for both TAp63γ and ΔNp63γ (lanes 5 and 6). This result was also repeated in H1299 cells (data not shown). Also of note, TAp63γ bound to βTrCP1 much more efficiently than to ΔNp63γ (compare lanes 5 and 11 to 6 and 12). Next, to determine if TAp63γ and βTrCP1 interacted directly *in vitro*, I performed GST-pulldown assays with purified his-TAp63γ (Figure 3.7, A) and GST-βTrCP1 (Figure 3.7, B). As shown in Figure 3.7, C, I found that TAp63γ precipitated on the GST-βTrCP1 bead, but not on the GST0 bead (compare last lane with middle lane), when probed with a p63 specific antibody. This result demonstrates that TAp63γ and βTrCP1 interact directly *in vitro*. I then determined if βTrCP1 could bind other p63 isoforms, using co-IP, as in Figure 3.6, with p63α constructs. HEK293 cells were transfected with a combination of Flag-βTrCP1, myc-TAp63α, myc-ΔNp63α, and empty vector, as noted. Again, both the TAp63α and the ΔNp63α isoforms bound to βTrCP1 (Figure 3.8).

Since I observed that p63α and p63γ could interact in cells, and p63γ could interact *in vitro*, with βTrCP1, I then tested whether these interactions could exist endogenously using co-IP in HEKn-E6/E7 cells, a human keratinocyte line which has high levels of p63 and βTrCP1 (Figure 3.9, A). By co-IP, βTrCP1 immunoprecipitated with ΔNp63α and TAp63γ, but not with TAp63β endogenously (lane 2, Figure 3.9, B). Also, p63 immunoprecipitated with βTrCP1 endogenously (lane 1). These results were also repeated using HaCaT and HeLa cell lysates (data not shown). Therefore, βTrCP1 is able to interact with several isoforms of p63 both exogenously and endogenously.

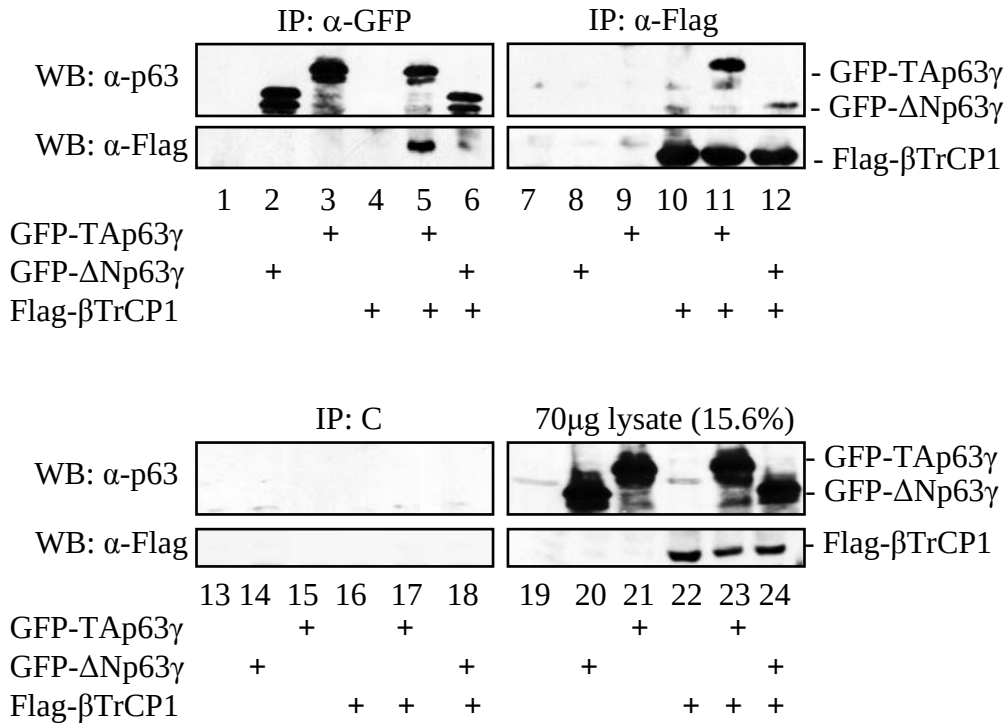


Figure 3.6 - TAp63γ and ΔNp63γ bind βTrCP1 in cells. GFP-TAp63γ, GFP-ΔNp63γ, and Flag-βTrCP1 were expressed in HEK293 cells. 448 μg clarified whole cell lysate co-immunoprecipitated per sample, with 15.6% of the pre-bound lysate loaded as input. WB was performed using the indicated antibodies.

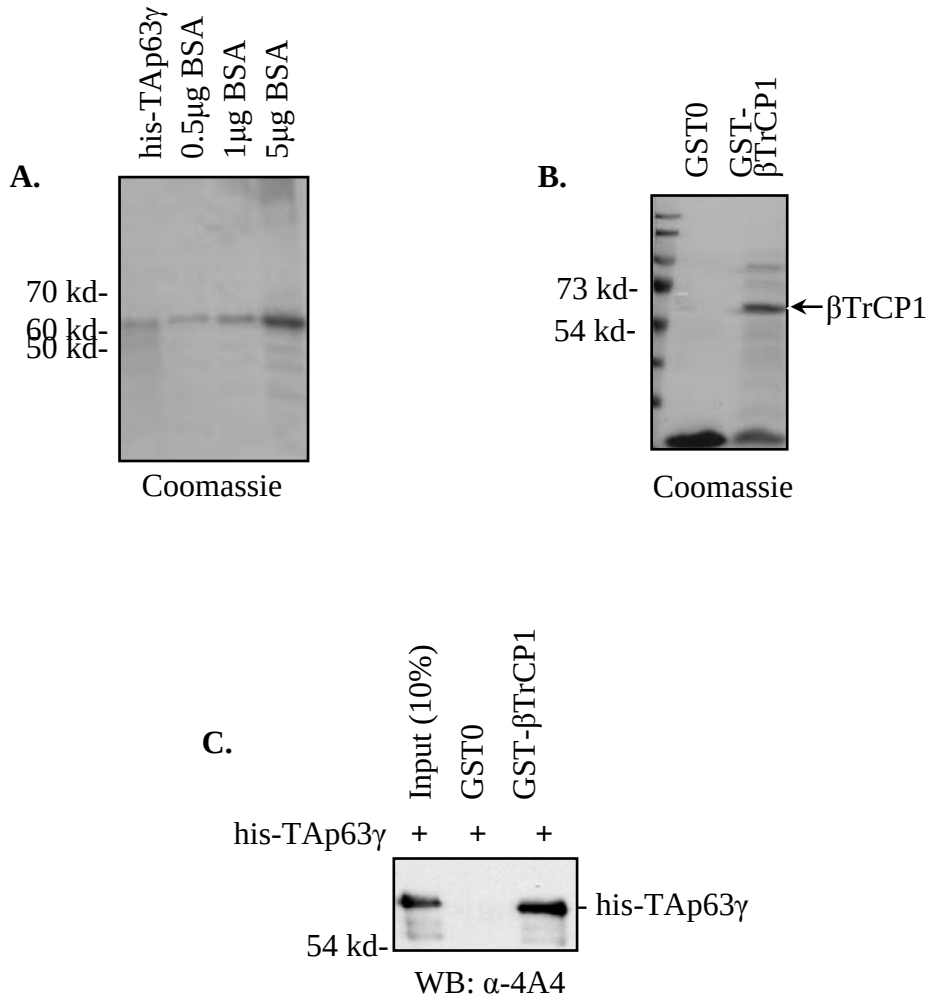


Figure 3.7 - TAp63γ binds βTrCP1 *in vitro*. A. Purification of his-TAp63γ. His-TAp63γ was run beside noted BSA standards. B. Generation of GST0 and GST-βTrCP1. GST0 and GST-βTrCP1 bead were generated and equalized. Shown are the controls for GST normalization used in C. C. *In vitro* binding assay. 60 ng purified his-TAp63γ was bound to GST0 or GST-βTrCP1 bead. 10% input, and binding samples were analyzed by WB.

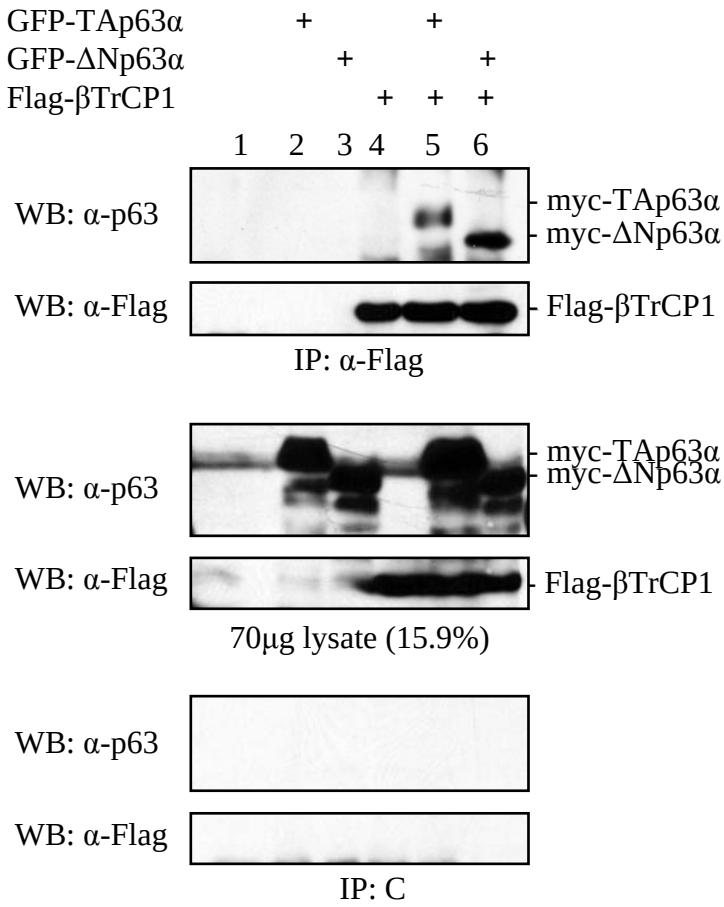


Figure 3.8 - TAp63α and ΔNp63α bind βTrCP1 in cells. myc-TAp63α, myc-ΔNp63α, and Flag-βTrCP1 were expressed in HEK293 cells. 448 μg clarified whole cell lysate co-immunoprecipitated per sample, with 15.6% of the pre-bound lysate loaded as input. WB was performed using the indicated antibodies.

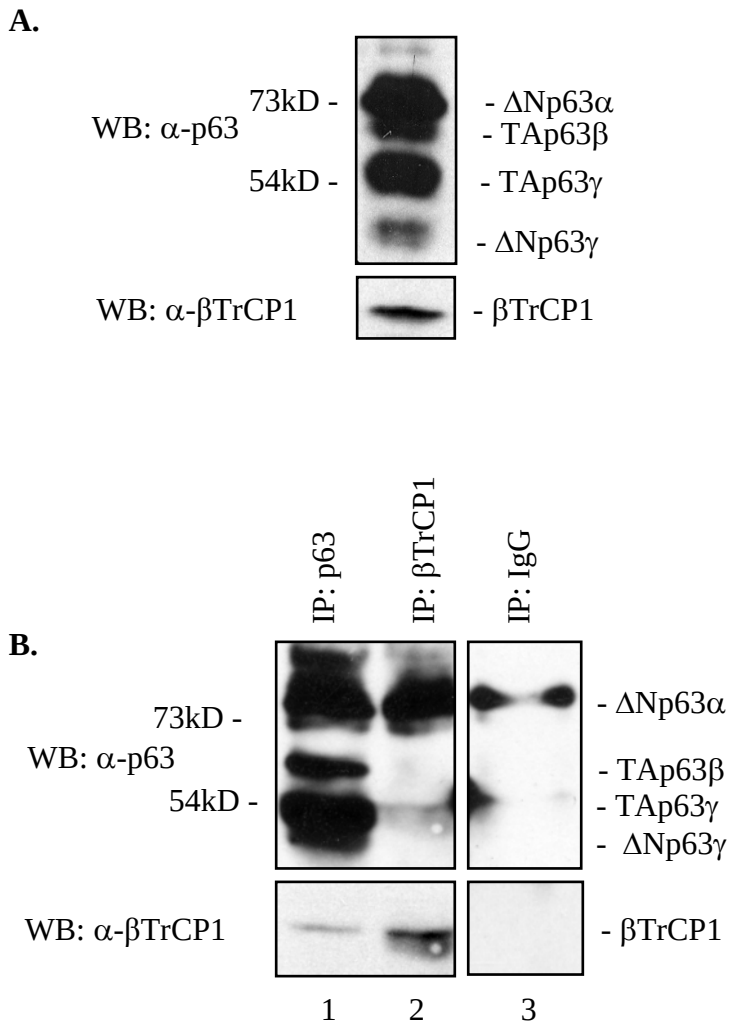


Figure 3.9 - ΔNp63α and TAp63γ, but not TAp63β bind βTrCP1 endogenously. A. HEKn-E6/E7 cells have endogenous p63 isoforms and βTrCP1. 100μg whole cell lysis from the passage previous to the experiment below and WB with noted antibodies. B. Co-immunoprecipitation of p63 and βTrCP1. HEKn-E6/E7 cells were harvested and 850 μg of total lysate was immunoprecipitated using the noted antibodies (left). The panel on the right shows the IgG control taken from the same gel, same exposure.

3.3 βTrCP1 binds TAp63γ's N- and C-termini

In order to further characterize the interaction of TAp63γ with βTrCP1, I mapped the regions where TAp63γ contacts βTrCP1 *in vitro*. I generated four his-tagged deletion mutants of TAp63γ, termed TAp63γF1-F4 (Figure 3.10). The purification of these fragments is shown by Comassie in Figure 3.11, A. TAp63γF1, the TA-containing deletion mutant (lane 6), bound strongly to GST-βTrCP1, while TAp63γF3 and TAp63γF4, the C-terminus-containing mutants, also bound, but less efficiently than TAp63γF1 (lanes 10 and 12), but not the DBD domain containing mutant, TAp63γF2 (Figure 3.11, B). These results suggest that the N-terminus and, to a lesser extent, a region in the last 59 residues of the protein were important in βTrCP1 binding; this region contains the residues unique to TAp63γ. These results are also consistent with the weaker interaction shown between ΔNp63γ and βTrCP1 in Figure 3.6.

3.4 TAp63γ's upregulation of p21, but not Bax, is amplified by βTrCP1 expression

Since TAp63γ was stabilized by βTrCP1 and they interacted directly, I then asked whether βTrCP1 could affect TAp63γ's function as a transcription factor. Two well described target genes of TAp63γ are *p21* and *Bax* (12, 101, 112), so I examined the effect of TAp63γ and βTrCP1 co-expression on p21 and Bax. Consistent with our previous reports (173), TAp63γ alone increased *p21* mRNA levels by RT-PCR (Figure 3.12, A, lane 2), and had little effect on Bax (lane 4). By qRT-PCR of the assay shown in 3.12, A (Figure 3.12, B), when TAp63γ was co-expressed with βTrCP1, *p21* mRNA levels increased 55% (Figure 3.12, lane 4 and graph). Triplicate qRT-PCR experiments were also highly significant Figure 3.12, C). Additionally, the TAp63γ mRNA

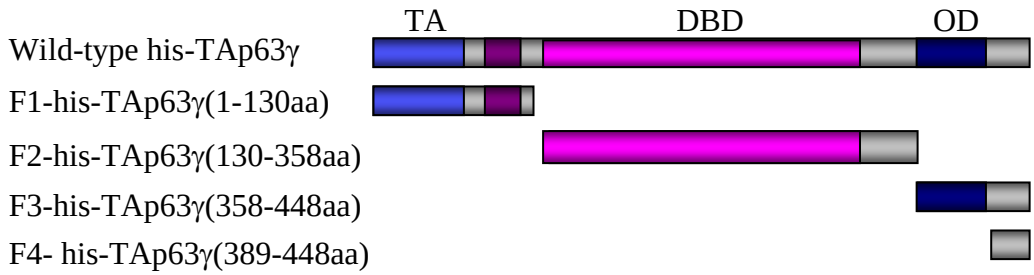


Figure 3.10 - Schematic of the TAp63γ truncation mutants. The TA, DBD and OD are indicated. The fragments (F) 1-4 and the encompassing amino acid residues are noted.

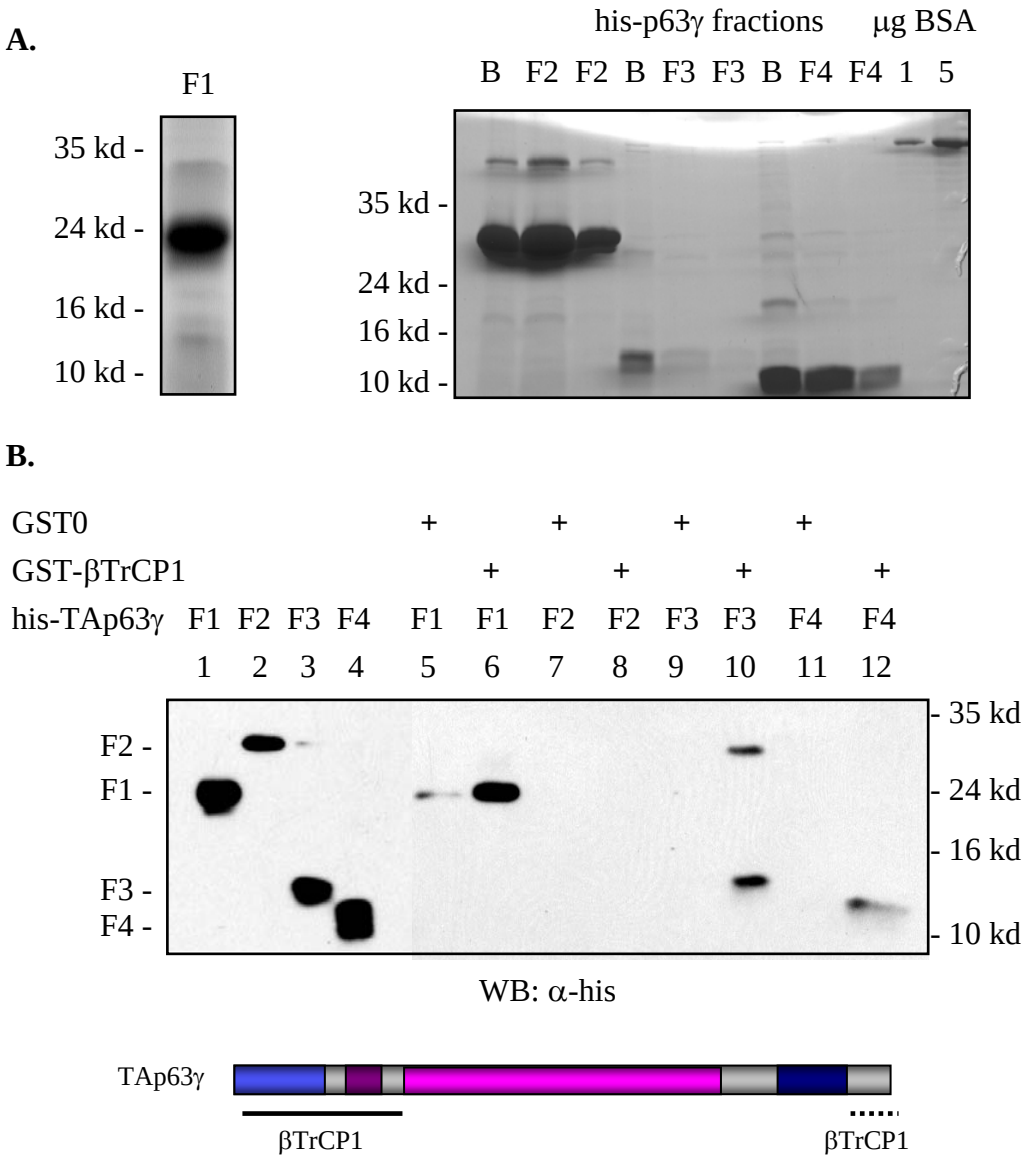


Figure 3.11 - βTrCP1 binds the N- and C-termini of TAp63γ *in vitro*. A. TAp63γ fragment purification. TAp63γ fragments were purified and concentration was determined by Coomassie stain. On TAp63γF2-F4 stain, B = bead with purified protein, fragments labeled TAp63γF2-F4 are unbound protein fractions. BSA standard is noted. B. *In vitro* binding of βTrCP1 and TAp63γ fragments. βTrCP1 was incubated with 40 ng of his-TAp63γF1-F4, as indicated (bottom). 100% input was loaded as a control. The upper band in lanes 3 and 10 is non-specific. Below is a summary of the binding of βTrCP1 to TAp63γ. TAp63γ is shown as in A, with the stronger and weaker interaction with βTrCP1 shown as the solid and dashed lines, respectively.

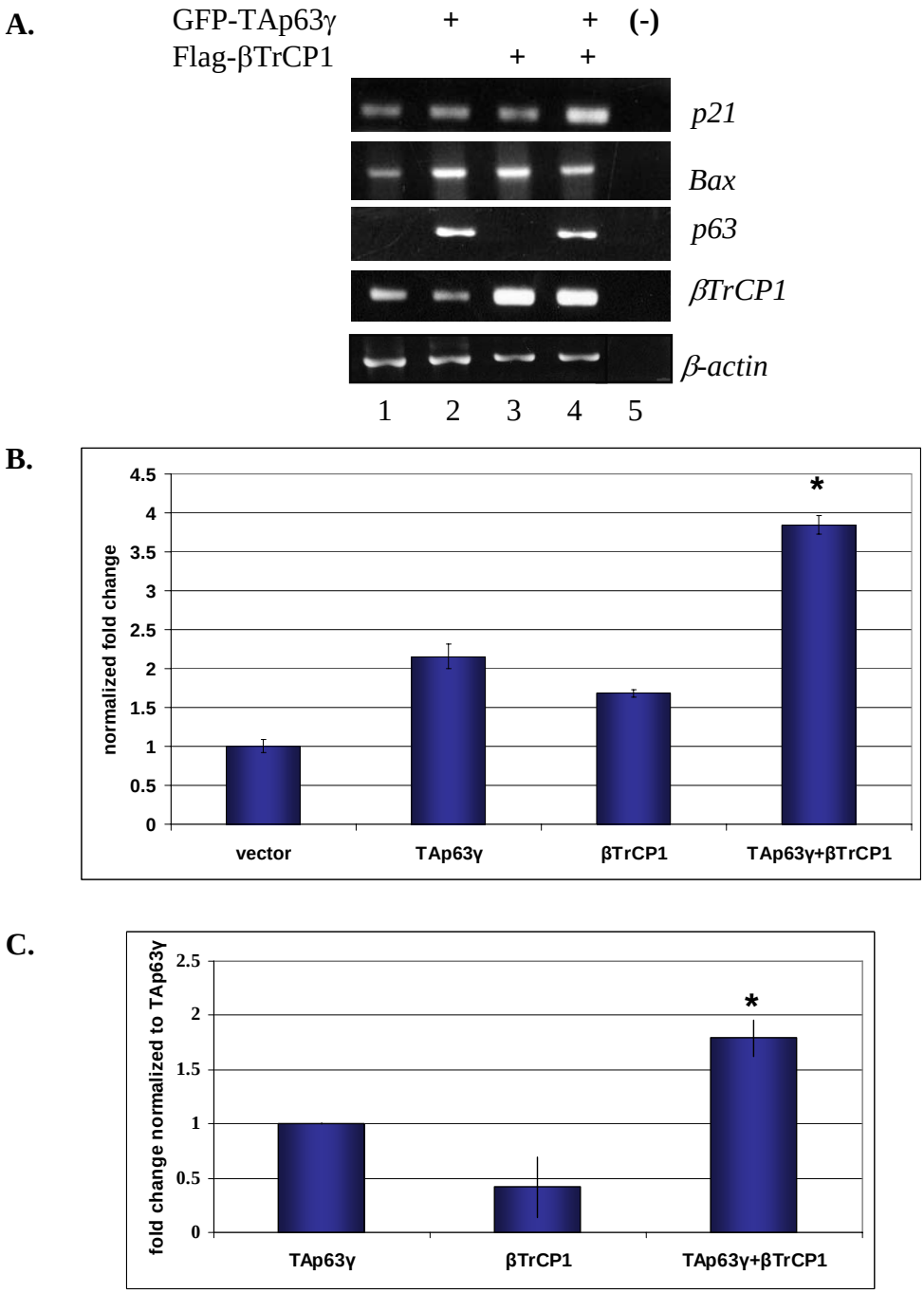


Figure 3.12 - TAp63γ's activation of *p21*, but not *Bax*, is increased by βTrCP1 expression at the mRNA level. A. *p21* increases with co-expression of TAp63γ and βTrCP1 by RT-PCR. RT-PCR for *p21*, *Bax*, *βTrCP1*, *p63*, and *β-actin* was performed in H1299 cells as shown. B. *p21* increases with co-expression of TAp63γ and βTrCP1 by qRT-PCR. qPCR was also performed on samples in A. and *p21* was normalized to *GAPDH.* Representative data are shown, as in Gallegos, JR *et al.* * = P < 0.0002. C. qRT-PCR triplicate quantification for *p21.* qPCR was also normalized to TAp63γ alone * = P = 0.0013. Cells were harvested at 24 hrs.

expression levels do not change in the presence of βTrCP1, again suggesting that the upregulation of the protein level is occurring through a post-translational mechanism, supporting my endogenous result in Figure 3.1.

