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Purification of Recombinant ▲ Np63 α and Characterization of Peptide Binding

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PURIFICATION OF RECOMBINANT ΔNP63α AND
CHARACTERIZATION OF PEPTIDE BINDING

A thesis submitted in partial fulfillment
of the requirements for the degree
of Master of Science

By

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B.S., King Saud University, 2008

2015
Wright State University

WRIGHT STATE UNIVERSITY

GRADUATE SCHOOL

August 19, 2015

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Amal Abdulah Albati ENTITLED Purification of Recombinant ΔNP63α and Characterization of Peptide Binding BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science

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ABSTRACT

Albati, Amal Abdulah. M.S. Department of Biochemistry and Molecular Biology, Wright State University, 2015. Purification of Recombinant ΔNp63α and Characterization of Peptide Binding

ΔNp63α, the primary p63 isoform of the p53 transcription factor family, is a proto-oncogene implicated in non-melanoma skin cancers. Expressed in the basal layer of the epidermis, ΔNp63α promotes cell survival and proliferation. Inhibition of this protein could potentially be beneficial in non-melanoma skin cancer patients.

The first goal of this project was the purification of recombinant ΔNp63α in *Escherichia Coli.* Recombinant ΔNp63α was expressed as GST-ΔNp63α followed by GST cleavage using GST trap affinity column chromatography yielding pure ΔNp63α. The second objective of this project was to test the binding capabilities of peptides previously identified by phage display to ΔNp63α. Six 12-mer peptides were previously identified by phage display using filamentous phage M13 followed by biopanning and amplification of target bound peptides. The amplified pools were sequenced and studied. In this study, the identified peptides were linked to biotin, the affinity and specificity of these peptides was evaluated using two different methods, streptavidin Dynabeads and ELISA. Through these methods, the biotinylated peptide P5 was found to be the most specific peptide to bind ΔNp63α. Future results utilizing these techniques may reveal promising therapeutic agents for the treatment of non-melanoma skin cancer.

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Huge thanks, love and appreciation go to my little family: my husband Bader, daughter Tala and son Abdulrahman. As for my husband I find it difficult to express my appreciation because it is so boundless. He is my most enthusiastic cheerleader and my

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best friend. I am grateful to him because he has given up so much to make my career a priority in our lives.

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I. INTRODUCTION

A. P63 AND ITS ROLE IN EPIDERMAL DEVELOPMENT

The skin protects the body from ultraviolet radiation, microorganisms and dehydration (Vanbokhoven et al, 2011). The major compartments in the skin are the dermis and the epidermis. The latter is multilayered and is important to form the skin barrier (Candi et al, 2006). Symmetric division in the basal layer of the epidermis generates new keratinocytes. Some keratinocytes divide asymmetrically and separate from the basal layer to undergo differentiation and generate cornified cells. Disruption of this controlled process causes pathological conditions such as inflammation, disruption of the skin barrier and cancer (Vanbokhoven et al, 2011). The p63 transcription factor, a p53 family member, plays important roles in epidermal morphogenesis (Candi et al, 2006; Pellegrini et al, 2001; Perez & Pietenpol, 2007). Studies have shown the importance of the p63 in development, apoptosis and tumorigenesis (Caserta et al, 2006; Dotsch et al, 2010; Schavolt & Pietenpol, 2007; Trink et al, 2007). Mutations in p63 lead to limb and skin defects in humans. p63 knock out mice show several abnormalities such as truncated limbs, absence of hair, teeth, nails, and neonatal death due to dehydration (Candi et al, 2006; Caserta et al, 2006; Dotsch et al, 2010; Marinari et al, 2009; Restelli et al, 2015)

The human p63 gene is located on chromosome 3q28 and contains 16 exons. It is structurally homologous to the p53 tumor suppressor gene, but does not function as a

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classical tumor suppressor gene. The homology between p53 and p63 is mainly present in a shared N-terminal transactivation domain (TA), a C-terminal oligomerization domain (OD) and a central DNA binding domain. p63 is expressed in multiple isoforms which perform different functions, thus resulting in a complex contribution to tumorigensis (Moll & Slade, 2004). As shown in Figure 1, all p63 isoforms contain a DNA-binding domain and an oligomerziation domain. p63 isoforms either contain a full-length transactivation (TA) domain or a truncated N-terminal transactivation version (denoted ΔN). The expression of p63 is initiated from two different transcriptional start sites denoted P1 and P2 which generate two different p63 isoforms: TAp63 isoforms which includes a full-length N-terminal transactivation domain (TA) and ΔNp63α versions which contains a short unique N-terminal domain (Caserta et al, 2006; Dotsch et al, 2010; Marinari et al, 2009; Moll & Slade, 2004; Vanbokhoven et al, 2011; Yang et al, 1998). Full length α or truncated (β, γ) isoforms are generated by alternative 3′-end splicing (Courtois et al, 2004; Dotsch et al, 2010; Yang et al, 1998). α-isoforms contain a sterile alpha motif (SAM) domain which mediates protein-protein interactions and a trans-inhibitory domain (TI) which blocks the transactivation by covering a few residues on the N-terminal TA domain (Caserta et al, 2006; Dotsch et al, 2010; Marinari et al, 2009; Moll & Slade, 2004; Vanbokhoven et al, 2011; Yang et al, 1998). ΔNp63α, is primarily expressed in the basal layer and downregulated in keratinocytes (Guo & Mills, 2007; Koster et al, 2004; Moll & Slade, 2004; Vanbokhoven et al, 2011). Both TAp63 and ΔNp63 isoforms are conserved in human and mice.

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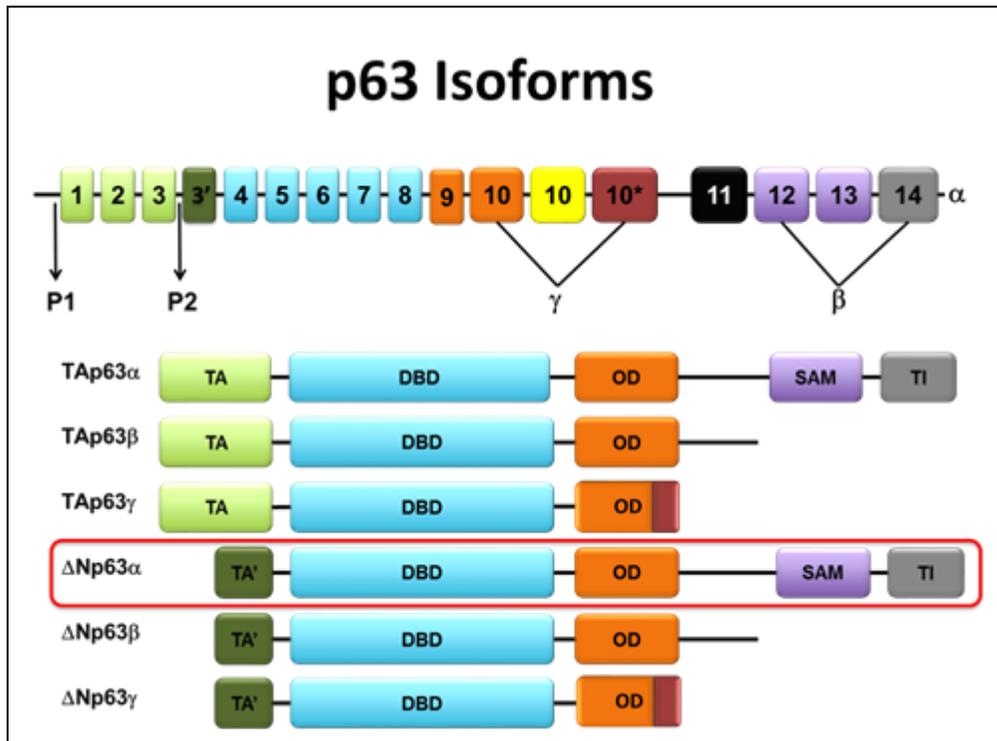
p63 Isoforms

Figure 1: Gene architecture of p63 isoforms. Alternative promotors (P1, P2) and alternative splicing (α, β, γ) are indicated. All p63 isoforms consist of TA, DBD and OD domains. The α isoforms contain SAM and TI domains. Alternative promotor usage P1 and P2 gives rise to transactivation isoform (TA) and N-terminal truncated (ΔN) isoform respectively.

B. P63 AND ITS ROLE IN CANCER

p63 is involved in the regulation of development, proliferation, tissue regeneration, morphogenesis, cell adhesion and cell signaling (Pellegrini et al, 2001; Perez & Pietenpol, 2007; Vanbokhoven et al, 2011). The TA and ΔN p63 isoforms have opposing roles in blocking or enhancing proliferation, and inducing or blocking cell death, but the mechanism by which these isoforms elicit their various functions is not currently fully understood.

p63 function is frequently lost or amplified in human cancer (Flores, 2007; Leonard et al, 2011; Trink et al, 2007). The p63 locus is frequently amplified in squamous cell carcinoma (Deyoung & Ellisen, 2007; Guo & Mills, 2007). ΔNp63α, the major isoform expressed in the suprabasal cells of the epidermis (Carroll et al, 2006; Moll & Slade, 2004; Pellegrini et al, 2001), is found to be amplified in various squamous cell carcinomas including head, neck and lung (Crook et al, 2000; Flores, 2007; Trink et al, 2007). Further, studies have shown that ΔNp63α inactivates p53. ΔNp63α promotes cell proliferation by downregulation of p53 target gene p21 (Deyoung & Ellisen, 2007; Flores, 2007; Trink et al, 2007). It was also shown that ΔNp63α preventing cell apoptosis through binding to the p53 response elements in p21 and 14-3-3σ (Westfall et al, 2003).

C. PHAGE DISPLAY

Phage display is a laboratory technology first described by George P. Smith in 1985 (Zwick et al, 1998) which is used to determine the interaction partners of a given protein. It is a potent technique that identifies new functions for peptides, and hence it is an important tool used in drug discovery. In this approach, a library of phages is generated which express a diversity of exogenous peptides. The filamentous phage M13 is the most

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used bacteriophage to produce random display peptides library. The library is then utilized to identify specific peptide ligands which bind a specific target (Barbas, 1993; Pande et al, 2010; Smith & Petrenko, 1997). The foreign DNA fragment that encodes different peptides is inserted into the filamentous phage genome. Then, the expressed peptides are fused on the surface of the coat proteins of the phage.

Screening for the specific target binding peptides is done by a process called biopanning. Biopaning is performed by exposing enriched phage clones to the immobilized desired target followed by washing off unbound peptides. Next, the target bound peptides are eluted and amplified followed by repeating biopanning process three to six times. Then, each clone is characterized by DNA sequence to identify target bound peptides (Figure 2). Phage display has been used to identify new protein-protein, protein-peptide, and protein-DNA interactions (Azzazy & Highsmith, 2002; Pande et al, 2010; Sergeeva et al, 2006; Smith & Petrenko, 1997; Takakusagi et al, 2010; Takami et al, 2011).

Phage display has been applied in different studies such as identification of peptides, antibodies, and vaccines (Dai et al, 2014; Kang et al, 2013; Ladner et al, 2004; Loi et al, 2013; Portefaix et al, 2002; Smith & Petrenko, 1997; Takami et al, 2011; Uchiyama et al, 2005; Wang et al, 2013; Winter et al, 1994).Using phage display, it is possible to identify peptides with high affinity and specificity to a target protein. These peptides can be used as drug candidate to inhibit the activity of target protein (Ladner et al, 2004). In our laboratory, it was used to identify six peptides that bind to ΔNp63α. In this study, two methods were used to determine if the identified peptides have the capacity to bind ΔNp63α using biotinylated peptides bound to either streptavidin Dynabeads pull down or 96-well streptavidin-based ELISA assays.