This result was also examined at the protein level. When TAp63γ was co-expressed with βTrCP1 protein levels increased significantly, approximately 50% over the TAp63γ control (Figure 3.13, A, lane 4 and 3.13, B). In contrast, Bax expression did not increase over the TAp63γ control at either the mRNA or protein level (Figure 3.13 and 3.14, lane 4). Therefore, the co-expression of TAp63γ with βTrCP1 causes significant upregulation of p21, but not Bax, over the level of either TAp63γ or βTrCP1 alone. Other downstream targets were also examined by mRNA and protein level, including MDM2, PIG3, and Noxa, but these displayed negative results (data not shown).

An important note for both of these assays is that they are taken at 24 hr and 48 hr time points for mRNA and protein level, respectively. Initially, time courses were run to find approximate kinetics for these experiments. The GFP-TAp63γ gene comes about 16 hrs after transfection. This is the earliest time point where the TAp63γ gene is detectible by RT-PCR, as in 3.13, βTrCP1 also is upregulated at about the same time. While the mRNA level is displayed at 24 hrs in these panels, mRNA assays were also taken at 48 hrs with little variation in the combined result with TAp63γ and βTrCP1 co-expression, though the overall expression load of both TAp63γ and βTrCP1 decreases slightly between 24-48 hrs. Maximum protein expression occurs between 36-48 hrs. This timing is reasonable for both p21 and Bax levels in an exogenous system (173) and also an endogenous developmental or tumorigenic setting where the timing of the reactions tend to occur more slowly (42, 108). However, it is important to note that the kinetics of these

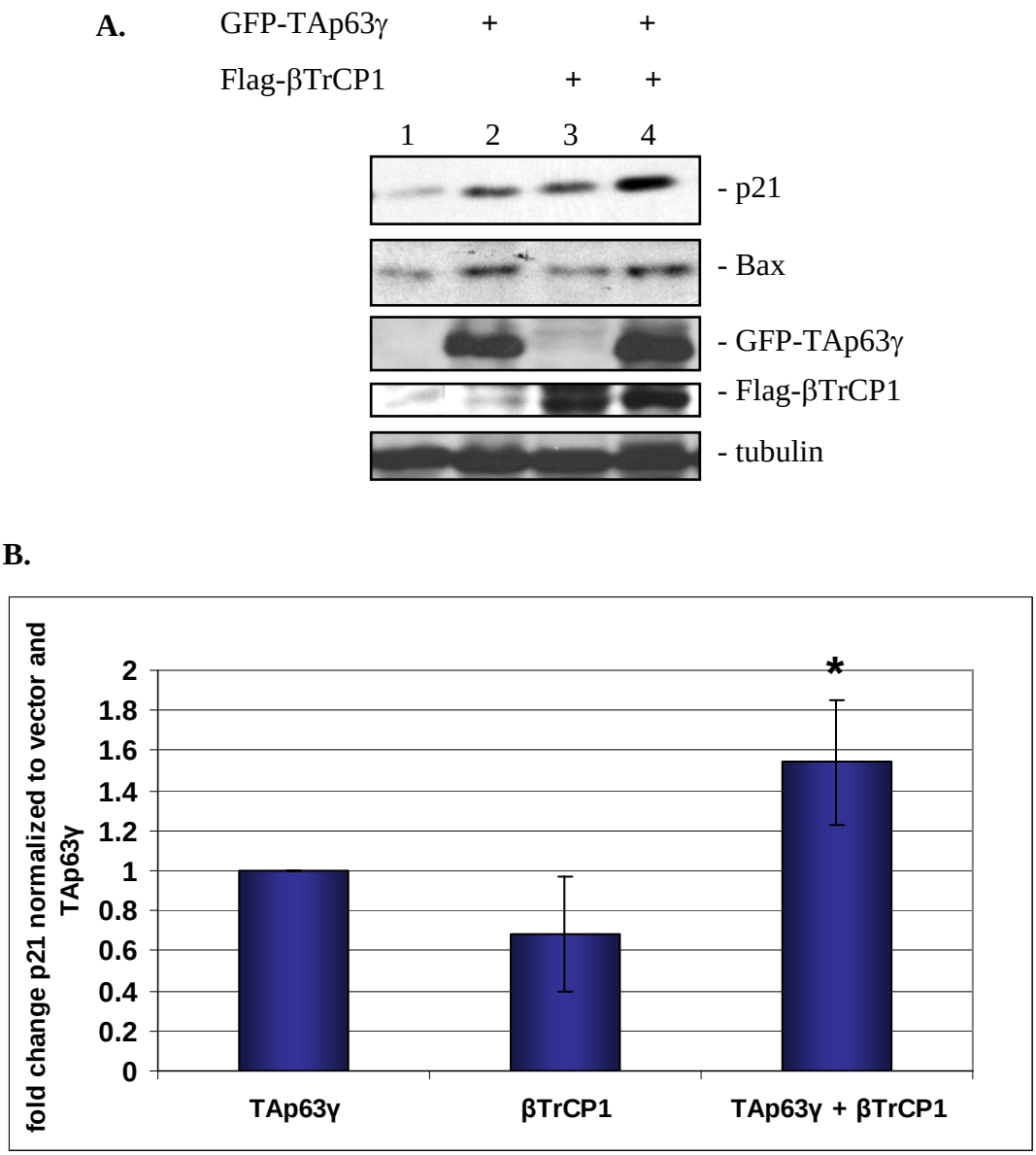


Figure 3.13 - TAp63γ's activation of p21, but not Bax, is increased by βTrCP1 expression at the protein level. A. p21 increases with co-expression of TAp63γ and βTrCP1. 70 μg whole cell lysate was analyzed by WB (top). B. Compilation of repeat WB experiments. Triplicate WBs were quantified with Adobe Photoshop software, normalized to tubulin and p63 and graphed. * = P = 0.048

responses would be expected to differ significantly from this timing in an endogenous stress situation (on the order of minutes to hours, versus days).

3.5 TAp63γ's binding at the *p21* promoter increases in the presence of βTrCP1 in cells

Since βTrCP1 stabilized TAp63γ, and βTrCP1 also augmented TAp63γ's activation of p21 protein and mRNA levels, I next asked if βTrCP1 could enhance TAp63γ's presence at the *p21* promoter. As an initial assay to determine if βTrCP1 could enhance the level of TAp63γ on chromatin, I performed a fractionation assay where sonicated whole cell lysates were loaded beside a soluble fraction (non-chromatin) and an insoluble (chromatin) fraction. As shown in Figure 3.14, where there is little visible change in the levels of p63 and βTrCP1 between the whole and soluble fractions, there is an enrichment of p63 in the insoluble fraction in the presence of βTrCP1, though the level of βTrCP1 in the insoluble fraction is consistent with the other fractions. This result suggests that while there is a large soluble pool of both TAp63γ and βTrCP1, as would be expected in the case of exogenous expression, that βTrCP1 is able to increase the amount of TAp63γ on chromatin.

Therefore, I continued to look specifically at TAp63γ's interaction with the *p21* promoter region. The regions on the promoter that were used for analysis began at amino acids -2283, -20, and +7878 and spanned 50 bp downstream in each case. These regions are called P1, P2, and P3, respectively (Figure 3.15). The P1 primers include a high affinity site for the p53 family members, while the P3 primers are a negative control. Lastly, sequence analysis of the *p21* promoter revealed both CATG sequences, as well as CGTG sequences which may be favorable for p63 binding, near the P2 primer set (112).

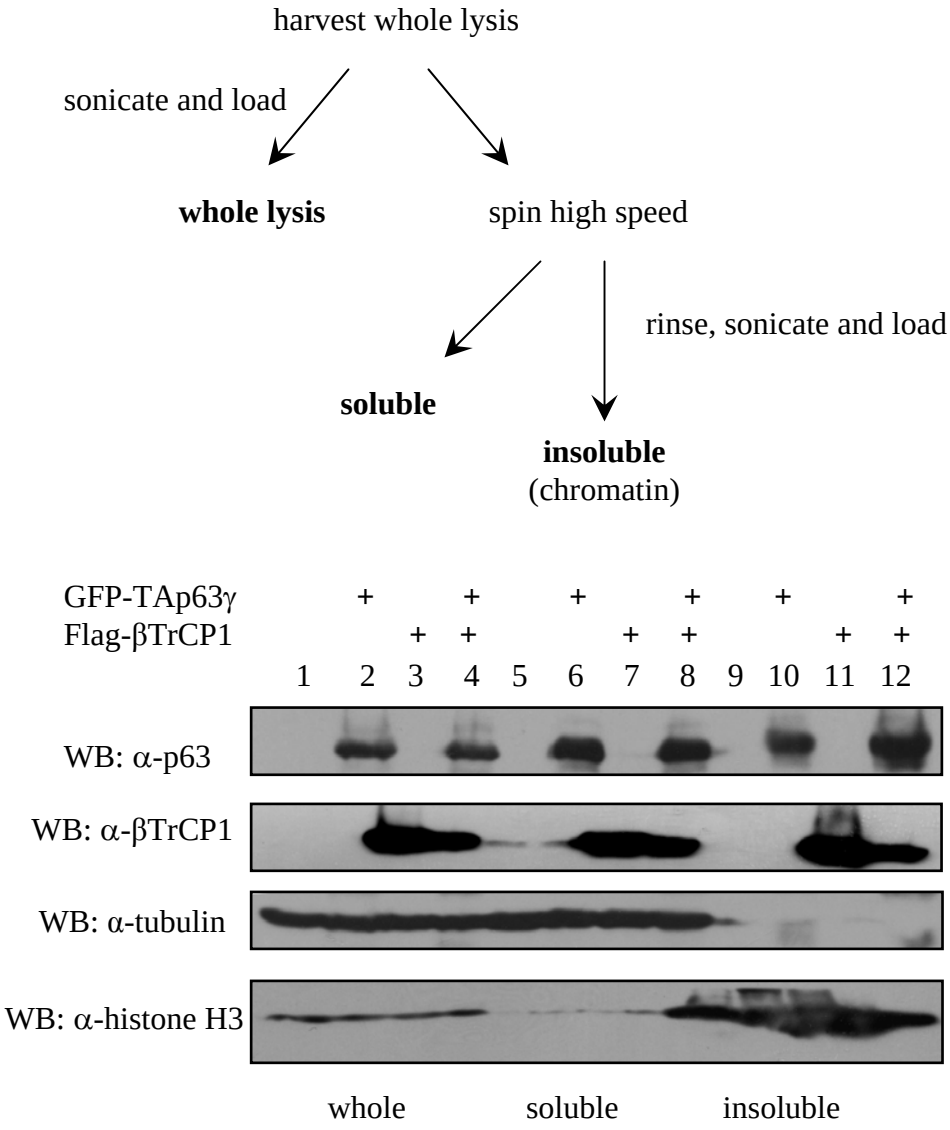


Figure 3.14 - βTrCP1 co-expression increases the amount of TAp63γ bound to chromatin *in vivo*. H1299 cells were transfected as noted and fractionated, as shown by the diagram above, into whole sonicated lysate, the soluble (non-chromatin) fraction, and insoluble (chromatin) fraction, run on a gel, and WB with indicated antibodies.

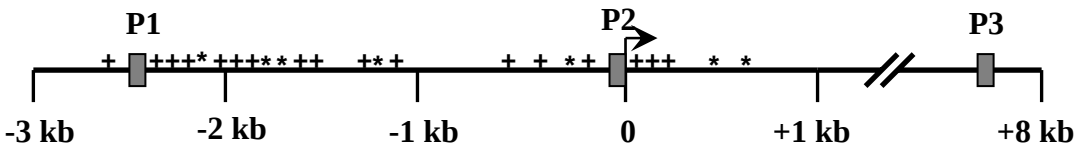


Figure 3.15 - Schematic of the *p21* promoter. The primer regions used for ChIP at the p21 promoter are shown by grey boxes with labels according to primer set. + = a CATG core sequence, * = a CGTG core sequence. qPCR primer sets P1, P2, P3 noted and shown by shaded boxes, as described in the text.

Thus, I also tested this region for TAp63γ binding. Consistent with our earlier results, TAp63γ alone resulted in a 20 fold increase of promoter binding over the vector alone control, while co-expression of TAp63γ with βTrCP1 resulted in an increase of over 50 fold of TAp63γ bound to the *p21* promoter (Figure 3.16, A). The P3 primers in the gene were negative, as were other primer pairs between -2 and -0.5 kb (data not shown). Interestingly, the P2 primers also detected an active site of TAp63γ recruitment on the *p21* promoter (Figure 3.16, A), demonstrating the existence of a novel site for p63 binding at the *p21* promoter. The data for triplicate experiments is shown in Figure 3.16, B. Therefore, the increased stability of TAp63γ in the presence of βTrCP1 leads to the increase of *p21* promoter-bound TAp63γ at both a canonical p53 family binding region and a novel p63 binding region.

In this case, I forewent the use of the luciferase, or other similar reporter assay as a test of active transcription of p63 on *p21*. As noted in the introduction, *p21* is a well established target gene of p63 and the other p53 family members (reviewed in (101)). However, it should be noted in the case of these studies that luciferase assay was performed successfully with TAp63γ on both MDM2 and IGFBP3 reporter constructs, but the results showed no additional increase with co-expression of βTrCP1, similar to mRNA assays. Considering that there have been no studies documenting an effect of p53 family members or βTrCP1 on *p21* mRNA stability, as of yet there are no known endogenous micro RNA which might be encoded from the expression of p63 or βTrCP1 which would indirectly activate p21 transcription, and that p21 levels are well-established as being controlled at the protein level (see Discussion), it is a more than reasonable conclusion given the results of our mRNA expression, protein expression, and our ChIP

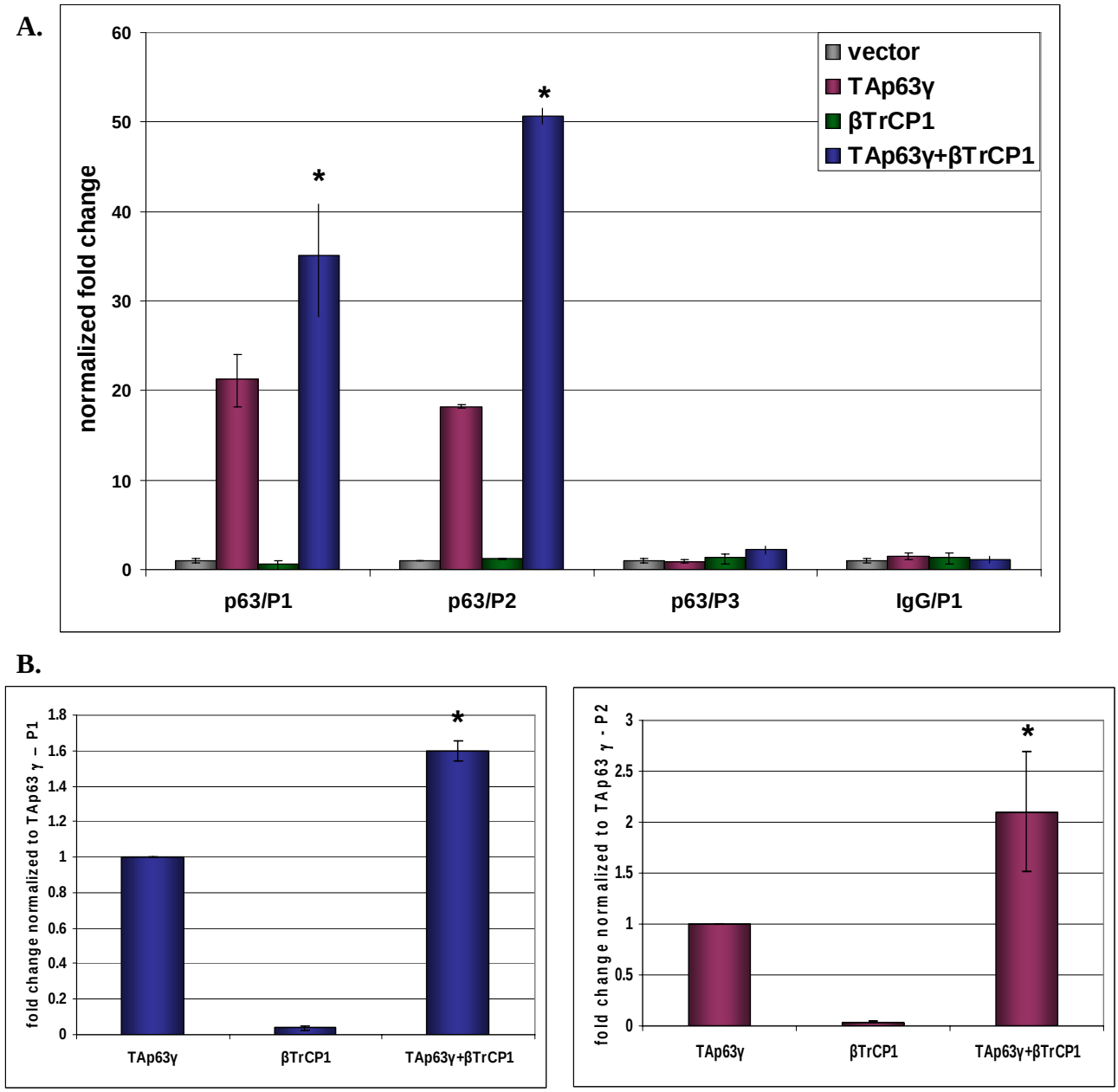
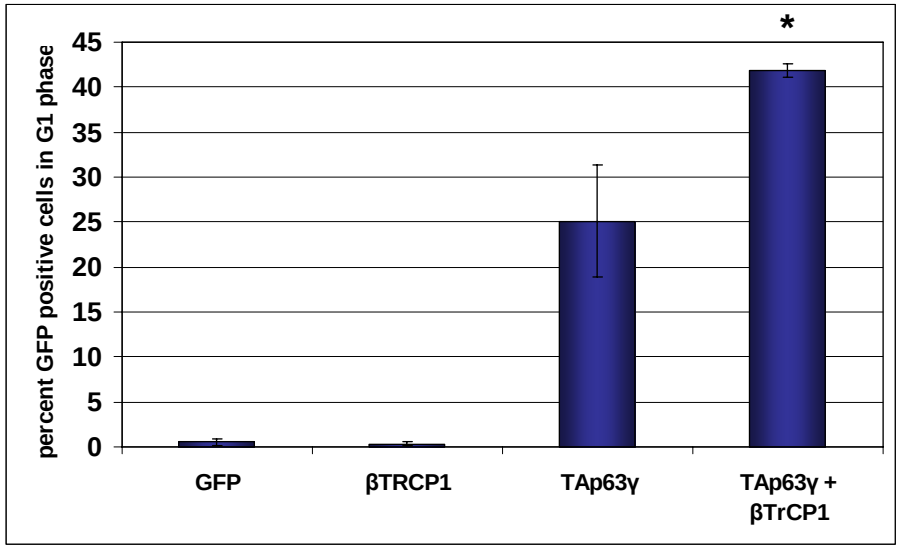


Figure 3.16 - TAp63γ binding at the *p21* promoter increases in the presence of βTrCP1 in cells. A. Quantitative ChIP analysis of TAp63γ at the *p21* promoter. H1299 cells were transfected and ChIPed using the indicated ChIP antibody/qPCR primer combinations. Representative data is shown. * = $P \leq 0.02$ (top) as in Gallegos, JR *et al*. B. ChIP repeat experiments. Triplicate repeats for P1 and P2 primers normalized to p63, left and right bottom, respectively * = $P \leq 0.04$. The above data was measured 24 hrs post-transfection.

experiments that increased p63 binding at the *p21* promoter directly equates to an increase of p21 through a transcriptional mechanism.

3.6 TAp63γ's activation of growth arrest is increased by βTrCP1 expression

Since our data clearly pointed to a role of βTrCP1 in the enhancement of TAp63γ's increase of p21 expression, and p21 is an established regulator of cell cycle arrest, I asked whether the upregulation of p21 protein would translate to a functional role in G1 cell cycle arrest. To examine the cell cycle profile, transfected H1299 cells were treated with 150 ng/ml nocodazole, which induces a G2 block, for 16 hrs and looked for cells that remained in G1 by FACS. Consistent with my previous data, while TAp63γ alone increased the proportion of cells in G1 (26%), the cells co-expressing TAp63γ and βTrCP1 showed a significant increase (42%) (Figure 3.17). Therefore, the increased activity of p21 upon TAp63γ co-expression with βTrCP1 translated to an equally striking upregulation of G1 cell cycle arrest.



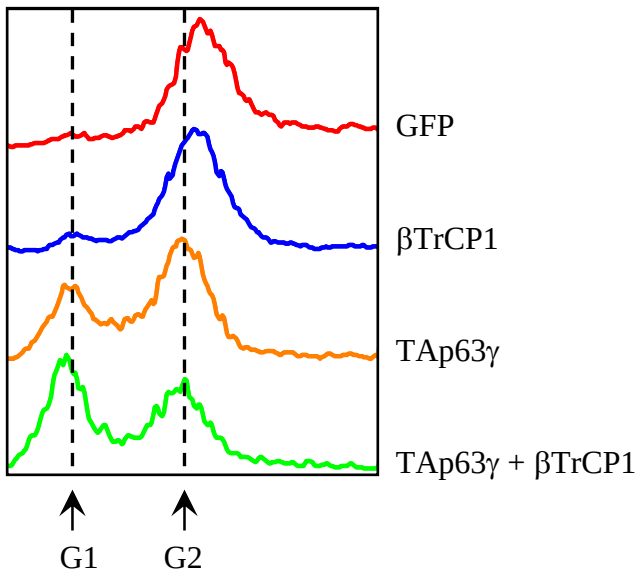


Figure 3.17 - TAp63γ's activation of growth arrest is increased by βTrCP1 expression. Representative curves of H1299 cells treated with nocodazole, subjected to FACS and gated for GFP positive signal are shown below as generated by FloJo software and plotted to scale on the same axis, as indicated. G1 = 2n peak by PI staining, G2 = 4n by PI staining. The percent of GFP-positive cells in each phase of the cell cycle was determined and data for three experiments were plotted (top). * = P = 0.02

CHAPTER 4

UBIQUITYLATION OF p63 BY βTRCP1 ACTIVATES p63

4.1 p63γ is ubiquitylated by $SCF^{\beta TrCP1}$

Our data in Chapter 3 identifies βTrCP1 as a positive regulator of TAp63γ which acts by increasing TAp63γ through a post-translational mechanism. Since $SCF^{\beta TrCP1}$ is an active member of the SCF complex, I examined whether $SCF^{\beta TrCP1}$ could ubiquitylate TAp63γ and whether this modification could be responsible for p63 activation. First, I wished to examine the general ubiquitylation of p63 in H1299 cells. Using a Ni-NTA binding assay, GFP-TAp63γ, myc-TAp63α, and myc-ΔNp63γ showed a shift to higher weight moieties in the presence of ubiquitin (Figure 4.1 left, lanes 4, 6 and 8), but not when transfected alone, demonstrating that all of the isoforms in this case are ubiquitylated. Interestingly, the ΔNp63γ isoform, though expressed at similar levels to the TAp63γ (compare WB, lanes 5-8), has less ubiquitylation than the TAp63 isoforms, as shown by the much lighter laddering. Therefore, several isoforms of p63 are ubiquitylated, with the TA isoforms ubiquitylated to a greater degree, suggesting that ubiquitin may be one way to account for the differences in individual p63 isoform activity in the cell. Next, to ask if βTrCP1 could ubiquitylate p63, I first performed an *in vitro* ubiquitylation assay using a purified SCF complex, p63, E1, E2, and ubiquitin. In fact, in the presence of all the ubiquitylation components, TAp63γ shifted to several ubiquitylated forms of the protein, which were absent in the control lanes (Figure 4.2, lane 7). I then tested whether Flag-βTrCP1 expression would increase the ubiquitylation of TAp63γ in H1299 cells. In this assay, I used Flag-βTrCP1, or the F-

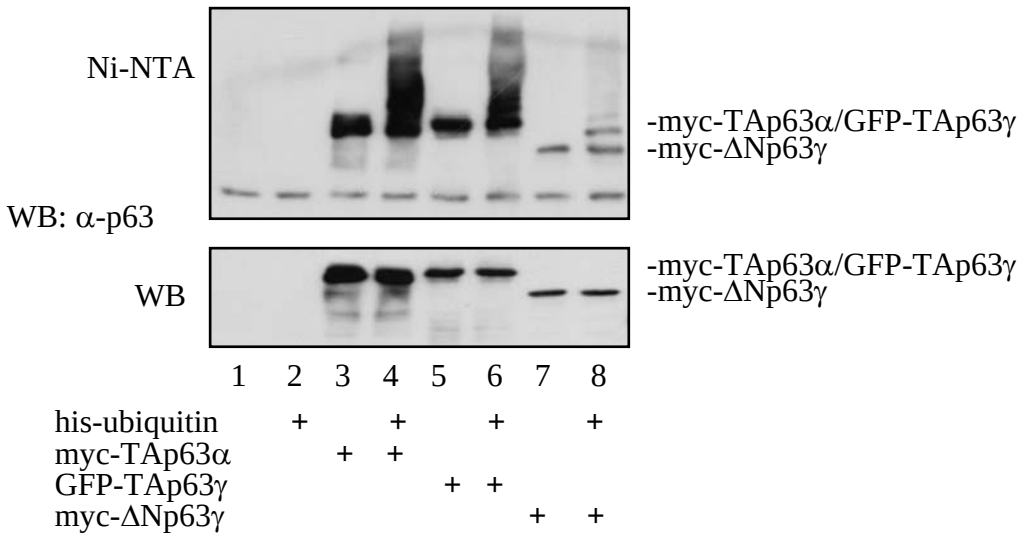


Figure 4.1 - Δp63 is ubiquitylated less than TAp63 in cells. H1299 cells were analyzed by WB with 50 μg whole cell lysis (bottom) or by Ni-NTA precipitation (top). JRG repeated, final figure by Joel Litersky.

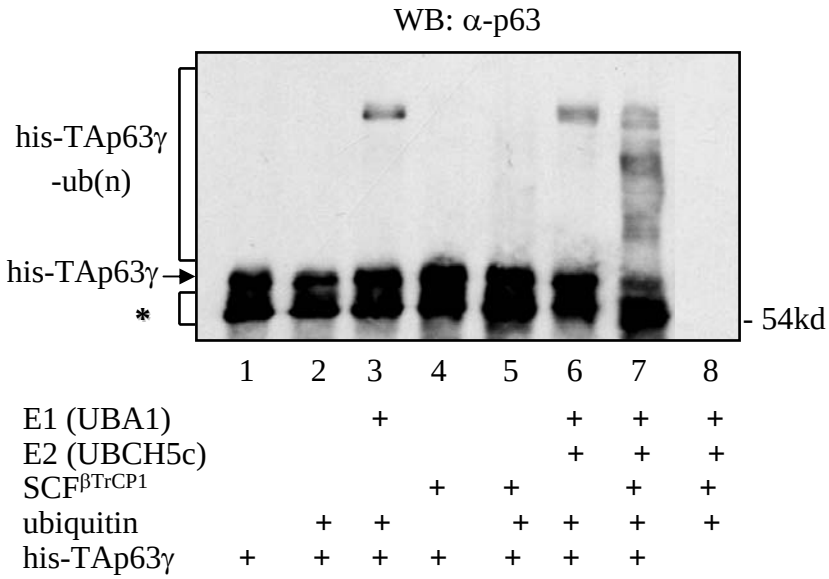


Figure 4.2 - SCF$^{\beta TrCP1}$ ubiquitylates TAp63γ *in vitro*. 100 ng purified his-TAp63γ was added to an ATP reaction buffer with noted substrates and analyzed with α-p63 antibody. * = degradation of purified TAp63γ.

box mutant of βTrCP1, Flag-ΔFβTrCP1. ΔFβTrCP1 is a dominant-negative truncation mutant of βTrCP1 which lacks the F-box. Therefore, the ΔFβTrCP1 protein can bind βTrCP1's substrates, but those substrates are not presented to the SCF complex for ubiquitylation (data not shown) (8, 34). Consequently, the level of generally ubiquitylated TAp63γ precipitated in cells (Figure 4.3, lane 3) increased specifically in the presence of Flag-βTrCP1 (lane 5), and decreased in the presence of the Flag-ΔFβTrCP1 (lane 7). Further, as an additional control to ensure that this result was not due to the function of another SCF complex, I also performed experiments co-expressing TAp63γ with another SCF substrate-recognition component, such as Skp2, and these experiments were negative (data not shown). Also, p53 and p73α were not ubiquitylated by βTrCP1 under similar experiments in cells, suggesting that this ubiquitylation is specific to p63 (data not shown). Thus, this data demonstrates that $SCF^{\beta TrCP1}$ could act as a specific E3 ubiquitin ligase for TAp63γ and that the ubiquitylation of TAp63γ requires the presence of the entire SCF complex. Therefore, while also acting functionally as a regulator of TAp63γ, βTrCP1 acts as an E3 ubiquitin ligase for TAp63γ.

4.2 Characterization of TAp63γ's ubiquitylation by $SCF^{\beta TrCP1}$

As mentioned in Chapter 1, the function of ubiquitylation can be mediated by both the position of the placement of the ubiquitin on the substrate and the type of ubiquitin linkage present. In order to determine the location of the ubiquitylation of TAp63γ by βTrCP1, I used the same his-TAp63γ fragments as in Chapter 3 (Figure 3.10) for *in vitro* ubiquitylation. As shown in Figure 4.4, the TAp63γF1 fragment (lane 2), comprising the N-terminus, was ubiquitylated, compared to both the TAp63γF1 alone or the substrate negative controls (lanes 1 and 3). In this case because of the various tags

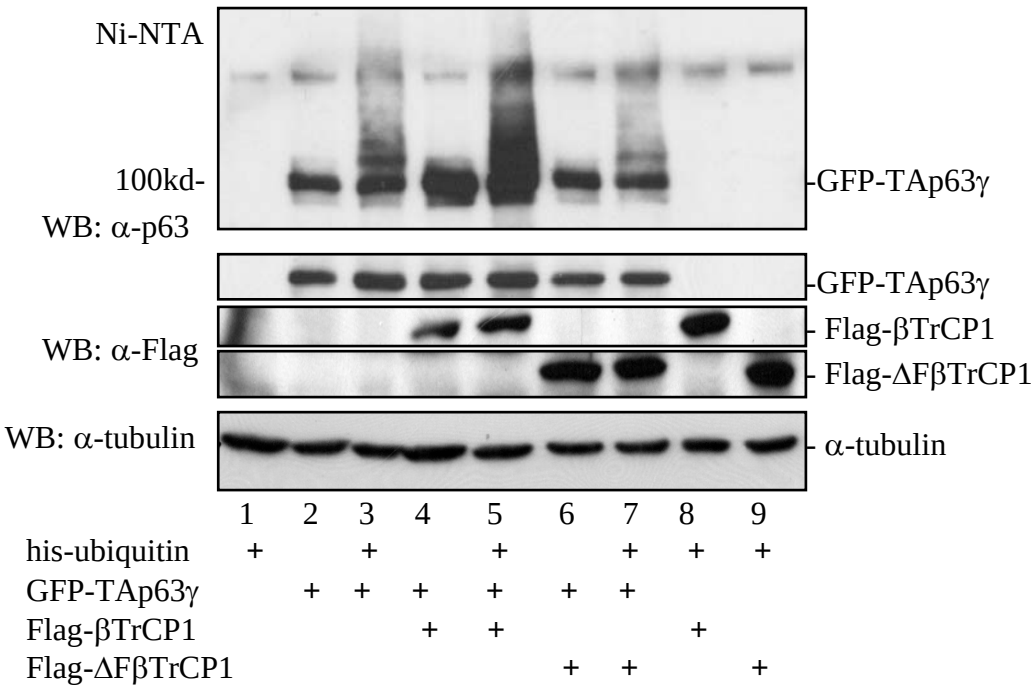


Figure 4.3 - SCF$^{\beta TrCP1}$, but not ΔFβTrCP1 ubiquitylates TAp63γ in cells.
Ubiquitylation was analyzed as in 4.1. JRG repeated, final figure by Joel Litersky.

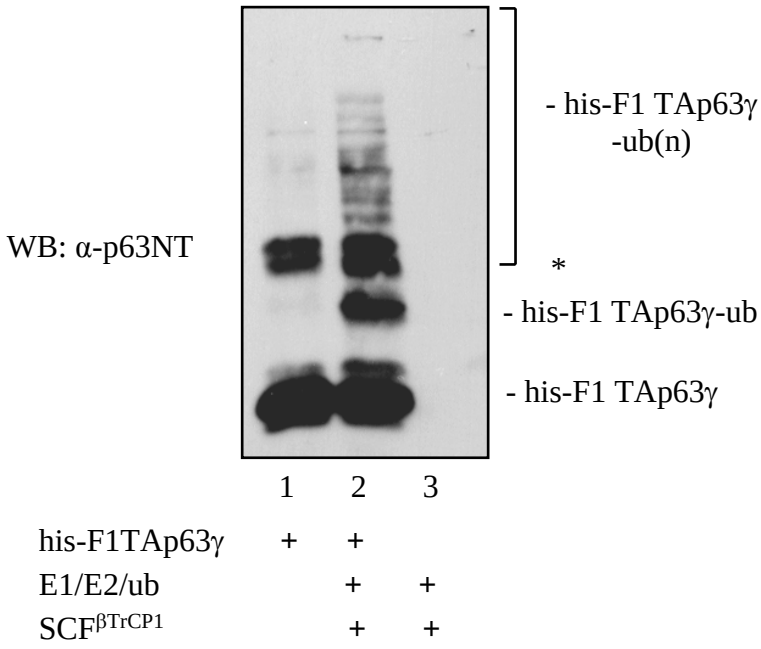


Figure 4.4 - SCF$^{\beta TrCP1}$ ubiquitylates the N-terminal of TAp63γ *in vitro.* His-F1TAp63γ was purified and used in an *in vitro* ubiquitylation reaction as in 4.2. * denotes a band which co-purifies with his-F1TAp63γ.

on the SCF complex itself, I used a p63 antibody generated in our lab against p63's N-terminus (p63-NTA). When this reaction was attempted with the TAp63γF2-F4 fragments, I were unable to discern a result due to lack of an antibody specific for those regions (the 4A4 antibody did not recognize the F2 fragment) that would allow us to separate the his-TAp63γ signal from that of the his-Skp1, which is also ubiquitylated.

Further, I also examined the affect of point mutations of several lysine mutations of our GFP-TAp63γ construct on TAp63γ/βTrCP1 signaling. Of the 19 lysine residues in pTAp63γ, K to A mutations in 9 of them, both single and in combination, were not sufficient to ablate ubiquitylation of TAp63γ (mutated K's shown in red italics, see Figure 2.1) in cells (data not shown). Therefore since βTrCP1 is able to bind both the N- and C- termini of TAp63γ and I mutated half of the K residues in the molecule, that TAp63γ's ubiquitylation by βTrCP1 is likely not restricted to a specific residue under the conditions of our assay. It is also worth noting that K49, which is the only lysine residue close to a putative βTrCP1 binding site I identified in TAp63γ's N-terminus (see below and Figures 2.1 and 4.10) was included in several of these mutant constructs, also supporting the result that βTrCP1 binds and may ubiquitylate TAp63γ on both its N- and C-termini.

I then proceeded to test the ubiquitin linkage on TAp63γ to determine if there was a correlation between the linkage and the activation I observed in our earlier assays (Chapter 3). βTrCP1 has only been documented to ubiquitylate substrates through a K48-mediated linkage. However, in some cases a K63-mediated ubiquitin linkage has been associated with activity, as noted in the Introduction. Therefore to test for the type of ubiquitin linkage, I repeated the ubiquitylation assays using his-K48R or his-K63R

ubiquitin mutants. These mutants will ablate a poly-ubiquitin chain attached at the specified linkage but not other types of linkages. As seen in Figure 4.5, the co-expression of TAp63γ with his-ubiquitin showed that basal ubiquitylation (lane 3) was increased in the presence of βTrCP1 (lane 6). Strikingly, the baseline level of TAp63γ ubiquitylation was drastically reduced in the presence of his-K48R ubiquitin, but not his-K63R ubiquitin (compare lanes 4 and 7 with lanes 5 and 8). Therefore, most of the ubiquitin linkages on TAp63γ are K48 poly-ubiquitin linkages and this is also true in the presence of βTrCP1. Also, in the presence of βTrCP1, the level of K63 linked ubiquitylation TAp63γ ubiquitylation in the presence of βTrCP1 decreases slightly (compare lanes 5 and 8). This decrease most likely signifies that a small portion of the linkages on TAp63γ are K63 mediated and could be mediated through βTrCP1. However, this effect is still minor compared with reduction of the K48 linkage.

4.3 p63γ's activation by $SCF^{\beta TrCP1}$ is mediated by its ubiquitylation

Since I found that βTrCP1 could both activate and ubiquitylate TAp63γ, I next wished to determine if the activation was related to the ubiquitin modification. Therefore, I repeated some of the activation assays using the Flag-ΔFβTrCP1 mutant. First, I examined TAp63γ half-life in H1299 cells in a similar manner to Figure 3.3, with the addition of the ΔFβTrCP1 mutant. Where co-expression of GFP-TAp63γ and Flag-βTrCP1 stabilized the TAp63γ (Figure 4.6, lanes 7-12), co-expression of GFP-TAp63γ with Flag-ΔFβTrCP1 at the same expression ratio as in Figure 4.3 did not stabilize the TAp63γ when compared with the GFP-TAp63γ alone (lanes 13-18).

In order to observe if this loss of TAp63γ stabilization is also correlated to a decrease in mRNA expression, I continued by examining the level of *p21* expression by

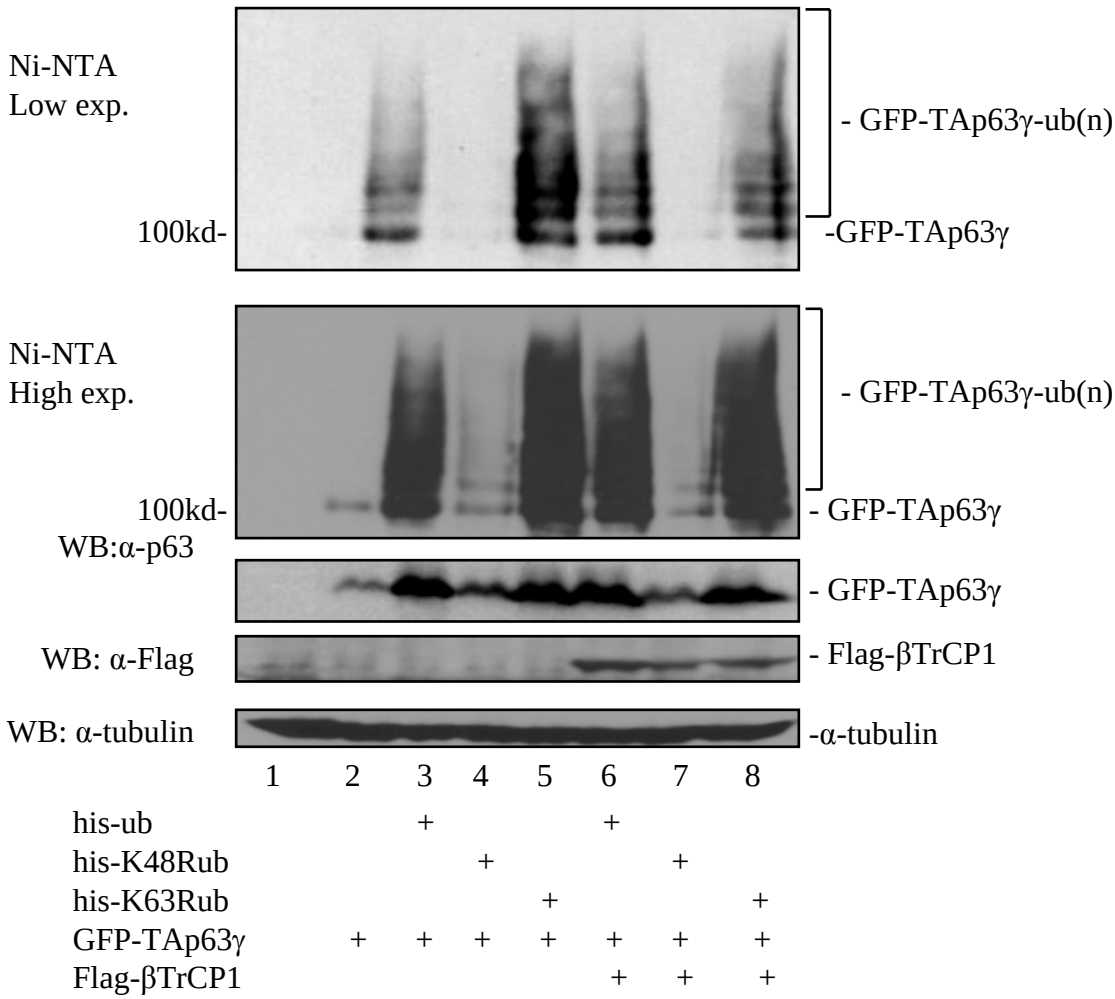
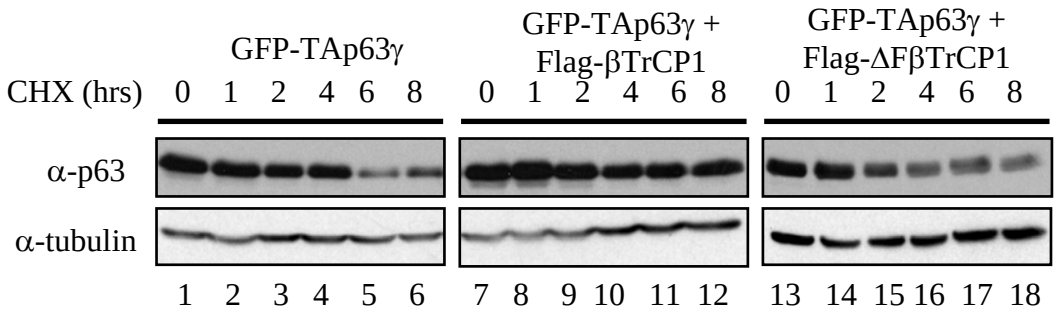


Figure 4.5 - The ubiquitylation of TAp63γ by SCF$^{\beta TrCP1}$ is through a K48 ubiquitin linkage. H1299 cells were transfected with noted plasmids, analyzed by Ni-NTA purification as in 4.1, and blotted with noted antibodies.



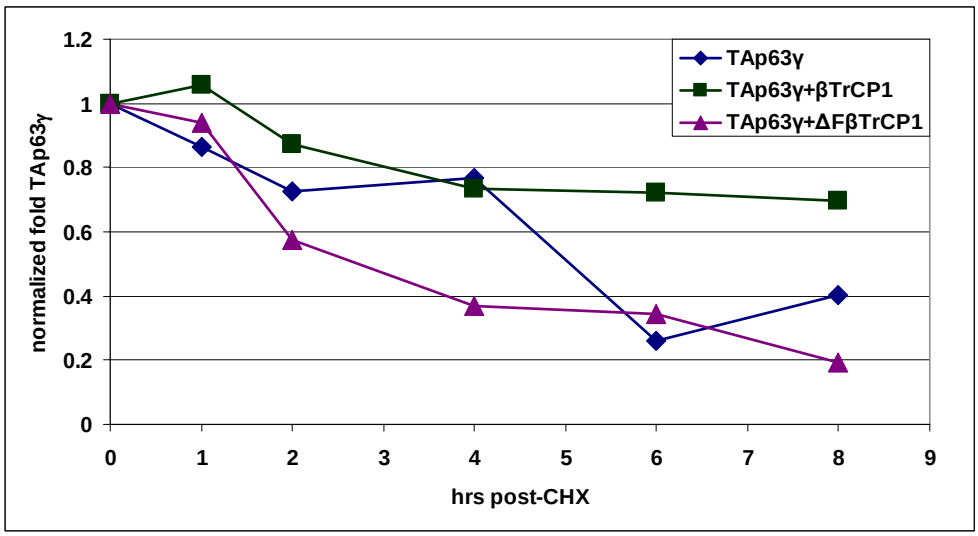


Figure 4.6 - ΔFβTrCP1 co-expression does not stabilize TAp63γ. H1299 cells were transfected with GFP-TAp63γ, Flag-βTrCP1 and Flag-ΔFβTrCP1. 48 hrs later they were treated with cycloheximide and harvested over the indicated time course. Representative data plotted below.

qPCR. When GFP-TAp63γ and the Flag-ΔFβTrCP1 were co-expressed, there was no significantly increased expression of *p21* compared to the *p21* induction correlated with the expression of GFP-TAp63γ alone, while *p21* expression was significantly increased in the presence of both GFP-TAp63γ and Flag-βTrCP1 (Figure 4.7, A; B is triplicate experiments). Therefore, I wanted to see if this loss of *p21* expression was also true at the level of promoter binding. Again, I used H1299 cells and performed ChIP. As shown in Figure 4.8, A, while the GFP-TAp63γ has the expected increase of binding to the *p21* P1 and P2 promoter regions (top and bottom, respectively), and GFP-TAp63γ and Flag-βTrCP1 co-expression leads to a significant increase in promoter binding, the level of GFP-TAp63γ bound to the *p21* promoter in the presence of Flag-ΔFβTrCP1 significantly decreases compared with GFP-TAp63γ alone on both the P1 and P2 regions (Figure 4.8, B). The P3 results were similarly negative, as in Figure 3.16 (data not shown). Triplicate experiments are shown in Figure 4.8, B. Concomitantly, these data demonstrate that the increased stabilization and activation of TAp63γ by βTrCP1 are direct results of the ubiquitylation itself, as the ΔFβTrCP1 mutant is still able to bind TAp63γ, but not to ubiquitylate it.

Also in support of this conclusion are preliminary assays I carried out using two point mutants of TAp63γ in a putative βTrCP1 binding site on TAp63γ's N-terminus (Figure 4.9). This site (61-DSDLSD-66) of p63 is not conserved in p53 and p73, as would be expected by lack of ubiquitylation on either of those molecules, noted previously. But the site is conserved in mouse. The deletion of this site would not be expected to ablate binding, since βTrCP1 also binds to TAp63γ's C-terminus. However, deletion of this site would be expected to alter the degree of ubiquitylation of the

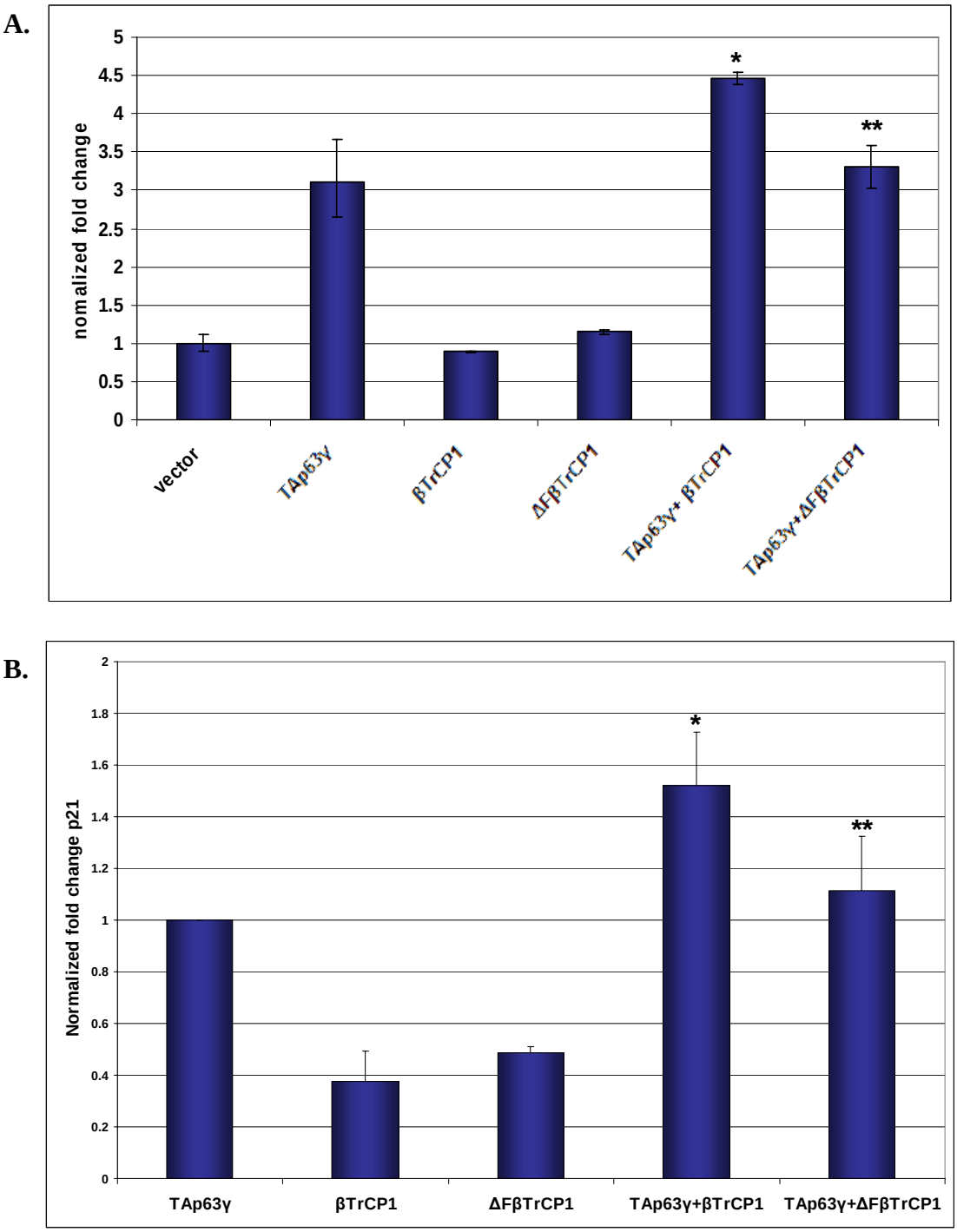
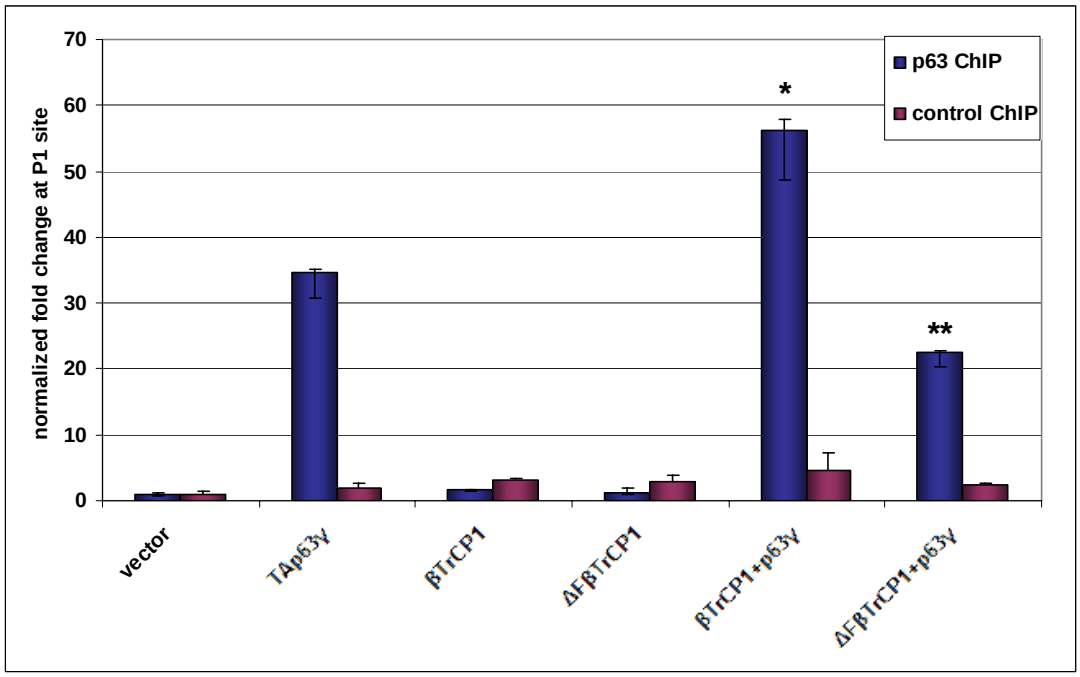


Figure 4.7 - ΔFβTrCP1 co-expression does not upregulate *p21*. qPCR was performed on *p21*, as in Fig. 4. A. Representative data. * = P = 0.022 increase from TAp63γ alone control, ** = P < 0.003 decrease from TAp63γ+βTrCP1 sample, as shown in Gallegos, JR *et al*. B. * = P = 0.004 increase from TAp63γ alone control, ** = P = 0.03 decrease from TAp63γ+βTrCP1 sample.