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Binding of phage peptides to immobilized target P63

Buffer washes

Non-specific phages

Elution

Amplification and analysis
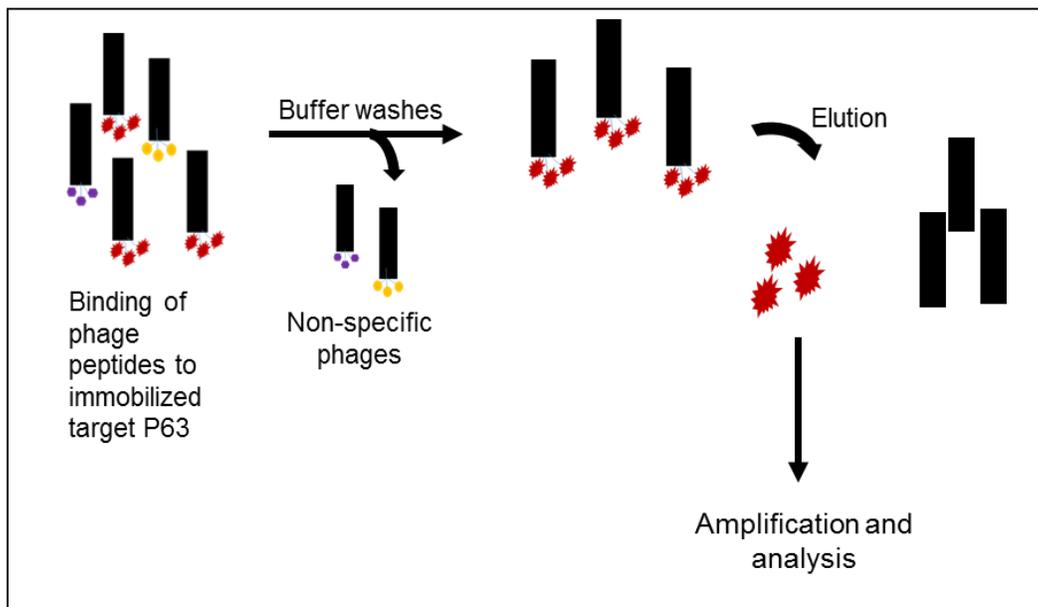
Figure 2: Schematic showing steps in screening a phage library to select p63 specific peptides by biopanning. The biopanning process starts with target immobilization. The unbound target is then washed off followed by elution of target bound phages. The eluted phages are amplified and the biopanning process is repeated three to four times under stringent conditions following which the phages are analyzed by DNA sequencing to identify the sequence of the target binding peptides.

D. RATIONALE AND SPECIFIC AIMS

ΔNp63α isoform of p63, is overexpressed in many cancers, and has oncogenic activity due to its ability to induce cell proliferation and inhibit cell apoptosis (Ram Kumar et al, 2014). This dissertation describes the identification of novel peptides which bind to ΔNp63α by phage display technology. Phage display technology has been used to show that peptides which bind specifically to the oncogenic peptide MDM2 can be used to target its function and prevent its binding to p53 (Bottger et al, 1996). Another phage display study demonstrated the ability of peptide P12 to bind to fibroblast growth factor (FGF8b), and interrupt binding to its receptor (Wang et al, 2013).

In this dissertation research, we completed two specific aims. First, recombinant ΔNp63α was produced in *Escherichia coli* bacteria and purified using column chromatography. Second, the recombinant protein was used to evaluate the binding of peptides P1 through P6 identified by phage display to both recombinant and transfected ΔNp63α *in vitro*.

The research presented herein suggests that chosen peptides P3, P5 and P6 may be used to target endogenous ΔNp63α and modulate its oncogenic function. Further testing is needed to determine the therapeutic potential of these peptides in treating squamous cell carcinoma and other p63-mediated malignancies.

II.   MATERIAL AND METHODS

A. Recombinant ΔNp63α preparation

ΔNp63α cloned into the pGEX vector was a generous gift from Dr. Basu (Restelli et al, 2015). ΔNp63α was expressed as a GST tagged-ΔNp63α in *Rosetta Escherichia coli* and grown in Difco LB Broth, Miller (Luria-Bertin) in the presence of 100 μg/ml ampicillin, 25 μg/ml chloramphenicol and 1mM glucose. A fresh 100 ml starter culture was incubated overnight at 37°C at a shaking speed of 250 rpm and used to inoculate four large scale 500 ml cultures. When the cultures reached an optical density of 1 at 600nm (O.D$_{600}$), 1 OD equivalent of uninduced culture was collected and pelleted for induction of recombinant GST−ΔNp63α by adding 1mM isopropyl β- D -1thiogalactopyranoside (IPTG) (Life technologies, cat # 15529-019). The cultures were incubated for 3.5 hours following induction and collected by centrifugation at 5,000 rpm for 10 minutes. Another 1 OD equivalent of induced cells was collected following centrifugation at 15,000 rpm for 5 minutes. Phenylmethanesulfonyl fluoride (PMSF) (sigma Product # P7626) was added to the pellet at a final concentration of 0.5mM and the cells were frozen at -80°C until the day of purification (Figure 3A). The remaining cells were frozen as cell pellets for additional large scale preparations.

Small scale test for GST-ΔNp63α induction was performed as follows: 1 OD equivalent pellets from both uninduced and induced samples were resuspended in 100 μl 1X L&C

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A)

B)