A.



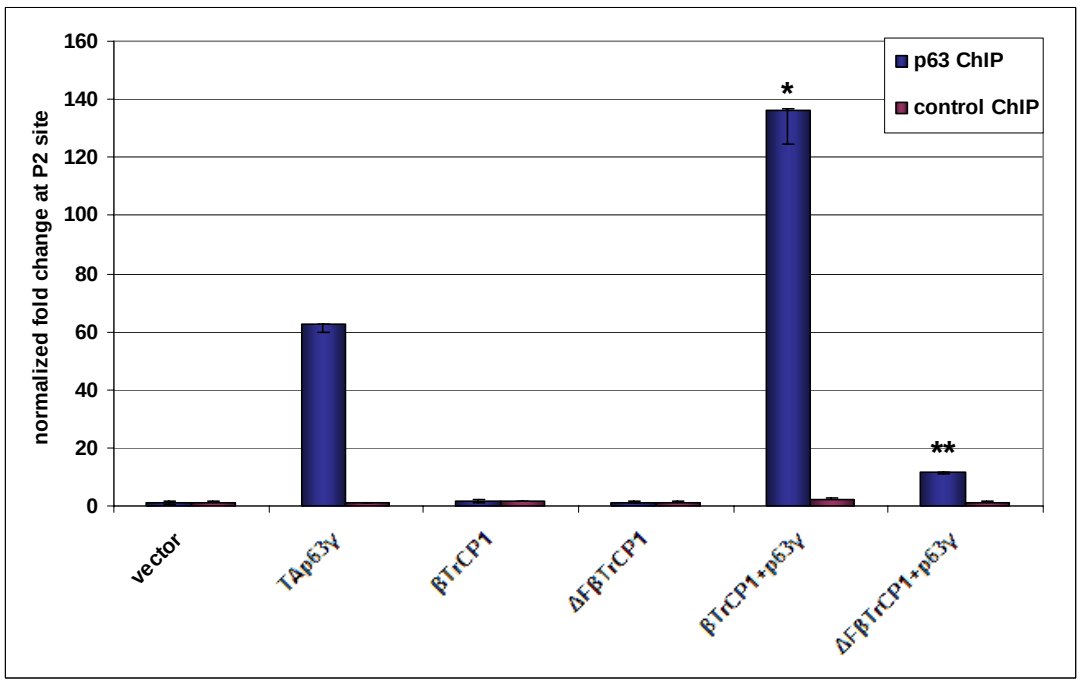
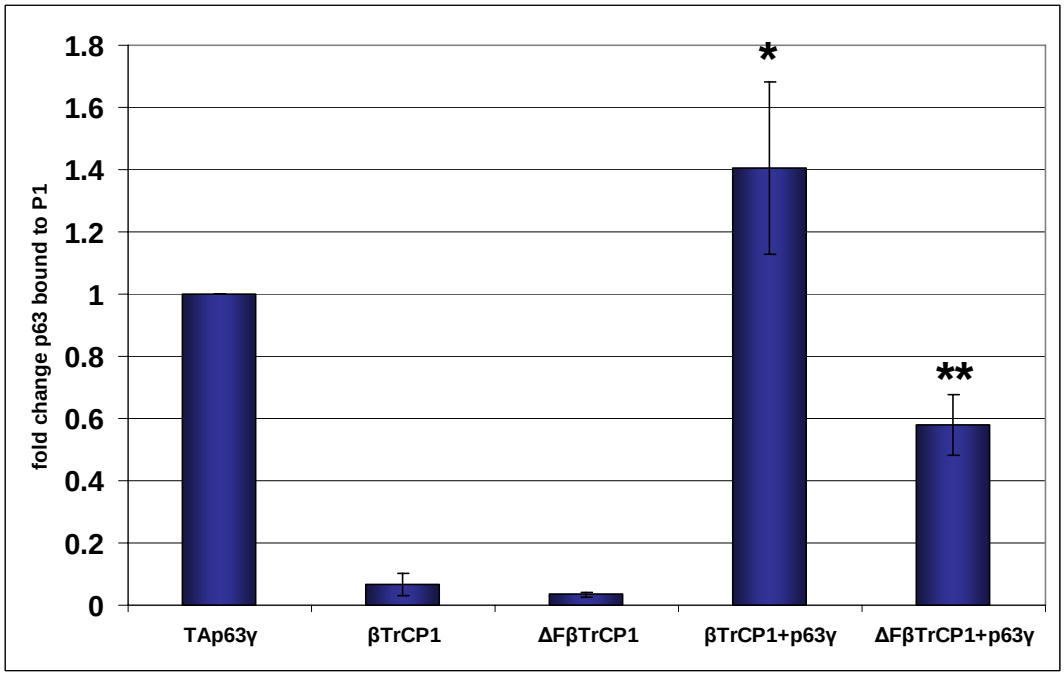


Figure 4.8 - TAp63γ binding to the *p21* promoter decreases in the presence of ΔFβTrCP1. Legend on continuing page.

B.



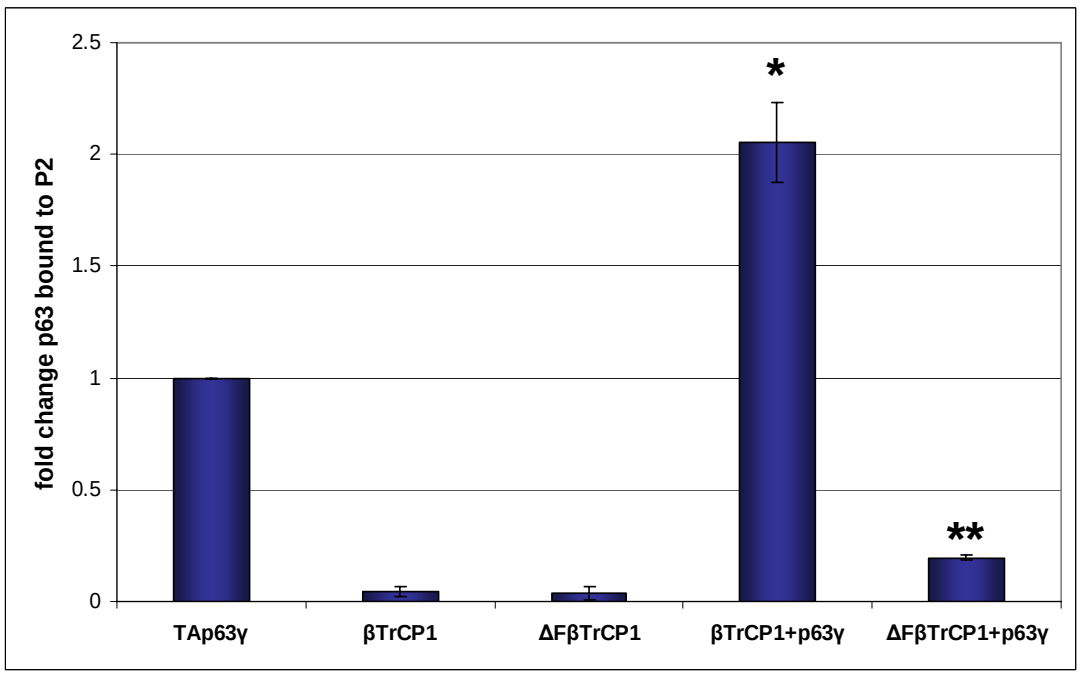


Figure 4.8 - TAp63γ binding to the *p21* promoter decreases in the presence of ΔFβTrCP1. Legend on continuing page.

Figure 4.8 - TAp63γ binding to the *p21* promoter decreases in the presence of ΔFβTrCP1. A. Representative ChIP experiments. H1299 cells were transfected and ChIPed using the indicated ChIP antibody/qPCR primer combinations. The top panel is data from the P1 primer set, the bottom panel is data from the P2 primer set on the same experiment. For the P1 graph: * = P = 0.006 increase from TAp63γ alone control, ** = P < 0.002 decrease from TAp63γ+βTrCP1 sample. For the P2 graph: * = P = 0.005 increase from TAp63γ alone control, ** = P = 0.013 decrease from TAp63γ+βTrCP1 sample, as shown in Gallegos, JR *et al*. B. Triplicate experiments normalized to TAp63γ alone. For the P1 graph: * = P = 0.006 increase from TAp63γ alone control, ** = P < 0.002 decrease from TAp63γ+βTrCP1 sample. For the P2 graph: * = P = 0.024 increase from TAp63γ alone control, ** = P = 0.003 decrease from TAp63γ+βTrCP1 sample.

molecule, since I observed that the N-terminus of TAp63γ bound βTrCP1 more efficiently than the C-terminus, that the N-terminus could be ubiquitylated *in vitro*, and that in other βTrCP1 substrates alteration of canonical sites by point mutation often changes the kinetics of ubiquitylation (34). The mutations I focused on were the TAp63γD61R and TAp63γS62A mutations, because mutation of these residues often greatly affects βTrCP1's activity with many substrates (34).

These mutations would not affect TAp63γ's binding to βTrCP1. In order to validate this prediction, I performed an IP in H1299 cells. As shown in Figure 4.10, the D61R and S62A mutants precipitate similar levels of βTrCP1 at equivalent expression level with TAp63γ (lanes 6-8) and also βTrCP1 precipitated similar levels of each of the mutants, compared with the standard TAp63γ construct (lanes 14-16). Therefore, TAp63γD61R and TAp63γS62A do not show any difference in binding to TAp63γ in cells. I next wanted to determine if the D61R and S62A mutations would affect the ubiquitylation of TAp63γ in cells. Therefore, I conducted similar ubiquitylation assays as described above. A very low exposure is shown in Figure 4.11 to show the differences in the ubiquitylation of TAp63γ between samples. In the presence of ubiquitin, the TAp63γ shows general ubiquitylation as in the previous assays (lane 2). This ubiquitylation is reduced dramatically with the TAp63γS62A mutant (lane 3), while it is increased dramatically in the presence of the TAp63γD61R mutant (lane 4). It should be noted that upon longer exposure, the TAp63γS62A mutant does show a ladder typical of poly-ubiquitylation, as in our several previous assays. Therefore, our data demonstrated that while having no effect on binding, that the TAp63γS62A and TAp63γD61R mutants showed striking differences in their degree of poly-ubiquitylation compared to TAp63γ

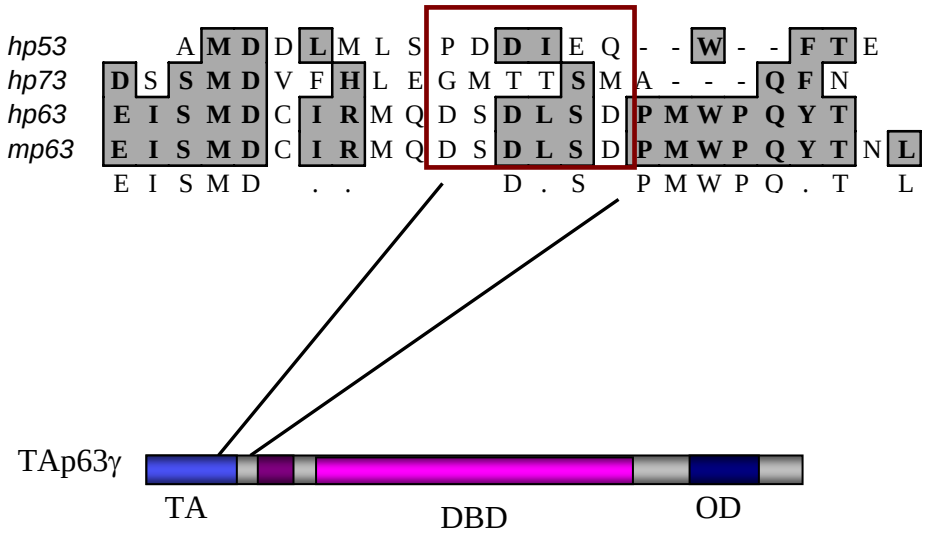


Figure 4.9 - Schematic of TAp63γ's putative canonical βTrCP1 binding domain and mutations. The canonical domain in p63 N- terminus in red box, with bars marking where it lies in the TA domain. The first D in the boxed sequence is D61.

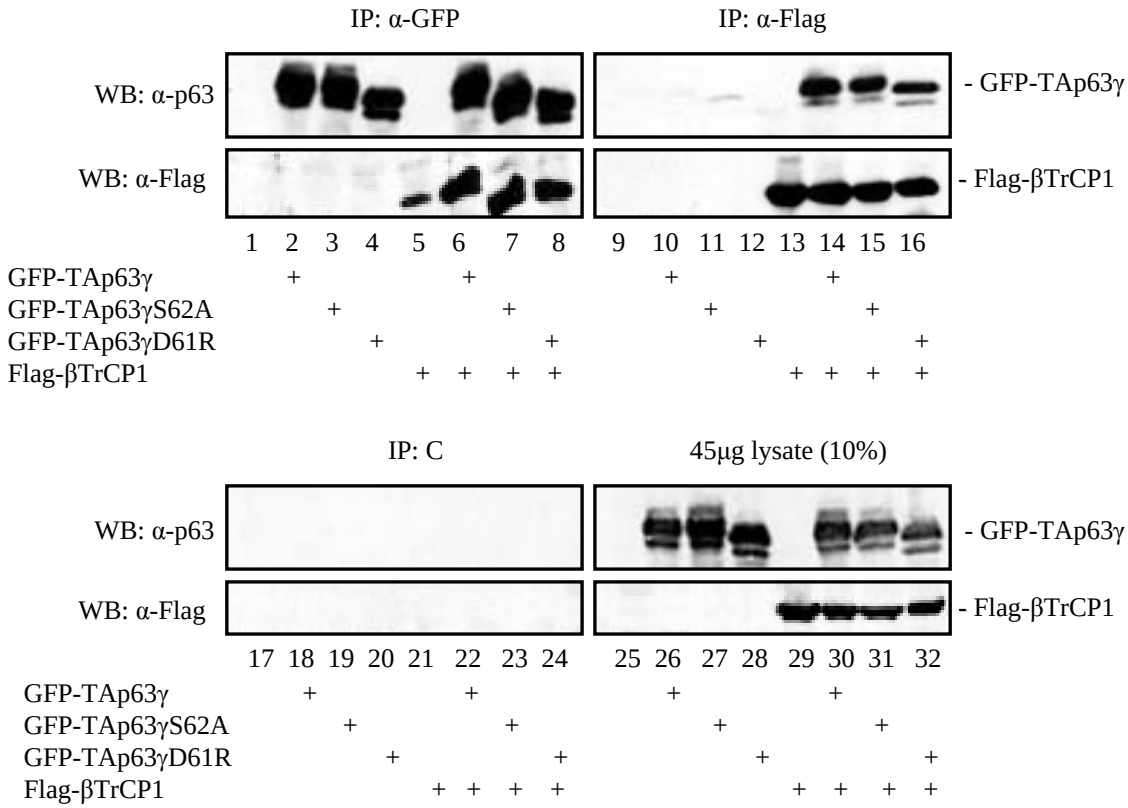


Figure 4.10 - The TAp63γD61R and TAp63γS62A mutants bind βTrCP1 with similar affinity to TAp63γ in cells. H1299 cells were transfected as noted and co-IP was conducted as in 3.7. WB with noted antibodies.

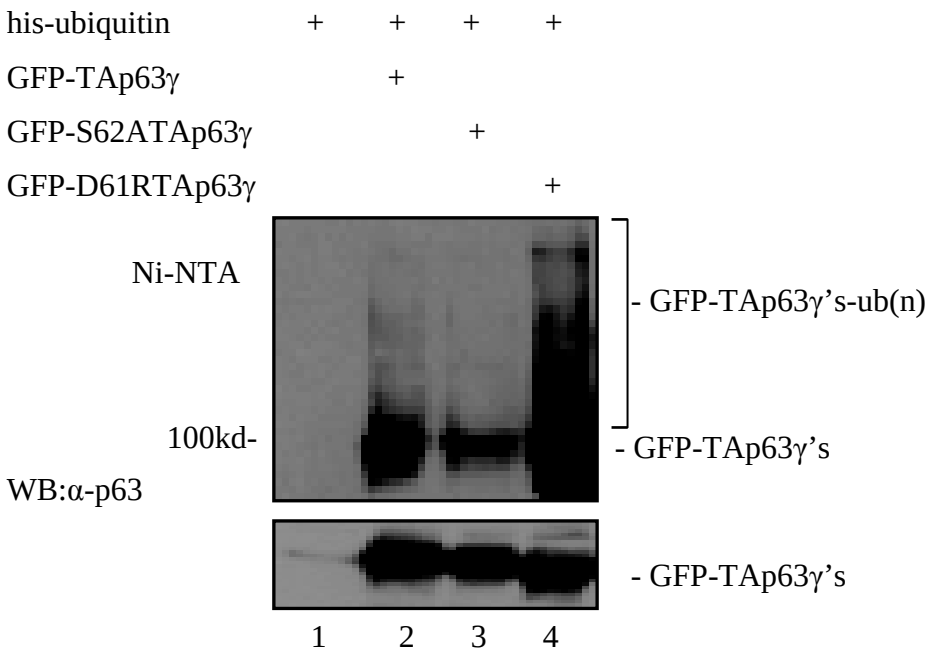
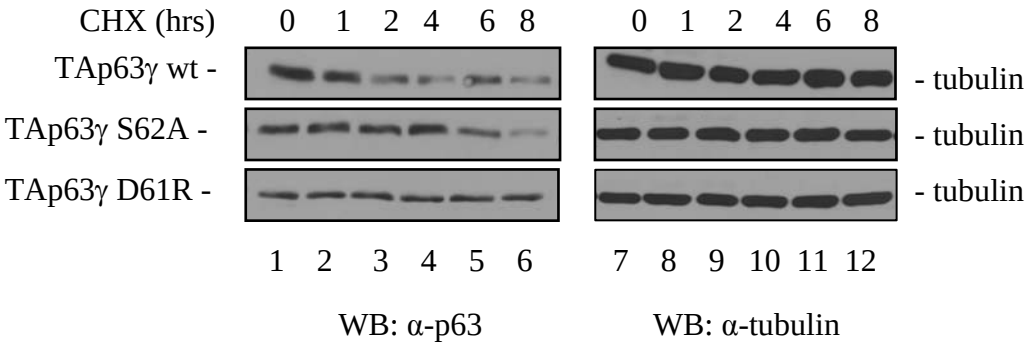


Figure 4.11 - The TAp63γD61R and TAp63γS62A mutants show striking ubiquitylation differences from TAp63γ in cells. H1299 cells were transfected as noted and analyzed by Ni-NTA ubiquitylation assay. WB was performed with indicated antibody.

alone. This difference in ubiquitylation has been shown on other substrates to be due to a differential recognition of substrate contact to the WD-domain binding domain of βTrCP1 which leads to differential ubiquitylation kinetics by the SCF complex, as noted in the Introduction (165).

Next, I wished to examine if this difference in modification would translate to a difference in stability. To this end, I performed stability assays with the TAp63γ versus the TAp63γD61R and TAp63γS62A mutants in H1299 cells. My preliminary experiment demonstrates that the TAp63γ half-life fell between 1.5-3 hrs, as in previous assays. Also, the TAp63γS62A mutant has an intermediate half-life of around 4-5 hrs, while the TAp63γD61R mutant is highly stable over the 8 hour time course (Figure 4.12). This result supports my earlier result with the ΔFβTrCP1 mutant showing that loss of ubiquitylation would not raise the stability of TAp63γ above that of TAp63γ alone, in that even the low residual level of poly-ubiquitylation on TAp63γ which is retained by the TAp63γS62R is sufficient to stabilize the protein over that of the TAp63γ alone, while the hyper-ubiquitylation conferred by the TAp63γD61R mutation is in fact able to render the protein highly stable. In accordance with this result, I also went on to see if the *p21* mRNA increase would also be conserved. Again, I co-expressed the TAp63γ constructs with βTrCP1 in H1299 cells and examined the *p21* mRNA levels by semi-quantitative PCR (Figure 4.13). In this preliminary experiment, I show that the level of *p21* mRNA is similarly upregulated with each of the TAp63γ mutants compared with TAp63γ (lanes 2-4 compared with lanes 6-8). This result suggests that even a minimal amount of ubiquitin is sufficient to confer a TAp63γ-mediated increase of *p21* mRNA. While further experiments would be needed to understand the mechanism of how these



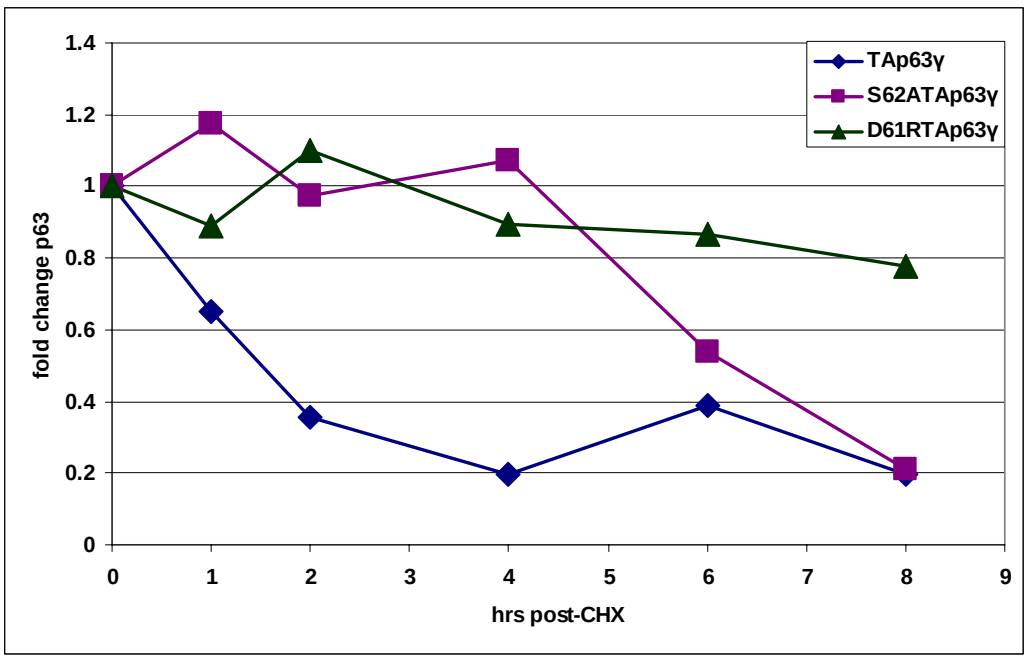


Figure 4.12 - The TAp63γD61R and TAp63γS62A mutants show altered stability that is greater than TAp63γ's stability in cells. H1299 cells were transfected as noted and treated with CHX over the labeled time course. WB was performed with noted antibodies.