C)
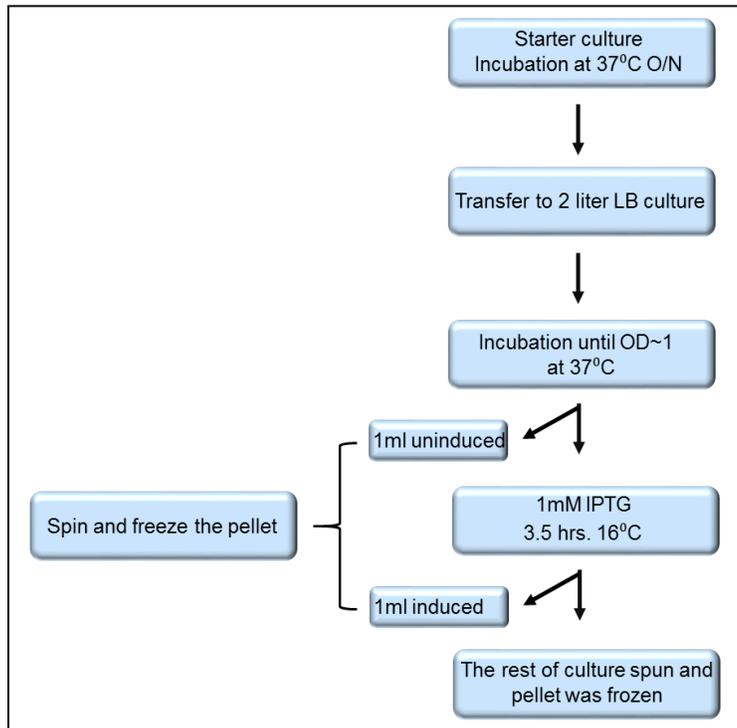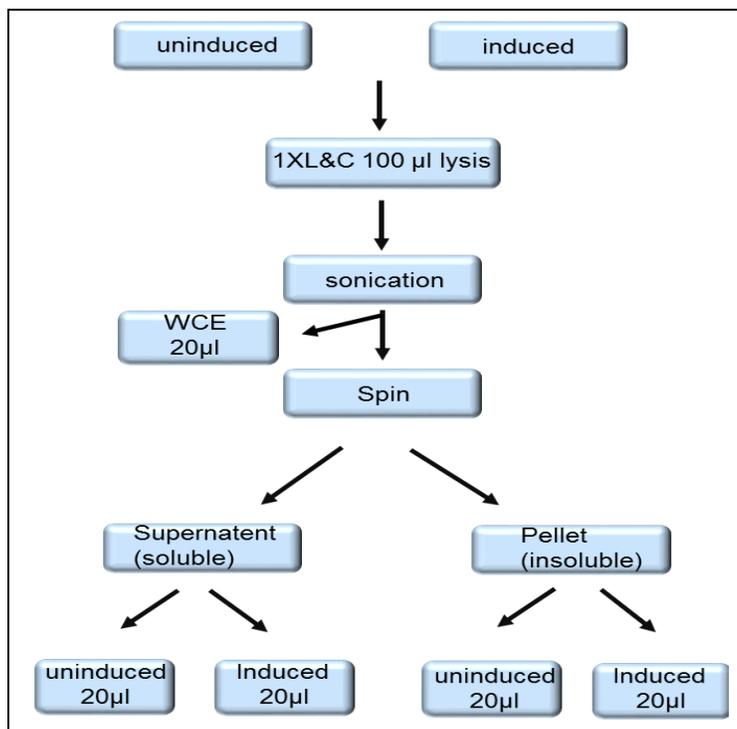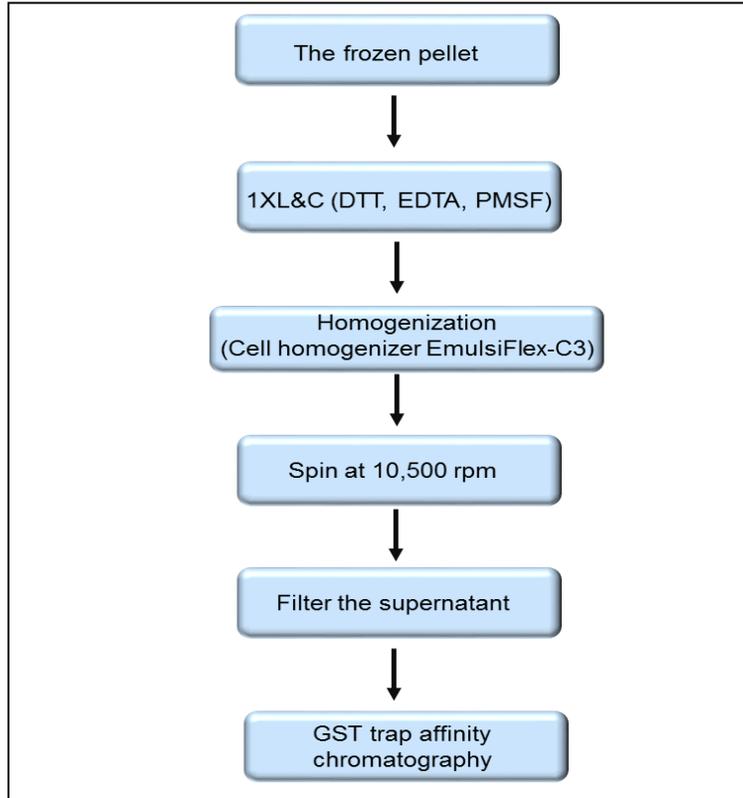
Figure 3: ΔNp63α purification workflow. A) A 100 ml starter culture was incubated overnight at 37ºC. The next day, the 100 ml starter culture was inoculated into a two liter culture and incubated at 37ºC until the optical density reached 1. A 1OD equivalent aliquot was collected and the rest of the culture was induced by IPTG and incubated for additional 3.5 hours. A 1 OD equivalent sample of induced cell aliquot was collected and the rest was spun and frozen at -80ºC. B) Expression of GST−ΔNp63α in 1 OD equivalent of uinduced and induced cells. Both culture samples were lysed in 1X L&C lysis buffer followed by sonication. A 20 microliter aliquot of whole cell extract was collected and the rest was spun for 5 minutes at 15,000 rpm speed. Supernatants (soluble fraction) were collected and the pellet (insoluble fraction) was resuspended using 1X L&C. 20 μl induced and uninduced samples were run on an SDS gel and stain with Coomassie Blue staining. C) After confirmation of expression of GST-ΔNp63α, the large scale frozen pellet was resuspended in 1X L&C and homogenized three times using an EmulsiFlex-C3 cell homogenizer. The cell lysate was then clarified at 10,500 rpm, filtered and purified by GST trap affinity chromatography.

lysis buffer (2X L&C: 40mM Tris pH 8.0, 600mM NaCl, 20% glycerol). The resuspended cell pellets were sonicated three times for 15 seconds each and an aliquot of the whole cell extract was collected from both induced and uninduced samples. Next, the samples were centrifuged at 15,000 rpm for 5 minutes and the supernatant was collected as the soluble fraction. The insoluble fraction (pellet) was resuspended in 100 μl of 1XL&C lysis buffer. 20 μl of whole cell extract, soluble and insoluble fractions from uninduced and induced conditions were run on 10% SDS gel after in 1X loading dye. GST-ΔNp63α expression was detected following staining the gel with instant blue stain (Expedeon, Product# 140904001) (Figure 3B) Following confirmation of induction on a small scale, frozen cell pellets from large scale preparations were thawed and resuspended in 100 ml 1X L&C buffer containing Protease Inhibitors Cocktail (PIC), homogenized three times using a Emulsiflex cell homogenizer (Avestin, Inc.) for 25 minutes, and spin-clarified by centrifuging the lysate at 10,500 rpm at 4°C for 30 minutes. The supernatant then was filtered through a 0.45 μm pore PES Filter (Figure 3C).