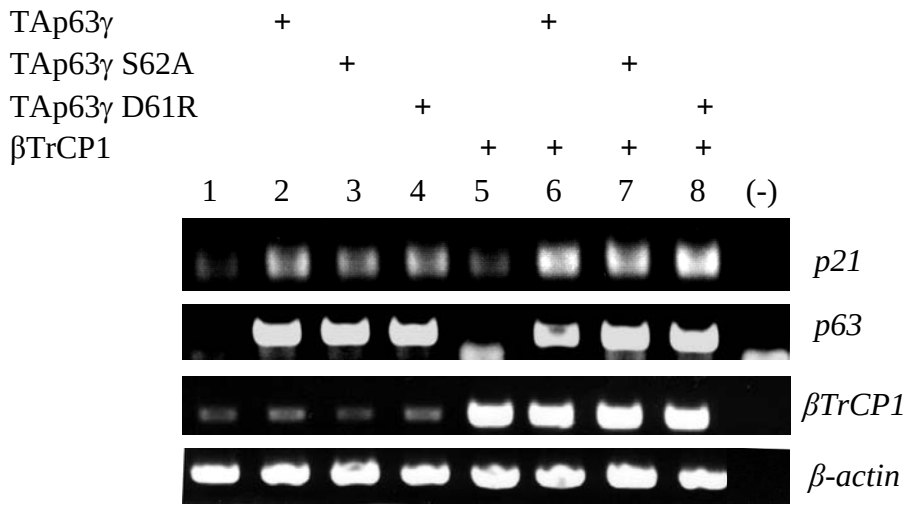


Figure 4.13 - The TAp63γD61R and TAp63γS62A mutants activate *p21* similarly to TAp63γ when co-expressed with βTrCP1. H1299 cells were transfected with the vectors above and analyzed by RT-PCR with the primers shown.

mutations effect p63γ ubiquitylation, stability, and its induction of *p21* mRNA, directly, the overall effect that these TAp63γ mutations have on stability demonstrates that while ubiquitylation works as a TAp63γ activating signal (as shown with the ΔFβTrCP1 mutant), that modulation of the degree of ubiquitylation has a direct affect on the length of TAp63γ's stability as well. Thus, while a minimal addition of poly-ubiquitin is necessary for TAp63γ's augmentation of p21 levels, the amount of global ubiquitylation on TAp63γ can elongate TAp63γ's stability.

CHAPTER 5

CONCLUSIONS, DISCUSSION AND FUTURE DIRECTIONS

p63 and βTrCP1 are involved in the control of important cell fate pathways and often deregulated in cancer. In the case of p63, its deregulation has been closely tied to its protein stability, and the major modification which controls the stability of all the p63 isoforms is ubiquitylation. βTrCP1 is a ubiquitin ligase which has many substrates in pathways which are critical to tumorigenesis, such as the NF-κB and Wnt pathways. Thus βTrCP1 may provide an important clue to p63 function and also provide a better understanding of how p63's activation intercalates with other major signaling pathways in cancer and development. In this dissertation, I provide evidence for a novel activation pathway between TAp63γ and βTrCP1, beginning with the observation that the endogenous and exogenous steady-state levels and the half-life of TAp63γ increase dramatically in the presence of βTrCP1. βTrCP1 binds to TAp63γ directly, and also to ΔNp63γ to a lesser degree, consistent with our data mapping βTrCP1 binding to the N- and C-termini of TAp63γ. This interaction is also present endogenously. The result of this interaction is the subsequent stabilization of TAp63γ and increased upregulation of *p21* mRNA and protein, though a mechanism which includes increased binding of TAp63γ to the *p21* promoter at both a well-characterized p53 family binding site and a novel binding site for p63 on the *p21* promoter. The observed increase of p21 protein is reflected functionally as an increase of cells in G1 arrest after the induction of a G2 block. Further, βTrCP1 is able to ubiquitylate TAp63γ *in vitro* and in cells and this ubiquitylation is needed for the functional activation of TAp63γ, as loss of the $SCF^{\beta TrCP1}$

ubiquitin ligase activity with the dominant-negative ubiquitylation-deficient mutant, ΔFβTrCP1, ablates TAp63γ's increased stability, prevents the upregulation of *p21* mRNA, and drastically decreases the amount of TAp63γ bound to both *p21* promoter regions. This data is supported by experiments using mutants of a putative canonical βTrCP1 binding site in p63's N-terminus (TAp63γD61R and TAp63γS62A). Mutations in this domain are able to change p63's ubiquitylation and stability without affecting TAp63γ's binding to βTrCP1 or its activation of *p21* in preliminary experiments, suggesting that a minimal ubiquitylation was necessary for its activity but that the overall signal length in vivo may be mediated by the degree of TAp63γ ubiquitylation.

In light of our data, I would like to propose a model (Figure 5.1) in which $SCF^{\beta TrCP1}$ associates with and stabilizes TAp63γ in the nucleus through the covalent addition of ubiquitin. The increased load of ubiquitylated TAp63γ at the *p21* promoter then leads to an increased level of p21 transcript and protein and a subsequent increase in G1 cell cycle arrest.

5.1 Ubiquitin as an activating signal

The literature on various E3 ligases in signaling, in terms not only of the regulation of protein degradation, but also in terms of protein activation, is increasing at an incredible rate. While our report is the first to demonstrate a direct role for $SCF^{\beta TrCP1}$ in stabilization and activation of its substrate through ubiquitylation, βTrCP1 has been characterized previously for its involvement in activation pathways, such as its well-established role in aiding in the activation of the NF-κB pathway both by degrading IκBα and by aiding in the proteolytic processing of NF-κB p100 (81). Additionally, the

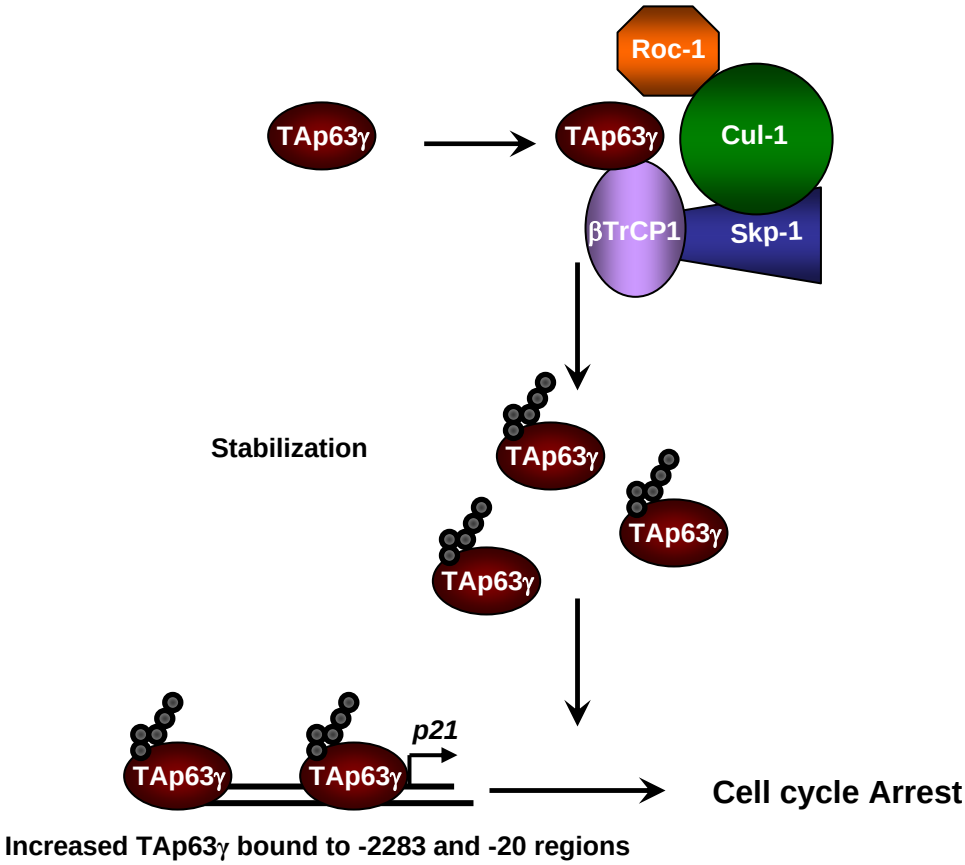


Figure 5.1 - Model for the ubiquitylation and activation of TAp63γ by $SCF^{\beta TrCP1}$. TAp63γ associates with $SCF^{\beta TrCP1}$ in response to an unknown signal. $SCF^{\beta TrCP1}$ then ubiquitylates TAp63γ on unknown sites, one which is on the N-terminal (shown), stabilizing it. The stabilized TAp63γ binds the endogenous *p21* promoter (bottom) at two sites. *p21* levels increase, due to the increased binding of TAp63γ to the promoter, most likely through a transcriptional mechanism, and elicit cell cycle arrest. $SCF^{\beta TrCP1}$ complex is shown in color, with ubiquitin represented by the gray circles.

association between transcriptional activators and SCF E3 ligases is also well-trodden experimental ground. For instance, another E3 ligase which forms a complex with the SCF core is Skp2. Skp2 co-activates the oncogene c-myc through a monoubiquitylation of c-myc on chromatin (68, 151). Our data clearly demonstrates that TAp63γ is stabilized exogenously at sub-saturating levels of both TAp63γ and βTrCP1 (Figures 3.2 and 3.3). Further, TAp63γ is destabilized endogenously by knockdown of βTrCP1 and βTrCP2 (Figure 3.5). Our endogenous data in primary MEFs shows that the TAp63γ protein level is markedly reduced with the loss of βTrCP1 (Figure 3.1). Consistent with this result, increased levels of βTrCP1 raise the steady-state levels of TAp63γ (Figure 3.2), while the mRNA levels remain unchanged (Figures 3.1, 3.2, and 3.12). Taken together, these data demonstrate that the stabilization and activation of TAp63γ by $SCF^{\beta TrCP1}$ is present endogenously.

An intriguing result is our data showing the clear effect of the ubiquitin modification itself on TAp63γ stability which could have impact on specific p63 isoform regulation. Our data shows that the p63 isoforms have a distinctly differing degree of ubiquitylation, demonstrating that ubiquitylation could be used as one mechanism to distinguish specific activition of each p63 isoform (Figure 4.1). Also, supporting this conclusion is our data with the TAp63γD61R and TAp63γS62A mutants where the degree of ubiquitylation, rather than the protein-protein interaction, mediates a differing length of stability (Figures 4.10 - 4.12). However, since our mRNA data, which did not show a difference in activity between TAp63γ and the TAp63γD61R and TAp63γS62A mutants (Figure 4.13), was taken at only a 24 hr time point I can not observe how these differences in signal length might affect the activation kinetics of TAp63γ. Future

experiments will characterize the activation of these mutants over a time course in order to determine if the activation length also varies similarly to the protein half-life.

Further, in terms of our assays with the βTrCP1 mutant, TAp63γ is ubiquitylated in the presence of βTrCP1, but not ΔFβTrCP1 (Figures 4.2 and 4.3). This result is important for our analysis, since the ΔFβTrCP1 mutants can still bind, but not ubiquitylate βTrCP1 substrates due to lack of recruitment of the SCF backbone. Our data clearly shows that significantly more TAp63γ is recruited to the chromatin fraction and to the *p21* promoter in the presence of βTrCP1 (Figures 3.14 and 3.16) after its stabilization, accounting for the significant increase of p21 at both the mRNA and protein level (Figures 3.12 and 3.13). This upregulation most likely occurs through direct transcriptional activation of TAp63γ on the *p21* promoter; characterization of the specifics of this mechanism will be discussed below. Yet in each case when the ubiquitylation activity is lost through expression of the ΔFβTrCP1 mutant, TAp63γ's stability (Figure 4.6), its upregulation of *p21* (Figure 4.7), and its binding to the *p21* promoter (Figure 4.8) are lost as well. The implications for this dominant-negative activity *in vivo* will be discussed below.

Further adding detail to our model is the fact that the stability of TAp63γ is likely initiated off chromatin, since our IP data is taken from a whole cell lysate that would not contain chromatin-bound proteins. In the case of my IP conditions I was also able to successfully detect endogenous cullin 1 by WB (data not shown), indicating that the SCF backbone was precipitating with the sample, since βTrCP1 and TAp63γ do not contact cullin directly. Further, I know that TAp63γ's stabilization and activation occur in the nucleus, since βTrCP1 and TAp63γ both are nuclear proteins, and are therefore aptly

placed for transcriptional regulation (34, 173). Also, preliminary data using immunofluorescence for GFP-TAp63γ and Flag-βTrCP1 showed that both proteins were nuclear, similar to their published expression patterns, and co-expression did not alter their localization (data not shown). Further, in the process of my ChIP assays I was unable to detect Flag-βTrCP1 bound to the same regions of the *p21* promoter which were highly positive for TAp63γ using a Flag-M2 antibody that works admirably for ChIP with other Flag-tagged proteins (data not shown). However, these findings do not rule out that the βTrCP1 is present bound to TAp63γ at the promoter, but not directly to DNA.

Important future experiments which would help to elucidate the exact sequence of events and better understand how the ubiquitylation itself might be potentiating the activity of TAp63γ on the *p21* promoter could be to precipitate the chromatin bound versus chromatin unbound TAp63γ complexes, to separate them by using a gel or column, as described below, and to identify them using mass spectroscopy, in order to compare the fractions for potential mediators of a larger transcription complex. Since each p63 isoform is predicted in using a different transactivation complex, elucidation of a TAp63γ/βTrCP1-specific signaling complex would provide more detailed information on one of these complexes and allow for future experiments to understand how TAp63γ could work with the other p53 family members on common target genes, as well as its specific transcriptional targets. Initial purification techniques which might allow for such separation of DNA bound complexes would include a large scale DAI (DNA affinity immunoblotting), where a biotin labeled DNA sequence of the *p21* promoter is mixed with cell lysate to IP out complexes, bound to magnetic avidin beads, and precipitated using magnetic attraction, followed by SDS-PAGE analysis, staining and identification of

complexes by mass spectrometry (92). An alternative to the above experiment would be an elution, followed by analysis of co-migrating fractions with the TAp63γ/βTrCP1 complex off of a DNA column. Both of these techniques might not precipitate all of the complex components, as they use oligo sequences of DNA. However once the proteins which precipitate with DNA-bound p63 are identified, further identification of proteins which interact with those components could continue elucidation of the macro-complexes. One protein which has been shown to be involved in the SCF^{Skp2} activation of c-myc and could also be involved in a TAp63γ/$SCF^{\beta TrCP1}$-containing transcription complex is p300. Our laboratory's earlier studies showed that p300 could bind and acetylate TAp63γ on the N-terminus and that this modification also served to upregulate p21 mRNA and protein levels. Therefore our study has provided an important starting point for more detailed experiments which could elucidate p63's differing and complex function on target genes such as *p21*, and since ubiquitylation has been traditionally studied for its role in cell cycle control, p21 is indeed a very apt place to begin to build such a signaling network.

While ubiquitin is traditionally considered a degradation signal, the list of proteins which use ubiquitin as an activating stimulus is growing and at the root of this debate is the concern over how important a specific type of poly-ubiquitin linkage is to signaling. Some ubiquitin linkages have been closely tied to activation, as is the case with the K63-ubiquitin linkage tested in this study. Our data showing that the ubiquitylation of TAp63γ by βTrCP1 occurs largely though a K48-mediated ubiquitin linkage is a very surprising and intriguing (Figure 4.5), as a K48 ubiquitin linkage has often been shown to mediate protein degradation, rather than stabilization (160). However, it is not as

surprising in the sense that βTrCP1 uses a K48 linkage to poly-ubiquitylate TAp63γ, since this E3 ubiquitin ligase has been documented to use this linkage for its other substrates to elicit their degradation (34, 81). Thus, our study, though quite preliminary and requiring further and close validation, suggests that βTrCP1 may work similarly to other SCF components, such as Skp2, which has been documented as using both K48 and K63-mediated linkages. Though how the SCF^{Skp2} is able to accommodate or select for one linkage versus another with all the core components remaining unchanged is under current investigation (56, 69, 152, 153). It would be interesting if a K48 ubiquitin linkage were used by βTrCP1 to mediate TAp63γ polyubiquitylation and stabilization as will be determined by further investigation.

One recent study which might explain this difference in signaling for βTrCP1 demonstrates that forked poly-ubiquitin chains created by some E2s, including UbcH5 (used in our *in vitro* assays), may also be a signal used for a function other than proteasomal degradation (66, 81). This study examined the linkage and chain branching of several types of E3s *in vitro*, using several E3's including the RING proteins MuRF1, a muscle-specific E3 critical in muscle atrophy, and MDM2, using a combination of *in vitro* ubiquitylation assays similar to those in our study followed by mass spectrometry. They found that MuRF1 and MDM2 did not show a preference for a specific ubiquitin linkage when mixed with various lysine mutants of ubiquitin, and that they were able to form branched chains containing many types of ubiquitin linkages when in the presence of wild-type ubiquitin (67). They also found that the HECT domain E3s they studied, E6AP and NEDD4 only make homogenous chains (K48 and K63 respectively) (67). Therefore, there is a possibility that the linkage might be altered *in vivo* depending on the

abundance or availability of a given E2 which could form a branched chain versus an unbranched chain. In fact, their data suggests this E2 dictation of chain branching is in fact the case, as MuRF1, an E3 ligase would form branched chains with UbcH5, but not with other families of E2s (67). This result is particularly striking when looking at the data on p63 degradation, since NEDD 4 is able to degrade p63, while MDM2 does not (157). Also of note, this study did not test UbcH3, another E2 which also works *in vivo* and *in vitro* with $SCF^{\beta TrCP1}$, and our *in vitro* assays showed equivalent activity to UbcH5c. However there is another paper which shows that the SCF complex is able to form non-K48 linked chains when functioning with UbcH5 (166). Our mutant assay is largely preliminary and limited to the very first tri-ubiquitin linkage off of the mono-ubiquitin attached to the molecule. Further examination of the branching in cells and in an *in vivo* system would be extremely challenging future experiments since the detection of ubiquitylated proteins, let alone poly-ubiquitin chains is difficult even with purified protein because of the small concentration of a given ubiquitylated moiety. Further, as the authors of the above mentioned studies conclude, deciphering how exactly these linkages and branches work *in vivo* to stall degradation and potentially mediate activity will be difficult and challenging experiments because of the milieu of other factors, such as caspases and deubiquitylases which are also working against chain stability *in vivo*. Therefore, if detailed biochemical studies were to proceed on the nature of βTrCP1's ubiquitylation of TAp63γ versus its other substrates, the preliminary studies would be limited to an *in vitro* system following a similar combination of *in vitro* ubiquitylation assays and mass spectrometry. However, the connection between ubiquitin linkage and

activity will continue to be an important question which will be no doubt be explored further as the techniques advance.

While TAp63γ is the first substrate known to be activated by $SCF^{\beta TrCP1}$-mediated ubiquitylation, the p53 family members can each be activated by ubiquitylation. Both MDM2 and NEDL2, a NEDD-4 related HECT-containing E3 ligase, stabilize p73, though NEDL2 is the only one which ubiquitylates p73 (157). Still, perhaps the most interesting of these studies is one paper showing that p53 is targeted for ubiquitylation by E4F1, and this modification slightly stabilizes it for a p21-specific activation cascade very similar to what I observe with p63 (88). While I tested several p53 family target genes, including *Bax*, *MDM2*, and *PIG3*, the only downstream regulation I observed was the regulation of *p21*. Albeit, while I tested a small subset of p63's known targets, lack of regulation of these pro-apoptotic target genes supports my observation that an increase of apoptotic cells was not detected by FACS analysis (Figure 3.17). This lack of apoptotic induction in conjunction with the presence of an activating ubiquitin modification was also similar to the p53/E4F1 study. Interestingly, the ubiquitylation of p53 by E4F1 also occurs through a K48-linked poly-ubiquitylation which they demonstrate as having a much different pattern of p53-ubiquitin moieties by WB than an MDM2-mediated ubiquitin linkage in cells (49, 88). Additionally, as far as the relationship between p63 and an SCF E3 ligase, it is unsurprising that p63 is targeted by an SCF complex, as p53 is also recognized by a cullin-containing complex during viral infection (123). The characterization of βTrCP1's ubiquitylation of TAp63γ would be an interesting future study, especially with the conservation of this stabilizing role of ubiquitin among two of the three p53 family members (101). For now, what is clear in

light of our data and earlier studies is that the relationship among p63's DNA binding, subsequent transactivation activity, and stability is most likely the result of subtle modifications and interactions tailored to each isoform.

5.2 p63 isoform specificity

As mentioned above, p63's six isoforms provide a mechanism of varied control of their signaling pathways. In the case of βTrCP1 interaction, both p63α and p63γ isoforms can bind βTrCP1 in cells exogenously and endogenously, but not p63β isoforms (Figures 3.6, 3.8 and 3.9). Also, βTrCP1 binds TAp63γ and ΔNp63γ isoforms *in vitro* and in cells at both the N- and C-terminal segments of the protein (Figures 3.6, 3.7 and 3.11), regions known to be important for moderating stability among the p53 family members. The interaction of the p63 isoforms in this signaling pathway could be explained by a thorough analysis of p63/βTrCP1 binding affinity and presence of βTrCP1 in the cell. These potential differences in p63 isoform competition for $SCF^{\beta TrCP1}$ binding could translate to a difference in ubiquitylation. In fact, a difference in p63 post-translational modification is often found in the current literature (see Introduction). For example, the sumoylation reaction that decreases p63 stability is unique to the p63α isoforms (37), as the ubiquitylation by Itch may also be present on only the p63α isoforms (128). In the case of our study, binding strength seems to be dependent on more than simply the availability of isoform-specific domains. Even though βTrCP1 binds to both the N- and C-terminal domains of p63, regions which, taken together are common to all the p63 isoforms, there is clearly a preference for binding to the p63α and p63γ isoforms. The reason for the lack of endogenous interaction with p63β could be that the p63β isoform has a slightly different folding pattern than the other isoforms, or it is

constitutively modified endogenously or bound in complexes in a way that would mask the charged areas needed for βTrCP1 interaction. Future proteomics studies, as mentioned above, and structural studies may reveal these differences in binding and complexes between each isoform.

Also, it is quite intriguing that βTrCP1 makes both N- and C- terminal contacts with p63 and has varying interaction at those regions because the difference in binding strength could easily provide a mechanism for the modulation of TAp63 versus ΔNp63 isoform activity. Other than conferring a selective TA isoform-specific advantage in binding to and subsequent activation by βTrCP1, this dual binding site may also serve three distinct purposes. First, the placement of the N-terminal site may occlude the binding of other substrates allowing for selective activation of specific downstream targets involved in growth arrest, but not apoptosis, as our later mRNA/protein activation and cell cycle profile suggests (Figures 3.12 and 3.17). In this respect the lack of binding in the case of endogenous TAp63β could be explained in that other interacting proteins may have a higher affinity for TAp63β at those sites (Figures 3.15 and 3.16). Again, characterization of isoform-specific translational complexes will provide more insight into this question. Secondly, it could also prevent export to the cytoplasm, in the case of MDM2 occlusion, or prevent immediate degradation by ligases, such as Itch. Lastly, it is also possible that βTrCP1's binding facilitates the placement of other post-translational modifications on p63. Our preliminary data has suggested that hyper-acetylation and hyper-phosphorylation works to block global p63 ubiquitylation (Appendix B), suggesting that perhaps under certain stimuli, TAp63γ stabilization by βTrCP1 may be replaced in favor of an acetylation or phosphorylation modification. Of note, the S62

residue of TAp63's potential βTrCP1 binding site is phosphorylated in response to UV (107), and our preliminary experiments with UVC exposure demonstrated that this stimulus caused a decrease in global ubiquitylation, suggesting that high levels of phosphorylation might block TAp63γ's binding to βTrCP1. Future experiments with mutant of p63 deficient in βTrCP1-mediated ubiquitylation, or ubiquitylation and activity assays in a *βTrCP1*$^{-/-}$ system would be challenging but would provide a careful analysis of the specific mechanisms involved with signaling at this site. Further, this data suggests the presence of a potential feedback mechanism (discussed below).