Recombinant GST-ΔNp63α protein was purified from contaminating proteins using GSTrap FF affinity columns (GE health care life science, product code 17-5130-01). The principle of column chromatography is shown in Figure 4A and 4B. In preparation for affinity purification, capillaries were rinsed at maximum speed with dH$_2$O and washed with 1X L&C buffer. A 1ml column was then installed and equilibrated with 1X L&C buffer at a flow rate of 1 ml/min. Next, the supernatant was loaded at a flow rate of 0.5 ml/min and washed with 1X L&C overnight at flow rate of 0.5ml/min at 4°C. The column was then equilibrated in PreScission buffer (50mM Tris HCl, 150mM NaCl, 1mM EDTA 1mM DTT, pH 8.0), followed by equilibration in PreScission protease dissolved in

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A)

Column Chromatography:

Principle GST-tag ΔNp63α purification

B)
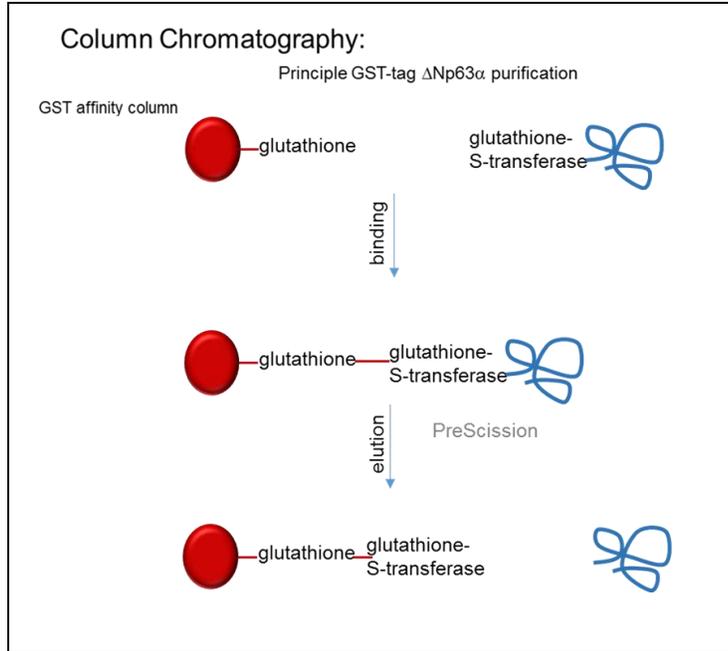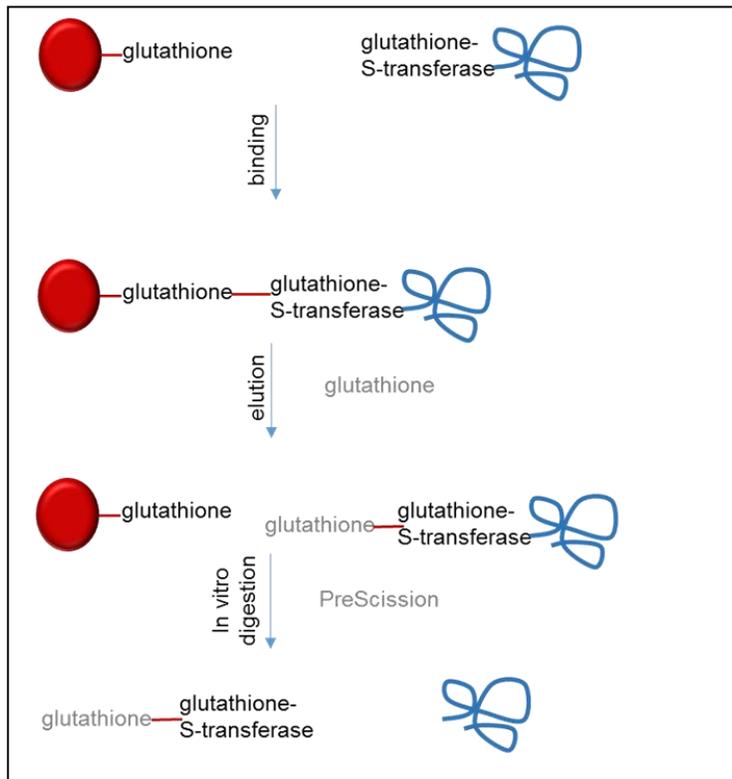
Figure 4: GST- ΔNp63α purification schematic. A) In column digestion: GST-ΔNp63α was loaded onto a GST affinity column followed by 16 hours incubation with PreScission protease, resulting in pure ΔNp63α. B) *In vitro* digestion of GST−ΔNp63α: GST−ΔNp63α was loaded to the column following by elution of GST-ΔNp63α using reduced glutathione. This step was followed by mixing GST-ΔNp63α and PreScission enzyme in an overnight incubation.

PreScission buffer and incubation for 16 hours. The bound GST tagged ΔNp63α was collected manually by injecting 1 ml of 10mM reduced glutathione (Sigma-aldrich, Product# G4251). GST tagged ΔNp63α was further concentrated and size selected by spinning the protein in a 50KD cutoff Amicon filter unit using a Sorvall Legend T/RT centrifuge. *In vitro* digestion of GST tagged ΔNp63α was performed with PreScission Protease in 1.7 ml Eppendorf tubes by incubating overnight (approximately 16 hours) at 4°C. Next, a second round of size selection was performed in a 50KD cutoff Amicon filter unit using a Sorvall Legend T/RT centrifuge. Purity of the recombinant ΔNp63α was evaluated on a 10% SDS gel stained with Instant blue stain (Expedeon, Inc., catalog # ISB1L).