Also, the intrinsic balance between the p63α and p63γ isoforms should be considered. As mentioned in Chapter 1, it has been demonstrated that ΔNp63α and TAp63γ are often inversely correlated and that the ΔNp63 isoforms are very potent inhibitors of the TAp63 isoforms (18). Our endogenous assay shows that more ΔNp63α precipitates with βTrCP1 than TAp63γ. I do not feel this result is a direct reflection of endogenous binding affinity. The most likely explanation for this result is that the non-denaturing conditions of the IP allowed a majority of the ubiquitylated TAp63γ complexes to precipitate out of solution, because while βTrCP1 is able to bind both p63α and p63γ isoforms, our data shows that the ubiquitylation of the p63α isoforms does not increase in the presence of βTrCP1 and that βTrCP1 co-expression actually decreases the half-life of those isoforms. I have not included this data into my study as described here because the regulation of p63α is clearly operating through another mechanism than what I describe for TAp63γ. Since ΔNp63 and TAp63 are not ubiquitylated by βTrCP1 even though they are still able to bind βTrCP1, it is possible that the p63α isoforms are involved with a competing βTrCP1 regulatory complex (see section 5.4 for discussion of

two such possible related pathways). However, it is important to note that the endogenous binding of both isoforms provides a clear mechanism for substrate competition that could allow for a balance of the TAp63γ and ΔNp63α isoforms. Clearly, future studies on specific isoform binding affinity and competition assays would need to be carried out to understand how much of this control is due to binding affinity and how much is due to other factors, such as variance of activation complexes and sub-cellular localization. Lastly, it would also be important to understand how this difference in interaction directly translates to competition on p63 target promoter regions, since there have yet to be biochemically detailed studies of promoter affinity or competition in a system with all of the family members. It is clear that TAp63γ is also the strongest transcription factor of the p63 isoforms (101). Therefore even a small adjustment that would alter the observed elevation of its activity would have impact on p63 function in the cell.

Further, in each of these cases, it is important to keep in mind that not only βTrCP1's affinity for the individual p63 isoforms, but also βTrCP1's affinity for its other substrates would play a critical role in its regulation of p63 under our model, as $SCF^{\beta TrCP1}$ protein levels have been demonstrated as a limiting factor in endogenous signaling (34, 81). Our data shows that βTrCP1 also binds TAp63α and ΔNp63α; this binding could provide another level of regulation through isoform competition. How this isoform competition might function in parallel with βTrCP1's other substrates is discussed below. Likewise, future studies will likely elucidate the effect of the specific balance of p63 isoforms, as well as the other p53 family members, on this pathway *in vivo*, since these studies focus largely on TAp63γ and our binding and ubiquitylation data suggests that

differences in binding and ubiquitylation would be important for the regulation of p63 signaling through this mechanism. Therefore, most likely a fine balance exists between the availability of both βTrCP1 and p63 impacting βTrCP1's ability to act as a regulator of p63.

5.3 The function of a second *p21* promoter binding region

p21 has long been known as a target gene of p53 and was later shown to be the major cell-cycle regulatory gene for p53, since it executes p53's primary cell growth arrest response (28). Several groups have shown that *p21* is also an active target of p63 and p73 as well (101). Since p21 is such an important cell cycle regulator, it is unsurprising that its control is complex and under the jurisdiction of many transcription factors, including the p53 family. The studies in MEFs null for each or a combination of the p53 family members showed that loss of *p63* or *p73* alone, lowered but did not ablate p53's transcription of *p21* or its binding to the *p21* promoter, suggesting that cell cycle regulation is both an ancient and at least partially redundant function of all three p53 family members (29). The TAp63 isoforms have long been known to activate *p21* transcription (101). Also, this response was varied according to the nature of the upstream stimulus, as UV was able to activate TAp63γ's control of p21 and elicit an apoptotic response, but other types of genotoxic stress showed much lower activity and apoptosis, leading them to conclude that perhaps TAp63γ induces a differentiation program under specific stress conditions (64). One group demonstrated that ectopic TAp63γ expression induced p21 in an erythroleukemia cell line to induce differentiation, rather than apoptosis (64). Though now it is known that this apoptotic effect most likely differs as a result tissue specific stimulus, as noted in Chapter 1. Also ΔNp63α has been

shown to be able to bind to the *p21* promoter and this binding decreases during keratinocyte differentiation along with binding to the 14-3-3σ promoter (94). Our laboratory's previous study shows that p300 acetylation is able to activate TAp63γ on *p21*, similarly to this study showing that βTrCP1 co-expression is able to upregulate p21 at the transcript and protein level. This control of p21 has implications in p63's roles in cellular senescence, differentiation and the cell stress response, which will be better understood with future analysis of how p63 regulates *p21* transcription.

In my study, I demonstrate that TAp63γ's ability to upregulate p21 at the mRNA and protein level is significantly enhanced in the presence of βTrCP1 (Figures 3.12 and 3.13). This upregulation is also associated with an increase of TAp63γ protein load at a well-established p53 family member site in the *p21* promoter (P1) and also a site near the promoter (P2) which has been shown by previous studies to not be a p53 binding site (38), but in the case of my study shows significant binding of TAp63γ which increases upon βTrCP1 co-expression (Figure 3.15). The binding activity at both *p21* promoter regions is significantly decreased compared to TAp63γ alone when co-expressed with the ΔFβTrCP1 mutant. Since the major focus of this study was the initial characterization of the pathway in terms of the ubiquitylation reaction and TAp63γ protein stability, I used both the RT-PCR analysis and ChIP as an indication of TAp63γ function. Since many other groups have shown that p63, particularly TAp63γ, is a potent transcriptional activator of p21 and that p21 levels are well-established to be under the control of protein stability through proteasome-mediated degradation, rather than mRNA degradation (58, 90, 106), it would be quite reasonable to argue that our observed upregulation of p21 is at the transcriptional level.

However, future experiments would need to expand on the mechanism of p21 transcription by p63 through the second promoter site. Through EMSA using sequences from the *p21* promoter it would be possible to isolate the sequence that TAp63γ binds near the -20 site. Further, since our ChIP assay makes approximately 1000 bp fragments, and the fact that the distribution of several p53 family- and p63-favored core binding sequences is at the start of the gene and into the gene sequence itself, this assay should also include regions at the beginning of the gene. In fact, recent genomic analysis showed that several p63 binding sites may also be within the p63 target gene sequence itself (169); therefore the fragment I amplified could be recognizing a region in the first ~1000 bp of the *p2*1 gene itself. Once the second binding sequence is determined by EMSA and DNA footprinting assays, that region could then be cloned into a vector for a luciferase or other type of reporter assay to test *de novo* transcription for a relative comparison of promoter strength of the P1 versus the P2 site. This information would be particularly useful in discerning the effect that p53 expression would have endogenously on this pathway. Since all of our assays (including the endogenous assays) were conducted in cell lines that have either lost p53, or have significantly reduced p53 levels, and p63 binding to promoters of target genes, including *p21*, is known to be partially controlled by both p53 and p73 activity, reporter assays would also provide valuable information as to the effect of the ubiquitin modification on promoter strength. Further, studies using nuclear run-on assays would allow a quantitative assessment of endogenous *p21* activation. Also, this technique along with other real-time kinetic measurements of gene activation are being currently adapted to the microarray chip technology, so soon the *de novo* kinetics of TAp63γ on the *p21* promoter will be able to be studied in a variety

of conditions so that I might better understand the details of how βTrCP1's activation of TAp63γ influences the *de novo* kinetics of *p21* transcription.

5.4 Potential *in vivo* effects – upstream signals and relationships to other pathways

One of the limitations of our study was that the assays involving p21 upregulation and promoter binding were done at a purely exogenous level. In order to study the endogenous role of this activation I would first need a defined upstream signaling stimulus that I would know to be specific for βTrCP1-mediated TAp63γ activation. As of yet, I do not know what upstream signal may contribute to regulation of the TAp63γ/βTrCP1 signaling pathway described here, though there are several possibilities in both developmental pathways and also cell stress pathways. Stimuli that have been shown to activate both βTrCP1 and the p53 family are UV irradiation and tumor necrosis factor α (TNFα) exposure (34, 101). UV irradiation while activating TAp63γ and its regulation of p21, has also been shown to trigger the proteasomal degradation of ΔNp63α. βTrCP1 is also activated by UV, since the regulation of some of its associated kinases are activated in response to UV irradiation. The most striking example of this regulation is the degradation of Cdc25a and b which is mediated by a UV triggered pathway driven by p38 and JNK phosphorylation (13, 57). In our preliminary studies, UVC irradiation 20-100 J/m^2 decreased the level of global p63 ubiquitylation in a dose-dependent fashion (data not shown). Similarly, our preliminary data with TNFα also showed no additional increase of TAp63γ ubiquitylation (data not shown). In the case of both of these experiments, it is possible that our observations are due to the fact that the expression level of exogenously expressed TAp63γ is too high to see a difference. The ideal scenario would be genetic experiments using stable knockdown specific for TAp63γ and

βTrCP1 or both under a variety of upstream stimuli to find an appropriate stimulus and dosage for TAp63γ/βTrCP1 pathway-mediated regulation of p21, versus the action of βTrCP1 or p63 with other signaling pathways that might give a misleading result. A very recent paper accomplished specific knockdown of βTrCP1 in cells (150), and isoform-specific siRNAs against p63 will likely be published soon (A. Mills, personal communication). Other than exploring dosages of the more endogenously applicable UVB exposure, other possibilities are hydrogen peroxide, which activates βTrCP1 and p53, or other forms of genotoxic stress from chemotherapeutic agents used to activate the p53-family members.

I did not explore developmental stimuli, but given what is known about p63 signaling and its importance to epithelial development it would be more than reasonable to test the role of TAp63γ's activation by βTrCP1 in development. As mentioned in Chapter 1, p63 has a major role in epithelial development which has been determined through the use of several knockout and transgenic mouse models. The TAp63 isoforms come on early in development (E7.5) and are involved in the commitment to stratification (94). They then continue in expression into adult tissue. After the single layer epidermis has committed to stratify, the ΔNp63 isoforms come on, at E9.5, to allow cells to respond to terminal differentiation signals and also to maintain the stem cell niche (94). It is during this stratification that the balance of most epithelial tissue shifts to favor the ΔNp63 isoforms, particularly ΔNp63α. So during development, there is an additional layer of temporal control dictating the TAp63γ/ΔNp63α isoform balance. This temporal control is also to balance the regulation of p21 during development because cell cycle arrest is a prerequisite for terminal differentiation. Thus TAp63γ expression can activate

p21, while ΔNp63α and TAp63α are known to inhibit p21 and 14-3-3σ, allowing the cells to resume amplification after commitment to terminal differentiation. Further feedback maintaining p21 activation is achieved through TAp63γ's transcriptional induction of Jagged-1, a Notch1 ligand, and also the activation of IKKα as a downstream target gene (94). When IKKα is active, this also allows for activation of NF-κB, which can also regulate p21 activity in growth arrest in a manner that can be altered by the p53 status of the cell depending on the stimulus and cell type (94). Further, our unpublished data suggests that IKKα and IKKβ also phosphorylate TAp63γ, and that the hyperphosphorylation induced by the IKK complex mimics that of exposure to a phosphatase inhibitor, okadaic acid (OA) (see Appendix B). This phosphorylation also causes a global drop in ubiquitylation of TAp63γ, suggesting that the βTrCP1 activation of TAp63γ might occur in a parallel pathway which is able to perturb the post-translational modification of TAp63γ.

Once a stimulus is determined, the next question that could be addressed would be how βTrCP1's activation of TAp63γ might play into this signaling pathway. It is likely, given the drastic reduction of ubiquitylation of TAp63γ following hyperphosphorylation and hyperacetylation, as shown in Appendix B, that βTrCP1's activation of TAp63γ would exist in parallel or in conjunction with its acetylation by p300. As mentioned in the Introduction, this N-terminal region is targeted by many binding proteins and the binding sites and potential modification sites of factors, such as βTrCP1, p300, and at least one unknown kinase overlap in that region. Since there is one lysine (K49) on TAp63γ's N-terminus and both p300 activation of TAp63γ and βTrCP1 activation of TAp63γ involve direct binding of each respective factor to the N-terminus, it is possible

that through shared modification of K49 of TAp63γ that there might be some overlap in function and from these two pathways, and another additional layer of regulation could be mediated by phosphorylation on nearby residues. In fact, in the case of p53's stabilization by E4F1, acetylation by PCAF, but not p300, acted as a specific inhibitory signal for E4F1-mediated ubiquitylation (88). As more downstream genes are found for p63 and the βTrCP1-specific ubiquitylation sites and those for TAp63γ acetylation are mapped, I will be able to know with more certainty how the ubiquitylation might relate to the acetylation. Further, given that βTrCP1 regulation is dictated largely by substrate competition, it is reasonable to propose the existence of such a feedback loop where TAp63γ is able to bind βTrCP1, to activate p21 to mediate a growth arrest, or stratification commitment program, which would later be downregulated by rising ΔNp63α levels that would compete for the βTrCP1 pool. A second source of downregulation would come from ΔNp63α's indirect activation of IKKα through GATA-3 which would increase the amount of phosphorylated IκB and NF-κB substrate (16) and compete away the pool of βTrCP1 bound to p63. Given the potential interplay and competition between the βTrCP1/TAp63γ signaling axis and the βTrCP1/IκBα/NF-κB signaling axis this regulation also provides a potential mechanism of chemoresistance because the interrelationship of these two pathways provides a way to uncouple the growth arrest and apoptotic function of the p53 family members. Therefore, if cells were arrested without undergoing apoptosis for an extended period of time, they could aquire additional mutations that could cause their transformation. This effect could be particularly amplified by the presence of inflammatory factors such as TNFα, which promotes an increase of p21 through the NF-κB pathway and the p63/βTrCP1 pathway;

both of these responses which do not trigger apoptosis, along with the concomitant degradation of p73 and accumulation of inactive p53 (27, 65). Increased levels of p21 have been historically associated with chemoresistance in some cancers. This scenario is one which could be assembled using the current literature, but of course would be very complex *in vivo* depending on the cell type and other factors since the p53 family, βTrCP1 and NF-κB can all be associated with apoptotic and anti-apoptotic roles.

Another axis that is related to both the p53 family and is also a target for βTrCP1 is the oncogene β-catenin. β-catenin is a transcription factor well characterized for its role in the Wnt signaling pathway, as well as an important component of cadherins junctions which regulate cell-cell adhesion. Under normal homeostasis β-catenin is bound by a complex containing GSK3β, axin, the APC complex and CK1. This complex phosphorylates β-catenin on its βTrCP1 canonical binding domain so that βTrCP1 can ubiquitylate it and degrade it (1). When this GSK3β complex is inhibited βTrCP1 can not degrade β-catenin, so free β-catenin can translocate to the nucleus and act as an oncogene (1). ΔNp63α is able to antagonize this loop and promote a nuclear accumulation of β-catenin (116). ΔNp63α has been shown to directly bind to the B56α subunit of PP2A and inhibit its activation of GSK3β, preventing β-catenin's recognition by βTrCP1 and allowing β-catenin to accumulate in the nucleus (116). Thus under cell stress it is quite possible that TAp63γ's stabilization by βTrCP1 and its upregulation of p21 would provide an important tumor-suppressor signal to counteract the oncogenic effects of β-catenin. In this case, the activation of β-catenin would cause a rise in the pool of free βTrCP1 in the nucleus, which would then activate TAp63γ and trigger growth arrest. Further, β-catenin activates p53 transcription and active p53 is able to

downregulate β-catenin levels (131). Therefore it is unsurprising that aberrant accumulation of β-catenin in tumors is often associated with p53-inactivating mutations.

TAp63γ's activation by βTrCP1 could provide an extra layer of redundancy in p53 tumor suppression in cells that have lost p53 function in this way, allowing them to arrest rather than remain in the replicative pool. In fact, in cancers such as colorectal cancer where increased expression of βTrCP1 is associated significantly with increased β-catenin and NF-κB levels, separate experiments have also showed that p53 is also often inactive (34, 143). It is also important to note that this regulation has also been shown to be particularly sensitive to the dominant-inhibitory effects of extreme βTrCP1 overexpression or F-box function compromised mutants that would act similarly to our ΔFβTrCP1 construct. Not surprisingly, increases in ΔFβTrCP1 are also associated with higher levels of β-catenin and NF-κB (34, 44). Therefore in cancers with upregulated or mutated βTrCP1 that have lost p53, TAp63γ signaling could also be compromised due to increased binding of a mutated βTrCP1 which would cripple its activation of p21. This scenario could manifest as an increase of the TAp63γ isoforms in the tumor as a result of a large pool of stable but inactive TAp63γ.

Since there was a postulated link between ΔNp63α and β-catenin which could explain ΔNp63α's pro-oncogenic role, two studies examined this relationship in human tumors. One study examined the relationship between ΔNp63α and β-catenin in a large tissue array of human neoplasms (75 each of several different tissue types, including breast, colon and lung). This study found no significant correlation between ΔNp63 and levels of nuclear accumulation of β-catenin (126). However a downfall of this study is that the authors did not examine the levels of any other regulators, including TAp63, p53,

and p73 status. It is more than possible that stabilization of the TAp63γ isoforms would overwhelm the levels of ΔNp63α. This increase in the TAp63 isoforms is seen in several epithelial tumor types, such as head and neck squamous cell carcinoma (79), and also non-epithelial tumor types, such as lymphomas (41). A more descriptive experiment using a similar broad tissue array would test for specific isoforms, as well as provide information on the p53 and p73 status of the tumors, and also provide the βTrCP1 levels along with those of β-catenin and provide information more applicable to the variety of mutations found in human tumors. In fact, a smaller, more specific study on both ΔNp63 and TAp63 in urothelial cancers found that reduced levels of ΔNp63α correlated with reduced levels of β-catenin (74) However, in this study they simply state that ΔNp63 is the predominant isoform, without a more intensive analysis of the samples.

Thus, if an accurate picture of p63 signaling is to be gleaned from the data, future *in vivo* studies should provide a more thorough analysis of specific isoform contribution in combination with what is known about p63 regulatory pathways. One way to begin this work would be to perform a thorough genomic and proteomic correlation between specific p63 isoform levels and the levels of members of pathways, such as the NF-κB and Wnt signaling pathways, in both normal and tumor samples. Needless to say, these experiments would be both very time- and labor-intensive, but they would be extraordinarily valuable in providing a solid basis for the analysis of the molecular data and the setup of future *in vivo* experiments concerning tumorigenesis and metastasis. Also, these trends could be correlated more closely with the data on upstream and downstream signaling stimuli and targets as they become available and provide a much

more detailed understanding of how the p53 family could influence cell fate and tumor progression.

Therefore our study showing ubiquitin-mediated TAp63γ activation by βTrCP1 provides valuable information for the study of both tumorigenic and developmental pathways. Perhaps the most important immediate experiments which would shed light on the regulation of p63 and how it works in conjunction with shared βTrCP1 signaling pathways would be biochemical studies comparing the binding affinity of the p63 isoforms with other known βTrCP1 substrates, and transcriptional studies examining the effect of βTrCP1 on *de novo* transcription of the p63 isoforms *in vivo*. This information would allow a better understanding of how p63 and βTrCP1 levels and competition might be perturbed in response to stress and developmental stimuli. Also, as a limited amount of information on upstream signaling and other post-translational modifications of p63 is available, the information obtained from the above proposed studies can be incorporated into a better understanding of what role this βTrCP1/p63 pathway has on *in vivo* signaling and provide a wider range of information in disease research.

SUMMARY

This dissertation describes a novel signaling pathway for p63 mediated by βTrCP1. This interaction is unique, as p63 is the only p53 family member which interacts with βTrCP1. However, the most striking observation of this study is that TAp63γ is stabilized and able to activate cell cycle arrest through p21 by way of an ubiquitin-mediated mechanism involving a non-canonical βTrCP1 binding site and an ubiquitin linkage that is typically used for degradation. Also, p63 is the first substrate which is stabilized by βTrCP1.

Our data shows that βTrCP1 expression stabilizes TAp63γ exogenously and endogenously, increasing its steady-state level through an increase in protein half-life and that loss of βTrCP1 lowers the steady-state of TAp63γ and decreases its half-life. This change was determined to occur through a post-translational mechanism, since the mRNA levels of p63 in all cases remained constant. This difference in stability was conferred through direct interaction of βTrCP1 with the N- and C- termini of p63, as p63α and p63γ isoforms interacted with βTrCP1 *in vitro* and in cells both exogenously and endogenously and that interaction was mapped *in vitro* to TAp63γ. The result of this increased stability was an increased upregulation of p21 mRNA and protein levels which corresponded to an increase of TAp63γ bound to the endogenous *p21* promoter at both a well-characterized p53 family binding site and a novel site for p63. This upregulation of p21 led to the induction of G1/S arrest. Strikingly, the cause for this increase in the stability and activity of TAp63γ was shown to be the ubiquitylation of TAp63γ by $SCF^{\beta TrCP1}$, as βTrCP1 was able to ubiquitylate TAp63γ *in vitro* and in cells. Also, this

ubiquitylation, along with the increase in stability, activity and promoter binding conferred by βTrCP1 was lost in the presence of the ΔFβTrCP1 mutant. This ubiquitylation was shown to occur through a K48 mediated ubiquitin linkage and, through studies with mutant p63s, that modulation of the degree of ubiquitylation was able to affect the stability of p63 and demonstrated that even a minimal level of ubiquitin was enough to mediate upregulation of *p21*.

This novel pathway is a mechanism that adds to our understanding of p63 regulation in several ways. It provides a pathway specific to the p63γ isoforms which might be regulated by the p63α isoforms and can activate the growth arrest function of p63 specifically. Further, it provides a mechanism of novel βTrCP1 interaction which could be used to understand the effect of βTrCP1 on regulation of its many substrates. Also, it provides another mechanism supporting the studies demonstrating that ubiquitin can function as activating signal as well as a degradation signal. This pathway has implications in both development and cancer and provides connections of the p53 family with the NF-κB pathway, the Wnt pathway, and others involved in the tight regulation of cell fate. Future studies will no doubt describe specific upstream signaling pathways which affect the ubiquitin-mediated activation of TAp63γ, add detail to the mechanism and how it might function in the endogenous micro-environment, and how perturbation or imbalance of this pathway directly relates to p63 deregulation in related disease phenotypes and cancer.

REFERENCES

1. **Aberle, H., A. Bauer, J. Stappert, A. Kispert, and R. Kemler.** 1997. beta-catenin is a target for the ubiquitin-proteasome pathway. Embo J **16:**3797-804.
2. **Amir, R. E., H. Haecker, M. Karin, and A. Ciechanover.** 2004. Mechanism of processing of the NF-kappa B2 p100 precursor: identification of the specific polyubiquitin chain-anchoring lysine residue and analysis of the role of NEDD8-modification on the SCF(beta-TrCP) ubiquitin ligase. Oncogene **23:**2540-7.
3. **Bakkers, J., M. Hild, C. Kramer, M. Furutani-Seiki, and M. Hammerschmidt.** 2002. Zebrafish DeltaNp63 is a direct target of Bmp signaling and encodes a transcriptional repressor blocking neural specification in the ventral ectoderm. Dev Cell **2:**617-27.
4. **Ballarino, M., A. Fruscalzo, M. Marchioni, and F. Carnevali.** 2004. Identification of positive and negative regulatory regions controlling expression of the Xenopus laevis betaTrCP gene. Gene **336:**275-85.
5. **Ballarino, M., M. Marchioni, and F. Carnevali.** 2002. The Xenopus laevis beta TrCP gene: genomic organization, alternative splicing, 5' and 3' region characterization and comparison of its structure with that of human beta TrCP genes. Biochim Biophys Acta **1577:**81-92.
6. **Barbareschi, M., L. Pecciarini, M. G. Cangi, E. Macri, A. Rizzo, G. Viale, and C. Doglioni.** 2001. p63, a p53 homologue, is a selective nuclear marker of myoepithelial cells of the human breast. Am J Surg Pathol **25:**1054-60.
7. **Barrera, F. N., J. A. Poveda, J. M. Gonzalez-Ros, and J. L. Neira.** 2003. Binding of the C-terminal sterile alpha motif (SAM) domain of human p73 to lipid membranes. J Biol Chem **278:**46878-85.
8. **Belaidouni, N., M. Peuchmaur, C. Perret, A. Florentin, R. Benarous, and C. Besnard-Guerin.** 2005. Overexpression of human beta TrCP1 deleted of its F box induces tumorigenesis in transgenic mice. Oncogene **24:**2271-6.
9. **Bergamaschi, D., Y. Samuels, B. Jin, S. Duraisingham, T. Crook, and X. Lu.** 2004. ASPP1 and ASPP2: common activators of p53 family members. Mol Cell Biol **24:**1341-50.
10. **Bode, A. M., and Z. Dong.** 2004. Post-translational modification of p53 in tumorigenesis. Nat Rev Cancer **4:**793-805.
11. **Boukamp P, P. R., Breitkreutz D, Hornung J, Markham A, Fusenig NE.** 1988. Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. J Cell Biol **106:**761-71.
12. **Bourdon, J. C., K. Fernandes, F. Murray-Zmijewski, G. Liu, A. Diot, D. P. Xirodimas, M. K. Saville, and D. P. Lane.** 2005. p53 isoforms can regulate p53 transcriptional activity. Genes Dev **19:**2122-37.
13. **Busino, L., M. Donzelli, M. Chiesa, D. Guardavaccaro, D. Ganoth, N. V. Dorrello, A. Hershko, M. Pagano, and G. F. Draetta.** 2003. Degradation of Cdc25A by beta-TrCP during S phase and in response to DNA damage. Nature **426:**87-91.