B. Quantitation of recombinant ΔNp63α

Protein concentration was determined by comparing samples against known concentrations of 1mg/ml bovine serum albumin solution ranging from 0.1 μg to 2μg per lane. Both standard BSA and diluted ΔNp63α (5 μl ΔNp63α diluted in 13 μl of sterile distilled) were mixed with 2 μl 10X loading dye (0.5M DTT, 10% SDS, 0.5M Tris pH=8, 50% glycerol, 0.2% bromophenol blue). The sample were heated at 97°C for 10 minutes and then run on a 10X SDS-PAGE gel. Purified ΔNp63α protein was detected by staining the gel with Coomassie blue stain (0.1% Coomassie brilliant blue, 10% acetic acid, 50% of 95% ethanol, 40% H_2O) at room temperature for 1 hour. Coomassie destaining solution (7.5% acetic acid, 20% methanol, 72.5%H_2O) was added to the gel and incubated from 15 minutes to overnight at 4°C as needed to visualize the protein bands. ΔNp63α concentration

was measured by densitometry using Multi Gauge software and plotting against a standard curve generated by the density of BSA standards.

C. Western blots

Purified ΔNp63α was run on a 10 % SDS-PAGE followed by transfer onto Immobilon-P PVDF membrane (Millipore Corporation, Billerica, MA) using transfer buffer (25mM Tris, 192mM glycine, 20% methanol and PH 8.3) at 0.35A for 60 minutes using transblot system (Bio-Rad). The blot was then blocked using 5% nonfat dry milk made in 1X Tris-Tween 20 buffer saline (1X TTBS: 50mM TRIS-Cl, 150mM NaCl, pH 7.6) for 1h at room temperature. ΔNp63α was detected using the following anti-p63 antibodies: polyclonal H-129 (1:1000), monoclonal 4A4 (1:4000) and monoclonal N2C1 (1:2000) (Santa Cruz biotechnology, Inc.) followed by incubation in the respective secondary antibody (1:2500 dilution of anti-mouse-HRP catalog # W4011 or anti-rabbit IgG catalog # W402B-HRP, Promega Corp.). Membranes were washed three times with 1X TTBS for 15 minutes each and then submerged in Super-signal West Pico Chemiluminescent substrate (Pierce, Rockford, IL) for 1 minute. Chemiluminescence was quantified using the Fuji Film LAS 4000 image reader (Fuji Medical System USA, Inc.).

D. Synthesis of biotinylated Peptides

Six 12-mer peptides (Table 1) were identified by a previous lab member (Dr. Pavyluk) using a Ph.D.-12 phage display peptides library kit (New England Biolabs, Beverly, MA, USA). These sequences were synthesized by Genscript USA, Inc. as biotin conjugated peptides. Each peptide vial (0.5mg pellet) was spun and reconstituted in 5000 μl Sterile PBS/0.01% Tween 20. The concentration of peptides used in the assay was 50 pmol.

Table 1: Sequence and molecular weights of biotin conjugated peptides identified by phage display.

Phage	Amino acids sequence	Mwt (Dalton)
P1	SLYHQYVTVMMHGG(lys){biotin}	1977.34
P2	HVVKQAMSNNMMGG(lys){biotin}	1858.25
P3	SSAHWRHFNWNLGG(lys){biotin}	2023.25
P4	VIAMDNYNGTTRGG(lys){biotin}	1823.07
P5	KPFHHHQMHLANGG(lys){biotin}	1965.28
P6	HHYNWNLPWLMSGG(lys){biotin}	2066.38

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E. Binding of ΔNp63α to ΔNp63α specific peptides identified by phage display using Streptavidin conjugated Dynabeads

Streptavidin M-280, M-270, Myone C1 and Myone T1 Dynabeads (Life Technologies, trial kit catalog # 65801D) were tested for use in binding studies. The streptavidin Dynabeads were washed with 0.01% PBS / Tween 20 followed by incubation with 50 and 100 pmol biotinylated peptides for 4 hours. Next, the beads were captured using DynaMag magnet separation (Life Technologies, Inc.) followed by washing 3 times with 0.01% PBS / Tween 20. The beads were then resuspended again with 0.01% PBS / Tween 20 buffer, incubated with 14.38 pmol ΔNp63α protein and incubated overnight at 4°C. The beads were collected using magnetic capture (as before), resuspended and washed with 0.01% PBS / Tween 20 buffer in three 10-minute washes. The beads were then resuspended and run onto 10 % SDS-PAGE gel and immunoblotted with a polyclonal H-129 p63-specific antibody as previously described (1:1000).

F. Enzyme-linked immunosorbent assay (ELISA)

The 6 different peptides (200 pmol) dissolved in Tris-buffered saline washing buffer (25mM Tris, 150mM NaCl, 0.1% BSA, 0.05% Tween 20, pH 7.2) were coated onto Streptavidin High Binding Capacity Coated 96-well Plate (Pierce, catalog #15500) and incubated overnight at 4°C. Excess unbound peptides were removed by washing the wells 3 times. Different concentrations of ΔNp63α were then added to each well in a total volume of 100μl of washing buffer. After incubation for 2 hours, the wells were washed three times with washing buffer. The bound ΔNp63α was then incubated with ΔNp63α specific antibodies, H-129 (1:1000), or N2C1 (1:2000) for 1 hour followed by three washes and

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incubation for an additional 1 hour with anti-rabbit IgG conjugated with horseradish peroxidase (1:2500) (Promega Corporation, catalog # W4011). After three washes, $100\mu l$ of substrate 3, 3´, 5, 5´- tetramethyl-benzidine (1-Step Ultra TMP-ELISA, Thermo Scientific, catalog # 34028) was added per well and the plate was incubated for 20 minutes. The reaction was terminated by adding $100\mu l$ / well of 2M H_2SO_4. The absorbance was measured at 450nm using a Safire monochromator microplate reader (Tecan Group Ltd.). Biotinylated peptides and GST were used as negative controls in the experiment.

G. Transfections

Human lung carcinoma H1299 cells were seeded at 3×10^5 per well. Next day, cells were transfected overnight with increasing concentrations of $\Delta Np63\alpha$ expression plasmid or empty vector control using Lipofectamine 2000 (Life Technologies, Inc., catalog # 11668019). At 24h post-transfection, cells were washed with 1X PBS, harvested with a 25% trypsin-ETDA solution and transferred and pelleted in Eppendorf tubes. Whole cell extracts were made by resuspending cell pellets in 100 μL of phosphate inhibitor buffer (PhIB) (50mM Tris pH 8, 120mM NaCl, 5mM EGTA, 1mM EDTA, 5mM sodium pyrophosphate decahydrate, 10mM NaF, 30mM para-nitro phenyl phosphate, 1mM benzamidine, 0.1%NP-40, and 1X sodium vanadate) plus 1% protease inhibitor cocktail (PIC) (Sigma-Aldrich Co.). Samples were vortexed in the PhIB and PIC every ten minutes for a total of 40 minutes while on ice and then centrifuged for 5 min at 14,000 rpm at 4°C. Protein concentration was determined using Pierce BCA reagent (Life Technologies, Inc., catalog # 23228) in a 96-well plate format. BCA standard curve was generated using Bovine Serum Albumin (BSA) in PBS at concentrations ranging from 1 μg to 13 μg. To

determine sample concentrations, 1 µl of sample was added to 99 µl of sterile distilled water for use in the BCA protein assay. To each well (standard or sample) 100µl of BCA reagent was added and the plate was incubated at 37°C for 15 minutes. Absorbance was measured using a Synergy H1 microplate spectrophotometer (BioTek Instruments, Inc.) at 562 nm and the protein concentration was calculated using a BSA standard curve.

A. Full length ΔNp63α protein purification:

i. Development of recombinant methodology to express ΔNp63α protein:
Recombinant ΔNp63α protein was successfully purified using a GST-tag based purification strategy. The initial (pilot) GST-ΔNp63α purification was performed using uninduced and induced 1OD equivalent of bacteria collected from two liter culture induced using a 3.5 hour incubation with IPTG. Assessment of GST-ΔNp63α expression by Coomassie Blue stained 10% SDS-PAGE gel indicated induction of a 100KD band corresponding to the expected molecular weight for GST-ΔNp63α (Figure 5).

ii. Large scale purification of ΔNp63α: After GST-ΔNp63α expression was confirmed in the pilot purification, we proceeded to purify ΔNp63α from a two liter cell culture. GST-trap affinity chromatography was utilized to capture the GST-ΔNp63α fusion protein and subsequently cleave the GST tag. In order to obtain pure ΔNp63α protein, elution was performed using PreScission protease as shown in the first two lanes (Figure 6). The third and fourth lanes demonstrate abundant levels of GST-ΔNp63α and ΔNp63α were eluted with reduced glutathione. This suffested that in-column digestion with Precission protease did not effectively elute untagged ΔNp63α. GST-ΔNp63α was also subjected to *in vitro* digestion with PreScission protease by mixing the GST-ΔNp63α with the appropriate amount of PreScission protease. The cleavage of GST was confirmed by Coomassie Blue staining which was indicated by an increase in the 73kDa protein and loss of the 100kDa GST-ΔNp63α band (Figure 7).