14. **Calabro, V., G. Mansueto, T. Parisi, M. Vivo, R. A. Calogero, and G. La Mantia.** 2002. The human MDM2 oncoprotein increases the transcriptional activity and the protein level of the p53 homolog p63. J Biol Chem **277:**2674-81.
15. **Calabro, V., G. Mansueto, R. Santoro, A. Gentilella, A. Pollice, P. Ghioni, L. Guerrini, and G. La Mantia.** 2004. Inhibition of p63 transcriptional activity by p14ARF: functional and physical link between human ARF tumor suppressor and a member of the p53 family. Mol Cell Biol **24:**8529-40.
16. **Candi, E., A. Terrinoni, A. Rufini, A. Chikh, A. M. Lena, Y. Suzuki, B. S. Sayan, R. A. Knight, and G. Melino.** 2006. p63 is upstream of IKK alpha in epidermal development. J Cell Sci **119:**4617-22.
17. **Celli, J., P. Duijf, B. C. Hamel, M. Bamshad, B. Kramer, A. P. Smits, R. Newbury-Ecob, R. C. Hennekam, G. Van Buggenhout, A. van Haeringen, C. G. Woods, A. J. van Essen, R. de Waal, G. Vriend, D. A. Haber, A. Yang, F. McKeon, H. G. Brunner, and H. van Bokhoven.** 1999. Heterozygous germline mutations in the p53 homolog p63 are the cause of EEC syndrome. Cell **99:**143-53.
18. **Chan, W. M., W. Y. Siu, A. Lau, and R. Y. Poon.** 2004. How many mutant p53 molecules are needed to inactivate a tetramer? Mol Cell Biol **24:**3536-51.
19. **Ciechanover, A., H. Gonen, B. Bercovich, S. Cohen, I. Fajerman, A. Israel, F. Mercurio, C. Kahana, A. L. Schwartz, K. Iwai, and A. Orian.** 2001. Mechanisms of ubiquitin-mediated, limited processing of the NF-kappaB1 precursor protein p105. Biochimie **83:**341-9.
20. **Crook, T., J. M. Nicholls, L. Brooks, J. O'Nions, and M. J. Allday.** 2000. High level expression of deltaN-p63: a mechanism for the inactivation of p53 in undifferentiated nasopharyngeal carcinoma (NPC)? Oncogene **19:**3439-44.
21. **Davis, M., A. Hatzubai, J. S. Andersen, E. Ben-Shushan, G. Z. Fisher, A. Yaron, A. Bauskin, F. Mercurio, M. Mann, and Y. Ben-Neriah.** 2002. Pseudosubstrate regulation of the SCF(beta-TrCP) ubiquitin ligase by hnRNP-U. Genes Dev **16:**439-51.
22. **Dellavalle, R. P., T. B. Egbert, A. Marchbank, L. J. Su, L. A. Lee, and P. Walsh.** 2001. CUSP/p63 expression in rat and human tissues. J Dermatol Sci **27:**82-7.
23. **Dellavalle, R. P., P. Walsh, A. Marchbank, T. E. Grayson, L. J. Su, E. R. Parker, J. DeGregori, K. Penheiter, M. Aszterbaum, E. H. Epstein, Jr., and L. A. Lee.** 2002. CUSP/p63 expression in basal cell carcinoma. Exp Dermatol **11:**203-8.
24. **Di Como, C. J., M. J. Urist, I. Babayan, M. Drobnjak, C. V. Hedvat, J. Teruya-Feldstein, K. Pohar, A. Hoos, and C. Cordon-Cardo.** 2002. p63 expression profiles in human normal and tumor tissues. Clin Cancer Res **8:**494-501.
25. **Di Iorio, E., V. Barbaro, A. Ruzza, D. Ponzin, G. Pellegrini, and M. De Luca.** 2005. Isoforms of DeltaNp63 and the migration of ocular limbal cells in human corneal regeneration. Proc Natl Acad Sci U S A **102:**9523-8.
26. **Dietz, S., K. Rother, C. Bamberger, H. Schmale, J. Mossner, and K. Engeland.** 2002. Differential regulation of transcription and induction of

programmed cell death by human p53-family members p63 and p73. FEBS Lett **525:**93-9.

27. **Drane, P., V. Leblanc, F. Miro-Mur, R. Saffroy, B. Debuire, and E. May.** 2002. Accumulation of an inactive form of p53 protein in cells treated with TNF alpha. Cell Death Differ **9:**527-37.
28. **el-Deiry, W. S., J. W. Harper, P. M. O'Connor, V. E. Velculescu, C. E. Canman, J. Jackman, J. A. Pietenpol, M. Burrell, D. E. Hill, Y. Wang, and et al.** 1994. WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. Cancer Res **54:**1169-74.
29. **Flores, E. R., S. Sengupta, J. B. Miller, J. J. Newman, R. Bronson, D. Crowley, A. Yang, F. McKeon, and T. Jacks.** 2005. Tumor predisposition in mice mutant for p63 and p73: evidence for broader tumor suppressor functions for the p53 family. Cancer Cell **7:**363-73.
30. **Flores, E. R., K. Y. Tsai, D. Crowley, S. Sengupta, A. Yang, F. McKeon, and T. Jacks.** 2002. p63 and p73 are required for p53-dependent apoptosis in response to DNA damage. Nature **416:**560-4.
31. **Fomenkov, A., Y. P. Huang, O. Topaloglu, A. Brechman, M. Osada, T. Fomenkova, E. Yuriditsky, B. Trink, D. Sidransky, and E. Ratovitski.** 2003. P63 alpha mutations lead to aberrant splicing of keratinocyte growth factor receptor in the Hay-Wells syndrome. J Biol Chem **278:**23906-14.
32. **Fong, A., and S. C. Sun.** 2002. Genetic evidence for the essential role of beta-transducin repeat-containing protein in the inducible processing of NF-kappa B2/p100. J Biol Chem **277:**22111-4.
33. **Fuchs, S. Y., A. Chen, Y. Xiong, Z. Q. Pan, and Z. Ronai.** 1999. HOS, a human homolog of Slimb, forms an SCF complex with Skp1 and Cullin1 and targets the phosphorylation-dependent degradation of IkappaB and beta-catenin. Oncogene **18:**2039-46.
34. **Fuchs, S. Y., V. S. Spiegelman, and K. G. Kumar.** 2004. The many faces of beta-TrCP E3 ubiquitin ligases: reflections in the magic mirror of cancer. Oncogene **23:**2028-36.
35. **Gallegos, J. R., J. Litersky, H. Lee, Y. Sun, K. Nakayama, K. Nakayama, and H. Lu.** 2007. SCFbeta TRCP1 activates and ubiquitylates TAp63gamma. J Biol Chem.
36. **Gerstein, A. V., T. A. Almeida, G. Zhao, E. Chess, M. Shih Ie, K. Buhler, K. Pienta, M. A. Rubin, R. Vessella, and N. Papadopoulos.** 2002. APC/CTNNB1 (beta-catenin) pathway alterations in human prostate cancers. Genes Chromosomes Cancer **34:**9-16.
37. **Ghioni, P., Y. D'Alessandra, G. Mansueto, E. Jaffray, R. T. Hay, G. La Mantia, and L. Guerrini.** 2005. The protein stability and transcriptional activity of p63alpha are regulated by SUMO-1 conjugation. Cell Cycle **4:**183-90.
38. **Gomes NP, B. G., Llorente B, Szostek SA, Emerson BM, Espinoza JM.** 2006. Gene-specific requirement for P-TEFb activity and RNA polymerase II phsophorylation within the p53 transcriptional program. Genes Dev **20:**601-612.
39. **Gostissa, M., M. Morelli, F. Mantovani, E. Guida, S. Piazza, L. Collavin, C. Brancolini, C. Schneider, and G. Del Sal.** 2004. The transcriptional repressor hDaxx potentiates p53-dependent apoptosis. J Biol Chem **279:**48013-23.

40. **Guardavaccaro, D., Y. Kudo, J. Boulaire, M. Barchi, L. Busino, M. Donzelli, F. Margottin-Goguet, P. K. Jackson, L. Yamasaki, and M. Pagano.** 2003. Control of meiotic and mitotic progression by the F box protein beta-Trcp1 in vivo. Dev Cell **4:**799-812.
41. **Guo, X., and A. A. Mills.** 2007. p63, cellular senescence and tumor development. Cell Cycle **6:**305-11.
42. **Hamstra, D. A., M. S. Bhojani, L. B. Griffin, B. Laxman, B. D. Ross, and A. Rehemtulla.** 2006. Real-time evaluation of p53 oscillatory behavior in vivo using bioluminescent imaging. Cancer Res **66:**7482-9.
43. **Harmes, D. C., E. Bresnick, E. A. Lubin, J. K. Watson, K. E. Heim, J. C. Curtin, A. M. Suskind, J. Lamb, and J. DiRenzo.** 2003. Positive and negative regulation of deltaN-p63 promoter activity by p53 and deltaN-p63-alpha contributes to differential regulation of p53 target genes. Oncogene **22:**7607-16.
44. **Hart, M., J. P. Concordet, I. Lassot, I. Albert, R. del los Santos, H. Durand, C. Perret, B. Rubinfeld, F. Margottin, R. Benarous, and P. Polakis.** 1999. The F-box protein beta-TrCP associates with phosphorylated beta-catenin and regulates its activity in the cell. Curr Biol **9:**207-10.
45. **Hatakeyama, S., M. Kitagawa, K. Nakayama, M. Shirane, M. Matsumoto, K. Hattori, H. Higashi, H. Nakano, K. Okumura, K. Onoe, R. A. Good, and K. Nakayama.** 1999. Ubiquitin-dependent degradation of IkappaBalpha is mediated by a ubiquitin ligase Skp1/Cul 1/F-box protein FWD1. Proc Natl Acad Sci U S A **96:**3859-63.
46. **He, N., C. Li, X. Zhang, T. Sheng, S. Chi, K. Chen, Q. Wang, R. Vertrees, R. Logrono, and J. Xie.** 2005. Regulation of lung cancer cell growth and invasiveness by beta-TRCP. Mol Carcinog **42:**18-28.
47. **Helton, E. S., J. Zhu, and X. Chen.** 2006. The unique NH2-terminally deleted (DeltaN) residues, the PXXP motif, and the PPXY motif are required for the transcriptional activity of the DeltaN variant of p63. J Biol Chem **281:**2533-42.
48. **Hibi, K., B. Trink, M. Patturajan, W. H. Westra, O. L. Caballero, D. E. Hill, E. A. Ratovitski, J. Jen, and D. Sidransky.** 2000. AIS is an oncogene amplified in squamous cell carcinoma. Proc Natl Acad Sci U S A **97:**5462-7.
49. **Hirano, Y., and Z. Ronai.** 2006. A new function for p53 ubiquitination. Cell **127:**675-7.
50. **Honda, R., H. Tanaka, and H. Yasuda.** 1997. Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS Lett **420:**25-7.
51. **Huang, Y. P., G. Wu, Z. Guo, M. Osada, T. Fomenkov, H. L. Park, B. Trink, D. Sidransky, A. Fomenkov, and E. A. Ratovitski.** 2004. Altered sumoylation of p63alpha contributes to the split-hand/foot malformation phenotype. Cell Cycle **3:**1587-96.
52. **Iordanov MS, C. R., Ryabinina OP, Dinh TH, Bright RK, Magun BE.** 2002. The UV (Ribotoxic) stress response of human keratinocytes involves the unexpected uncoupling of the Ras-extracellular signal-related kinase signalling cascade from the activated epidermal growth factor receptor. Molecular Cell Biology **15:**5380-94.

53. **Jacobs, W. B., G. Govoni, D. Ho, J. K. Atwal, F. Barnabe-Heider, W. M. Keyes, A. A. Mills, F. D. Miller, and D. R. Kaplan.** 2005. p63 is an essential proapoptotic protein during neural development. Neuron **48:**743-56.
54. **Jemal, A., T. Murray, E. Ward, A. Samuels, R. C. Tiwari, A. Ghafoor, E. J. Feuer, and M. J. Thun.** 2005. Cancer statistics, 2005. CA Cancer J Clin **55:**10-30.
55. **Jiang, J., and G. Struhl.** 1998. Regulation of the Hedgehog and Wingless signalling pathways by the F-box/WD40-repeat protein Slimb. Nature **391:**493-6.
56. **Jin, J., and J. W. Harper.** 2003. A license to kill: transcriptional activation and enhanced turnover of Myc by the SCF(kp2) ubiquitin ligase. Cancer Cell **3:**517-8.
57. **Jin, J., T. Shirogane, L. Xu, G. Nalepa, J. Qin, S. J. Elledge, and J. W. Harper.** 2003. SCFbeta-TRCP links Chk1 signaling to degradation of the Cdc25A protein phosphatase. Genes Dev **17:**3062-74.
58. **Jin, Y., M. S. Dai, S. Z. Lu, Y. Xu, Z. Luo, Y. Zhao, and H. Lu.** 2006. 14-3-3gamma binds to MDMX that is phosphorylated by UV-activated Chk1, resulting in p53 activation. Embo J **25:**1207-18.
59. **Jin Y, Z. X., Lee H, Lu H.** 2004. MDM2 mediates p300/CREB-binding protein-associated factor ubiquitination and degradation. J Biol Chem **279:**20035-43.
60. **Kadakia, M., C. Slader, and S. J. Berberich.** 2001. Regulation of p63 function by Mdm2 and MdmX. DNA Cell Biol **20:**321-30.
61. **Kaghad, M., H. Bonnet, A. Yang, L. Creancier, J. C. Biscan, A. Valent, A. Minty, P. Chalon, J. M. Lelias, X. Dumont, P. Ferrara, F. McKeon, and D. Caput.** 1997. Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. Cell **90:**809-19.
62. **Kanemori, Y., K. Uto, and N. Sagata.** 2005. Beta-TrCP recognizes a previously undescribed nonphosphorylated destruction motif in Cdc25A and Cdc25B phosphatases. Proc Natl Acad Sci U S A **102:**6279-84.
63. **Karin, M., and Y. Ben-Neriah.** 2000. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. Annu Rev Immunol **18:**621-63.
64. **Katoh, I., K. I. Aisaki, S. I. Kurata, S. Ikawa, and Y. Ikawa.** 2000. p51A (TAp63gamma), a p53 homolog, accumulates in response to DNA damage for cell regulation. Oncogene **19:**3126-30.
65. **Kikuchi, H., T. Ozaki, K. Furuya, T. Hanamoto, M. Nakanishi, H. Yamamoto, K. Yoshida, S. Todo, and A. Nakagawara.** 2006. NF-kappaB regulates the stability and activity of p73 by inducing its proteolytic degradation through a ubiquitin-dependent proteasome pathway. Oncogene **25:**7608-17.
66. **Kim HG, K. K., Lldias F, Kissalev AF, Scaglione KM, Skowyra D, Gygi SP, Goldberg AL.** 2007. Certain E2-E3 pairs synthesize nondegradable forked ubiquitin chains containing all possible isopeptide linkages. J Biol Chem**:**e-pub ahead of print.
67. **Kim, H. T., K. P. Kim, F. Lledias, A. F. Kisselev, K. M. Scaglione, D. Skowyra, S. P. Gygi, and A. L. Goldberg.** 2007. Certain pairs of ubiquitin-conjugating enzymes (E2s) and ubiquitin-protein ligases (E3s) synthesize nondegradable forked ubiquitin chains containing all possible isopeptide linkages. J Biol Chem **282:**17375-86.

68. **Kim SJ, H. A., Tworkowski KA, Salghetti SE, Tansey WP.** 2003. Skp2 Regulates Myc Protein Stability and Activity. Mol Cell **11:**1177-1188.
69. **Kim, S. Y., A. Herbst, K. A. Tworkowski, S. E. Salghetti, and W. P. Tansey.** 2003. Skp2 regulates Myc protein stability and activity. Mol Cell **11:**1177-88.
70. **King KE, P. R., Gerdes MJ, Tokino T, Yamashita T, Baker CC, Weinberg WC.** 2006. Unique domain functions of p63 isotypes that differentially regulate distinct aspects of epidermal homeostasis. Carcinogenesis **27:**53-63.
71. **Kitagawa, M., S. Hatakeyama, M. Shirane, M. Matsumoto, N. Ishida, K. Hattori, I. Nakamichi, A. Kikuchi, K. Nakayama, and K. Nakayama.** 1999. An F-box protein, FWD1, mediates ubiquitin-dependent proteolysis of beta-catenin. Embo J **18:**2401-10.
72. **Knudson, A. G., Jr.** 1989. The ninth Gordon Hamilton-Fairley memorial lecture. Hereditary cancers: clues to mechanisms of carcinogenesis. Br J Cancer **59:**661-6.
73. **Koch, A., A. Waha, W. Hartmann, A. Hrychyk, U. Schuller, A. Waha, K. A. Wharton, Jr., S. Y. Fuchs, D. von Schweinitz, and T. Pietsch.** 2005. Elevated expression of Wnt antagonists is a common event in hepatoblastomas. Clin Cancer Res **11:**4295-304.
74. **Koga, F., S. Kawakami, J. Kumagai, T. Takizawa, N. Ando, G. Arai, Y. Kageyama, and K. Kihara.** 2003. Impaired Delta Np63 expression associates with reduced beta-catenin and aggressive phenotypes of urothelial neoplasms. Br J Cancer **88:**740-7.
75. **Koike, J., N. Sagara, H. Kirikoshi, A. Takagi, T. Miwa, M. Hirai, and M. Katoh.** 2000. Molecular cloning and genomic structure of the betaTRCP2 gene on chromosome 5q35.1. Biochem Biophys Res Commun **269:**103-9.
76. **Koster, M. I., D. Dai, B. Marinari, Y. Sano, A. Costanzo, M. Karin, and D. R. Roop.** 2007. p63 induces key target genes required for epidermal morphogenesis. Proc Natl Acad Sci U S A **104:**3255-60.
77. **Koster, M. I., D. Dai, and D. R. Roop.** 2007. Conflicting roles for p63 in skin development and carcinogenesis. Cell Cycle **6:**269-73.
78. **Koster, M. I., S. Kim, A. A. Mills, F. J. DeMayo, and D. R. Roop.** 2004. p63 is the molecular switch for initiation of an epithelial stratification program. Genes Dev **18:**126-31.
79. **Koster, M. I., S. L. Lu, L. D. White, X. J. Wang, and D. R. Roop.** 2006. Reactivation of developmentally expressed p63 isoforms predisposes to tumor development and progression. Cancer Res **66:**3981-6.
80. **Koster, M. I., and D. R. Roop.** 2004. p63 and epithelial appendage development. Differentiation **72:**364-70.
81. **Krappmann, D., and C. Scheidereit.** 2005. A pervasive role of ubiquitin conjugation in activation and termination of IkappaB kinase pathways. EMBO Rep **6:**321-6.
82. **Kroll, M., F. Margottin, A. Kohl, P. Renard, H. Durand, J. P. Concordet, F. Bachelerie, F. Arenzana-Seisdedos, and R. Benarous.** 1999. Inducible degradation of IkappaBalpha by the proteasome requires interaction with the F-box protein h-betaTrCP. J Biol Chem **274:**7941-5.
83. **Kudo, Y., D. Guardavaccaro, P. G. Santamaria, R. Koyama-Nasu, E. Latres, R. Bronson, L. Yamasaki, and M. Pagano.** 2004. Role of F-box protein

betaTrcp1 in mammary gland development and tumorigenesis. Mol Cell Biol **24:**8184-94.

84. **Kulesz-Martin M, L. J., Fei S, Pelz C, Sears R, Powell MB, Halaban R, Johnson J.** 2005. Melanocyte and keratinocyte carcinogenesis: p53 family protein activities and intersecting mRNA expression profiles. J Investig Dermatol Symp Proc **10:**142-152.
85. **Kurata, S., T. Okuyama, M. Osada, T. Watanabe, Y. Tomimori, S. Sato, A. Iwai, T. Tsuji, Y. Ikawa, and I. Katoh.** 2004. p51/p63 Controls subunit alpha3 of the major epidermis integrin anchoring the stem cells to the niche. J Biol Chem **279:**50069-77.
86. **Lassot, I., E. Segeral, C. Berlioz-Torrent, H. Durand, L. Groussin, T. Hai, R. Benarous, and F. Margottin-Goguet.** 2001. ATF4 degradation relies on a phosphorylation-dependent interaction with the SCF(betaTrCP) ubiquitin ligase. Mol Cell Biol **21:**2192-202.
87. **Latres, E., D. S. Chiaur, and M. Pagano.** 1999. The human F box protein beta-Trcp associates with the Cul1/Skp1 complex and regulates the stability of beta-catenin. Oncogene **18:**849-54.
88. **Le Cam, L., L. K. Linares, C. Paul, E. Julien, M. Lacroix, E. Hatchi, R. Triboulet, G. Bossis, A. Shmueli, M. S. Rodriguez, O. Coux, and C. Sardet.** 2006. E4F1 is an atypical ubiquitin ligase that modulates p53 effector functions independently of degradation. Cell **127:**775-88.
89. **Lee, H., and D. Kimelman.** 2002. A dominant-negative form of p63 is required for epidermal proliferation in zebrafish. Dev Cell **2:**607-16.
90. **Lee, H., S. X. Zeng, and H. Lu.** 2006. UV Induces p21 rapid turnover independently of ubiquitin and Skp2. J Biol Chem **281:**26876-83.
91. **Li Y, Z. S., Landias I, Lu H.** 2007. Human SSRP1 has Spt16-dependent and -independent roles in gene transcription. J Biol Chem **282:**6936-45.
92. **Liu, Y., H. Asch, and M. F. Kulesz-Martin.** 2001. Functional quantification of DNA-binding proteins p53 and estrogen receptor in cells and tumor tissues by DNA affinity immunoblotting. Cancer Res **61:**5402-6.
93. **Lu, P., M. Barad, and P. D. Vize.** 2001. Xenopus p63 expression in early ectoderm and neurectoderm. Mech Dev **102:**275-8.
94. **Mack, J. A., S. Anand, and E. V. Maytin.** 2005. Proliferation and cornification during development of the mammalian epidermis. Birth Defects Res C Embryo Today **75:**314-29.
95. **MacPartlin, M., S. Zeng, H. Lee, D. Stauffer, Y. Jin, M. Thayer, and H. Lu.** 2005. p300 regulates p63 transcriptional activity. J Biol Chem **280:**30604-10.
96. **Maisse, C., P. Guerrieri, and G. Melino.** 2003. p73 and p63 protein stability: the way to regulate function? Biochem Pharmacol **66:**1555-61.
97. **Mantovani, F., and L. Banks.** 2003. Regulation of the discs large tumor suppressor by a phosphorylation-dependent interaction with the beta-TrCP ubiquitin ligase receptor. J Biol Chem **278:**42477-86.
98. **Margottin, F., S. P. Bour, H. Durand, L. Selig, S. Benichou, V. Richard, D. Thomas, K. Strebel, and R. Benarous.** 1998. A novel human WD protein, h-beta TrCp, that interacts with HIV-1 Vpu connects CD4 to the ER degradation pathway through an F-box motif. Mol Cell **1:**565-74.

99. **Melino, G., R. A. Knight, and G. Cesareni.** 2006. Degradation of p63 by Itch. Cell Cycle **5:**1735-9.
100. **Mills, A. A., B. Zheng, X. J. Wang, H. Vogel, D. R. Roop, and A. Bradley.** 1999. p63 is a p53 homologue required for limb and epidermal morphogenesis. Nature **398:**708-13.
101. **Moll, U. M., and N. Slade.** 2004. p63 and p73: roles in development and tumor formation. Mol Cancer Res **2:**371-86.
102. **Moore, J. E., C. B. McMullen, G. Mahon, and A. P. Adamis.** 2002. The corneal epithelial stem cell. DNA Cell Biol **21:**443-51.
103. **Muerkoster, S., A. Arlt, B. Sipos, M. Witt, M. Grossmann, G. Kloppel, H. Kalthoff, U. R. Folsch, and H. Schafer.** 2005. Increased expression of the E3-ubiquitin ligase receptor subunit betaTRCP1 relates to constitutive nuclear factor-kappaB activation and chemoresistance in pancreatic carcinoma cells. Cancer Res **65:**1316-24.
104. **Nakayama, K., S. Hatakeyama, S. Maruyama, A. Kikuchi, K. Onoe, R. A. Good, and K. I. Nakayama.** 2003. Impaired degradation of inhibitory subunit of NF-kappa B (I kappa B) and beta-catenin as a result of targeted disruption of the beta-TrCP1 gene. Proc Natl Acad Sci U S A **100:**8752-7.
105. **Nakayama K, H. S., Maruyama S, Kikuchi A, Onoe K, Good RA, Nakayama KI.** 2003. Impaired degradation of inhibitory subunit of NF-kappaB (IkappaB) and beta-catenin as a result of targeted disruption of the beta-TrCP1 gene. PNAS **100:**8752-8757.
106. **Nakayama, K. I., and K. Nakayama.** 2006. Ubiquitin ligases: cell-cycle control and cancer. Nat Rev Cancer **6:**369-81.
107. **Ogawa, E., R. Okuyama, S. Ikawa, H. Nagoshi, T. Egawa, A. Kurihara, M. Yabuki, H. Tagami, M. Obinata, and S. Aiba.** 2007. p51/p63 inhibits ultraviolet B-induced apoptosis via Akt activation. Oncogene.
108. **Ohtani, N., Y. Imamura, K. Yamakoshi, F. Hirota, R. Nakayama, Y. Kubo, N. Ishimaru, A. Takahashi, A. Hirao, T. Shimizu, D. J. Mann, H. Saya, Y. Hayashi, S. Arase, M. Matsumoto, N. Kazuki, and E. Hara.** 2007. Visualizing the dynamics of p21(Waf1/Cip1) cyclin-dependent kinase inhibitor expression in living animals. Proc Natl Acad Sci U S A **104:**15034-9.
109. **Orford, K., C. Crockett, J. P. Jensen, A. M. Weissman, and S. W. Byers.** 1997. Serine phosphorylation-regulated ubiquitination and degradation of beta-catenin. J Biol Chem **272:**24735-8.
110. **Orian, A., H. Gonen, B. Bercovich, I. Fajerman, E. Eytan, A. Israel, F. Mercurio, K. Iwai, A. L. Schwartz, and A. Ciechanover.** 2000. SCF(beta)(-TrCP) ubiquitin ligase-mediated processing of NF-kappaB p105 requires phosphorylation of its C-terminus by IkappaB kinase. Embo J **19:**2580-91.
111. **Ortt, K., and S. Sinha.** 2006. Derivation of the consensus DNA-binding sequence for p63 reveals unique requirements that are distinct from p53. FEBS Lett **580:**4544-50.
112. **Osada, M., H. L. Park, Y. Nagakawa, K. Yamashita, A. Fomenkov, M. S. Kim, G. Wu, S. Nomoto, B. Trink, and D. Sidransky.** 2005. Differential recognition of response elements determines target gene specificity for p53 and p63. Mol Cell Biol **25:**6077-89.

113. **Ougolkov, A., B. Zhang, K. Yamashita, V. Bilim, M. Mai, S. Y. Fuchs, and T. Minamoto.** 2004. Associations among beta-TrCP, an E3 ubiquitin ligase receptor, beta-catenin, and NF-kappaB in colorectal cancer. J Natl Cancer Inst **96:**1161-70.
114. **Ozaki, T., K. Watanabe, T. Nakagawa, K. Miyazaki, M. Takahashi, and A. Nakagawara.** 2003. Function of p73, not of p53, is inhibited by the physical interaction with RACK1 and its inhibitory effect is counteracted by pRB. Oncogene **22:**3231-42.
115. **Park, B. J., S. J. Lee, J. I. Kim, S. J. Lee, C. H. Lee, S. G. Chang, J. H. Park, and S. G. Chi.** 2000. Frequent alteration of p63 expression in human primary bladder carcinomas. Cancer Res **60:**3370-4.
116. **Patturajan, M., S. Nomoto, M. Sommer, A. Fomenkov, K. Hibi, R. Zangen, N. Poliak, J. Califano, B. Trink, E. Ratovitski, and D. Sidransky.** 2002. DeltaNp63 induces beta-catenin nuclear accumulation and signaling. Cancer Cell **1:**369-79.
117. **Paull, A. C., and D. R. Whikehart.** 2005. Expression of the p53 family of proteins in central and peripheral human corneal endothelial cells. Mol Vis **11:**328-34.
118. **Pellegrini, G., E. Dellambra, O. Golisano, E. Martinelli, I. Fantozzi, S. Bondanza, D. Ponzin, F. McKeon, and M. De Luca.** 2001. p63 identifies keratinocyte stem cells. Proc Natl Acad Sci U S A **98:**3156-61.
119. **Perez, C. A., J. Ott, D. J. Mays, and J. A. Pietenpol.** 2007. p63 consensus DNA-binding site: identification, analysis and application into a p63MH algorithm. Oncogene.
120. **Perez, C. A., and J. A. Pietenpol.** 2007. Transcriptional programs regulated by p63 in normal epithelium and tumors. Cell Cycle **6:**246-54.
121. **Pickart, C. M.** 2001. Mechanisms underlying ubiquitination. Annu Rev Biochem **70:**503-33.
122. **Ponassi, R., A. Terrinoni, A. Chikh, A. Rufini, A. M. Lena, B. S. Sayan, G. Melino, and E. Candi.** 2006. p63 and p73, members of the p53 gene family, transactivate PKCdelta. Biochem Pharmacol **72:**1417-22.
123. **Querido, E., P. Blanchette, Q. Yan, T. Kamura, M. Morrison, D. Boivin, W. G. Kaelin, R. C. Conaway, J. W. Conaway, and P. E. Branton.** 2001. Degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs via a novel mechanism involving a Cullin-containing complex. Genes Dev **15:**3104-17.
124. **Radu, E., O. Simionescu, T. Regalia, D. Dumitrescu, and L. M. Popescu.** 2002. Stem cells (p63(+)) in keratinocyte cultures from human adult skin. J Cell Mol Med **6:**593-8.
125. **Ray, D., E. C. Osmundson, and H. Kiyokawa.** 2006. Constitutive and UV-induced fibronectin degradation is a ubiquitination-dependent process controlled by beta-TrCP. J Biol Chem **281:**23060-5.
126. **Reis-Filho, J. S., P. T. Simpson, L. G. Fulford, A. Martins, and F. C. Schmitt.** 2003. P63-driven nuclear accumulation of beta-catenin is not a frequent event in human neoplasms. Pathol Res Pract **199:**785-93.
127. **Ribeiro-Silva, A., L. N. Ramalho, S. B. Garcia, D. F. Brandao, F. Chahud, and S. Zucoloto.** 2005. p63 correlates with both BRCA1 and cytokeratin 5 in

invasive breast carcinomas: further evidence for the pathogenesis of the basal phenotype of breast cancer. Histopathology **47:**458-66.

128. **Rossi, M., R. I. Aqeilan, M. Neale, E. Candi, P. Salomoni, R. A. Knight, C. M. Croce, and G. Melino.** 2006. The E3 ubiquitin ligase Itch controls the protein stability of p63. Proc Natl Acad Sci U S A **103:**12753-8.
129. **Rossi M, A. R., Neale M, Candi E, Salomoni P, Knight RA, Croce CM, Melino G.** 2006. The E3 ubiquitin ligase Itch controls the protein stability of p63. PNAS **103:**12753-58.
130. **Rossi, M., M. De Simone, A. Pollice, R. Santoro, G. La Mantia, L. Guerrini, and V. Calabro.** 2006. Itch/AIP4 associates with and promotes p63 protein degradation. Cell Cycle **5:**1816-22.
131. **Sadot, E., B. Geiger, M. Oren, and A. Ben-Ze'ev.** 2001. Down-regulation of beta-catenin by activated p53. Mol Cell Biol **21:**6768-81.
132. **Saitoh, T., and M. Katoh.** 2001. Expression profiles of betaTRCP1 and betaTRCP2, and mutation analysis of betaTRCP2 in gastric cancer. Int J Oncol **18:**959-64.
133. **Schmale, H., and C. Bamberger.** 1997. A novel protein with strong homology to the tumor suppressor p53. Oncogene **15:**1363-7.
134. **Serber, Z., H. C. Lai, A. Yang, H. D. Ou, M. S. Sigal, A. E. Kelly, B. D. Darimont, P. H. Duijf, H. Van Bokhoven, F. McKeon, and V. Dotsch.** 2002. A C-terminal inhibitory domain controls the activity of p63 by an intramolecular mechanism. Mol Cell Biol **22:**8601-11.
135. **Shirane, M., S. Hatakeyama, K. Hattori, K. Nakayama, and K. Nakayama.** 1999. Common pathway for the ubiquitination of IkappaBalpha, IkappaBbeta, and IkappaBepsilon mediated by the F-box protein FWD1. J Biol Chem **274:**28169-74.
136. **Shirogane, T., J. Jin, X. L. Ang, and J. W. Harper.** 2005. SCFbeta-TRCP controls clock-dependent transcription via casein kinase 1-dependent degradation of the mammalian period-1 (Per1) protein. J Biol Chem **280:**26863-72.
137. **Signoretti, S., M. M. Pires, M. Lindauer, J. W. Horner, C. Grisanzio, S. Dhar, P. Majumder, F. McKeon, P. W. Kantoff, W. R. Sellers, and M. Loda.** 2005. p63 regulates commitment to the prostate cell lineage. Proc Natl Acad Sci U S A **102:**11355-60.
138. **Signoretti, S., D. Waltregny, J. Dilks, B. Isaac, D. Lin, L. Garraway, A. Yang, R. Montironi, F. McKeon, and M. Loda.** 2000. p63 is a prostate basal cell marker and is required for prostate development. Am J Pathol **157:**1769-75.
139. **Sil, A. K., S. Maeda, Y. Sano, D. R. Roop, and M. Karin.** 2004. IkappaB kinase-alpha acts in the epidermis to control skeletal and craniofacial morphogenesis. Nature **428:**660-4.
140. **Spencer, E., J. Jiang, and Z. J. Chen.** 1999. Signal-induced ubiquitination of IkappaBalpha by the F-box protein Slimb/beta-TrCP. Genes Dev **13:**284-94.
141. **Sullivan, A., and X. Lu.** 2007. ASPP: a new family of oncogenes and tumour suppressor genes. Br J Cancer **96:**196-200.
142. **Suzuki, H., T. Chiba, M. Kobayashi, M. Takeuchi, T. Suzuki, A. Ichiyama, T. Ikenoue, M. Omata, K. Furuichi, and K. Tanaka.** 1999. IkappaBalpha ubiquitination is catalyzed by an SCF-like complex containing Skp1, cullin-1, and

two F-box/WD40-repeat proteins, betaTrCP1 and betaTrCP2. Biochem Biophys Res Commun **256:**127-32.

143. **Takami, K., I. Yana, H. Kurahashi, and I. Nishisho.** 1995. Multistep carcinogenesis in colorectal cancers. Southeast Asian J Trop Med Public Health **26 Suppl 1:**190-6.
144. **Tan, M., J. R. Gallegos, Q. Gu, Y. Huang, J. Li, Y. Jin, H. Lu, and Y. Sun.** 2006. SAG/ROC-SCF beta-TrCP E3 ubiquitin ligase promotes pro-caspase-3 degradation as a mechanism of apoptosis protection. Neoplasia **8:**1042-54.
145. **Tan, P., S. Y. Fuchs, A. Chen, K. Wu, C. Gomez, Z. Ronai, and Z. Q. Pan.** 1999. Recruitment of a ROC1-CUL1 ubiquitin ligase by Skp1 and HOS to catalyze the ubiquitination of I kappa B alpha. Mol Cell **3:**527-33.
146. **Tannapfel, A., S. Schmelzer, M. Benicke, M. Klimpfinger, K. Kohlhaw, J. Mossner, K. Engeland, and C. Wittekind.** 2001. Expression of the p53 homologues p63 and p73 in multiple simultaneous gastric cancer. J Pathol **195:**163-70.
147. **Tomimori, Y., I. Katoh, S. Kurata, T. Okuyama, R. Kamiyama, and Y. Ikawa.** 2004. Evolutionarily conserved expression pattern and trans-regulating activity of Xenopus p51/p63. Biochem Biophys Res Commun **313:**230-6.
148. **Trink, B., K. Okami, L. Wu, V. Sriuranpong, J. Jen, and D. Sidransky.** 1998. A new human p53 homologue. Nat Med **4:**747-8.
149. **Urist, M. J., C. J. Di Como, M. L. Lu, E. Charytonowicz, D. Verbel, C. P. Crum, T. A. Ince, F. D. McKeon, and C. Cordon-Cardo.** 2002. Loss of p63 expression is associated with tumor progression in bladder cancer. Am J Pathol **161:**1199-206.
150. **van Kerkhof, P., J. Putters, and G. J. Strous.** 2007. The ubiquitin ligase SCF(betaTrCP) regulates the degradation of the growth hormone receptor. J Biol Chem **282:**20475-83.
151. **von der Lehr N, J., Larsson L.** 2002. Implication of the Ubiquitin/Proteasome System in Myc-Regulated Transcription. Cell Cycle **2:**403-407.
152. **von der Lehr, N., S. Johansson, and L. G. Larsson.** 2003. Implication of the ubiquitin/proteasome system in Myc-regulated transcription. Cell Cycle **2:**403-7.
153. **von der Lehr, N., S. Johansson, S. Wu, F. Bahram, A. Castell, C. Cetinkaya, P. Hydbring, I. Weidung, K. Nakayama, K. I. Nakayama, O. Soderberg, T. K. Kerppola, and L. G. Larsson.** 2003. The F-box protein Skp2 participates in c-Myc proteosomal degradation and acts as a cofactor for c-Myc-regulated transcription. Mol Cell **11:**1189-200.
154. **Wang, W. K., M. Bycroft, N. W. Foster, A. M. Buckle, A. R. Fersht, and Y. W. Chen.** 2001. Structure of the C-terminal sterile alpha-motif (SAM) domain of human p73 alpha. Acta Crystallogr D Biol Crystallogr **57:**545-51.
155. **Wang, X., T. Arooz, W. Y. Siu, C. H. Chiu, A. Lau, K. Yamashita, and R. Y. Poon.** 2001. MDM2 and MDMX can interact differently with ARF and members of the p53 family. FEBS Lett **490:**202-8.
156. **Watanabe, N., H. Arai, Y. Nishihara, M. Taniguchi, N. Watanabe, T. Hunter, and H. Osada.** 2004. M-phase kinases induce phospho-dependent ubiquitination of somatic Wee1 by SCFbeta-TrCP. Proc Natl Acad Sci U S A **101:**4419-24.

157. **Watson, I. R., and M. S. Irwin.** 2006. Ubiquitin and ubiquitin-like modifications of the p53 family. Neoplasia **8:**655-66.
158. **Weber, A., U. Bellmann, F. Bootz, C. Wittekind, and A. Tannapfel.** 2002. Expression of p53 and its homologues in primary and recurrent squamous cell carcinomas of the head and neck. Int J Cancer **99:**22-8.
159. **Weinstein, M. H., S. Signoretti, and M. Loda.** 2002. Diagnostic utility of immunohistochemical staining for p63, a sensitive marker of prostatic basal cells. Mod Pathol **15:**1302-8.
160. **Weissman, A. M.** 2001. Themes and variations on ubiquitylation. Nat Rev Mol Cell Biol **2:**169-78.
161. **Westfall, M. D., D. J. Mays, J. C. Sniezek, and J. A. Pietenpol.** 2003. The Delta Np63 alpha phosphoprotein binds the p21 and 14-3-3 sigma promoters in vivo and has transcriptional repressor activity that is reduced by Hay-Wells syndrome-derived mutations. Mol Cell Biol **23:**2264-76.
162. **Winston, J. T., P. Strack, P. Beer-Romero, C. Y. Chu, S. J. Elledge, and J. W. Harper.** 1999. The SCFbeta-TRCP-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IkappaBalpha and beta-catenin and stimulates IkappaBalpha ubiquitination in vitro. Genes Dev **13:**270-83.
163. **Wojcik, E. J., D. M. Glover, and T. S. Hays.** 2000. The SCF ubiquitin ligase protein slimb regulates centrosome duplication in Drosophila. Curr Biol **10:**1131-4.
164. **Wu, C., and S. Ghosh.** 1999. beta-TrCP mediates the signal-induced ubiquitination of IkappaBbeta. J Biol Chem **274:**29591-4.
165. **Wu, G., G. Xu, B. A. Schulman, P. D. Jeffrey, J. W. Harper, and N. P. Pavletich.** 2003. Structure of a beta-TrCP1-Skp1-beta-catenin complex: destruction motif binding and lysine specificity of the SCF(beta-TrCP1) ubiquitin ligase. Mol Cell **11:**1445-56.
166. **Wu, K., A. Chen, and Z. Q. Pan.** 2000. Conjugation of Nedd8 to CUL1 enhances the ability of the ROC1-CUL1 complex to promote ubiquitin polymerization. J Biol Chem **275:**32317-24.
167. **Yang, A., M. Kaghad, Y. Wang, E. Gillett, M. D. Fleming, V. Dotsch, N. C. Andrews, D. Caput, and F. McKeon.** 1998. p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. Mol Cell **2:**305-16.
168. **Yang, A., and F. McKeon.** 2000. P63 and P73: P53 mimics, menaces and more. Nat Rev Mol Cell Biol **1:**199-207.
169. **Yang, A., Z. Zhu, P. Kapranov, F. McKeon, G. M. Church, T. R. Gingeras, and K. Struhl.** 2006. Relationships between p63 binding, DNA sequence, transcription activity, and biological function in human cells. Mol Cell **24:**593-602.
170. **Yang, L., N. Wang, Y. Tang, X. Cao, and M. Wan.** 2006. Acute myelogenous leukemia-derived SMAD4 mutations target the protein to ubiquitin-proteasome degradation. Hum Mutat **27:**897-905.
171. **Yaron, A., H. Gonen, I. Alkalay, A. Hatzubai, S. Jung, S. Beyth, F. Mercurio, A. M. Manning, A. Ciechanover, and Y. Ben-Neriah.** 1997. Inhibition of NF-

kappa-B cellular function via specific targeting of the I-kappa-B-ubiquitin ligase. Embo J **16:**6486-94.

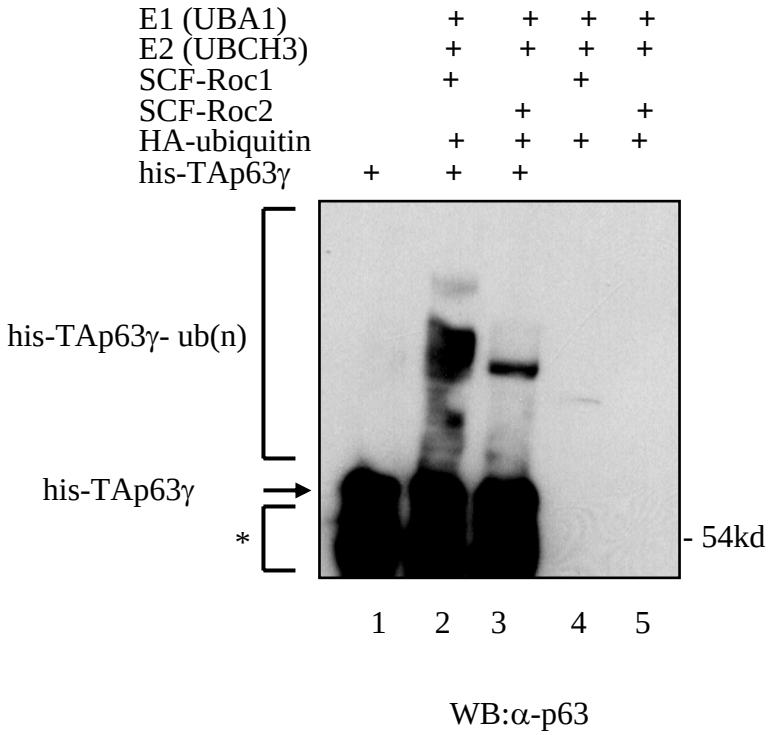
172. **Yaron, A., A. Hatzubai, M. Davis, I. Lavon, S. Amit, A. M. Manning, J. S. Andersen, M. Mann, F. Mercurio, and Y. Ben-Neriah.** 1998. Identification of the receptor component of the IkappaBalpha-ubiquitin ligase. Nature **396:**590-4.
173. **Zeng, S. X., M. S. Dai, D. M. Keller, and H. Lu.** 2002. SSRP1 functions as a co-activator of the transcriptional activator p63. Embo J **21:**5487-97.
174. **Zeng, X., Y. Zhu, and H. Lu.** 2001. NBP is the p53 homolog p63. Carcinogenesis **22:**215-9.

APPENDIX A

TAp63γ IS ALSO UBIQUITYLATED *IN VITRO* BY A SCF$^{\beta TrCP1}$ COMPLEX CONTAINING ROC2

Methods on creation of the SCF$^{\beta TrCP1}$-Roc2 complex are included in Chapter 2. The SCF$^{\beta TrCP1}$-Roc2 complex was generated as part of a co-authorship: M Tan, J Gallegos, H Lu, Y Sun (145).

In order to determine if SCF$^{\beta TrCP1}$-Roc2 was also able to ubiquitylate TAp63γ *in vitro*, purified TAp63γ was mixed with the noted components for in vitro ubiquitylation. As seen in Appendix A, SCF$^{\beta TrCP1}$-Roc2 was able to ubiquitylate TAp63γ similarly to SCF$^{\beta TrCP1}$-Roc1 (lane 3 versus lane 2), but TAp63γ alone or the substrate negative controls did not show the laddering characteristic of ubiquitylation.

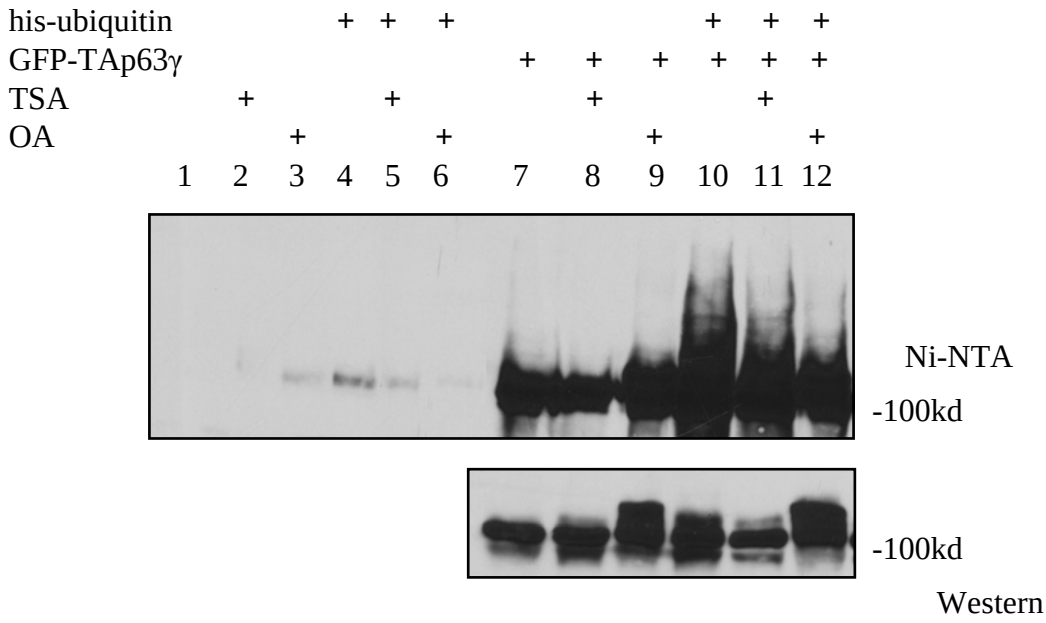


Appendix A: TAp63γ is also ubiquitylated *in vitro* by a SCF$^{\beta TrCP1}$ complex containing Roc2. SCF$^{\beta TrCP1}$-Roc2 (SCF-Roc2) was purified according to Methods and used in an *in vitro* ubiquitylation reaction as in 4.2. SCF-Roc1 refers to the SCF complex used throughout earlier experiments. WB performed with noted antibody. * = p63 degradation products.

APPENDIX B

HYPERACETYLATION AND HYPERPHOSPHORYLATION INCUCED BY TRICHOSTATIN A AND OKADAIC ACID INHIBITS THE UBIQUITYLATION OF TAp63γ

In order to determine the effect of phosphorylation and acetylation on TAp63γ's ubiquitylation, H1299 cells were treated with Trichostatin A (TA), a deacteylase inhibitor, or okadaic acid (OA), a phosphatase inhibitor overnight and for 4 hrs, respectively. The cells were then exposed to MG132 for 8 hrs, harvested, and assayed for ubiquitylation. Both TSA and OA treatment did not affect the overall amount of TAp63γ alone compared with untreated cells (lanes 7-9, Appendix B). However, in the presence of ubiquitin it was clear that both TSA and OA lowered the global ubiquitylation of TAp63γ compared with untreated (lanes 10-12), with TSA not having as great of an effect as OA.



WB: α-p63

Appendix B: Hyperacetylation and hyperphosphorylation induced by trichostatin A and okadaic acid inhibits the ubiquitylation of TAp63γ. H1299 cells were transfected with noted vectors and 10 μM TSA or 0.25 μM OA, as indicated. The ubiquitylation assay was blotted as indicated.