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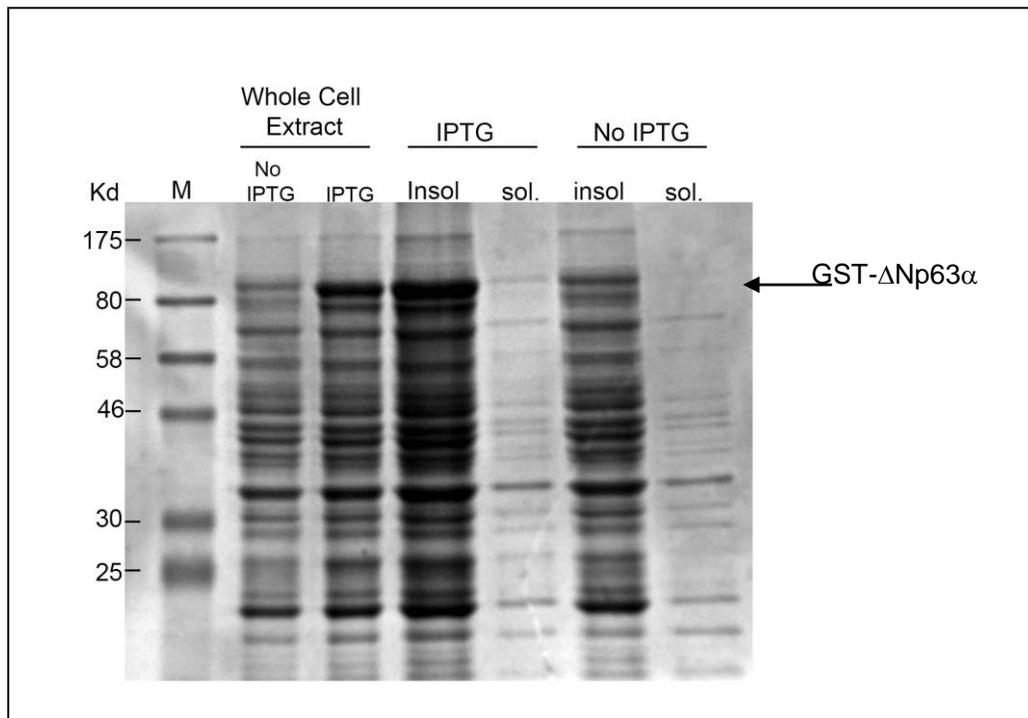
Figure 5 : Coomassie Blue staining gel demonstrates IPTG induction of recombinant GST−ΔNp63α (No IPTG or IPTG). Arrow indicates increased levels of GST- ΔNp63α was observed in the whole cell extracts and in the insoluble fraction, but not in the soluble fraction of IPTG induced sample.
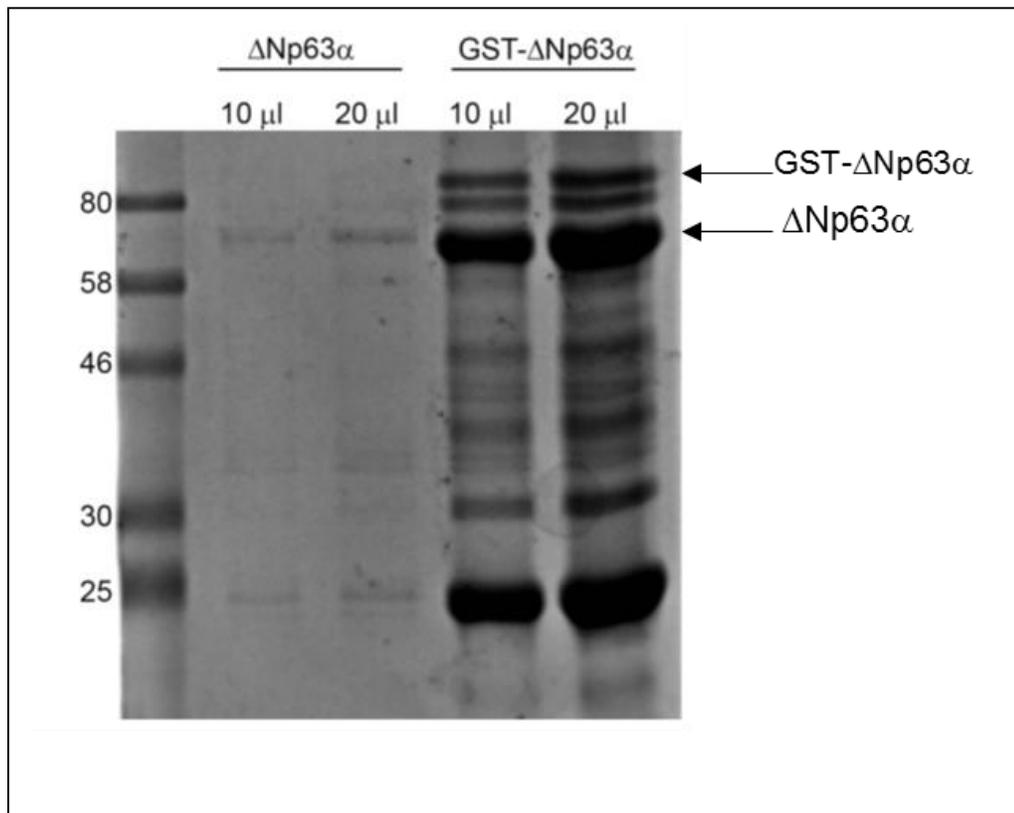
Figure 6: Large scale purification of GST-ΔNp63α followed by digestion with PreScission protease to cleave GST. Two different aliquots of preparations eluted by PreScission protease followed by reduced glutathione were loaded onto the gel. The majority of ΔNp63α was found in the fraction eluted by reduced glutathione.
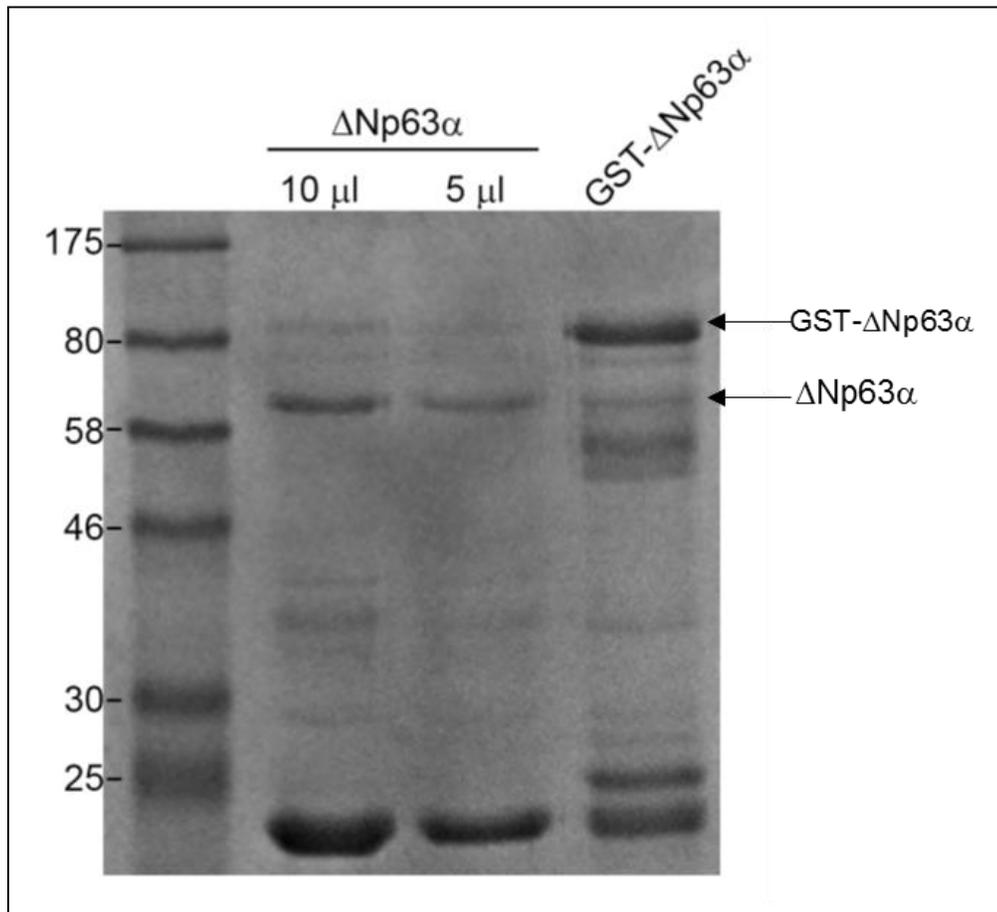
Figure 7: An aliquot of GST- ΔNp63α was subjected to *in vitro* **digestion by PreScission protease for GST cleavage**. Digested proteins were loaded onto an SDS-PAGE gel and stained with instant Coomassie Blue stain indicate efficient cleavage of GST (first 2 lanes). GST-ΔNp63α was used as a control.

Size selection performed using a 50 kDa cutoff Amicon filter successfully removed non-specific contamination from the crude recombinant ΔNp63α sample. As shown in Figure 8, size-selection removed most of the non-specific bands from the crude ΔNp63α and GST-ΔNp63α.

B. Quantitation of ΔNp63α:

Recombinant ΔNp63α was quantified by densitometry against a standard curve of Bovine Serum Albumin (BSA). BSA standards from 0.1 μg to 2 μg were run alongside ΔNp63α, and the gel was stained with instant blue stain and analyzed using Multi-Gauge (Figure 9A). The concentration of ΔNp63α was calculated using the BSA standard curve (Figure 9B) and found to be 116 ng / μl.

C. Detection of the lower band in recombinant ΔNp63α sample:

In order to evaluate the possibility that the lower band in Figure 8 not a breakdown product of p63 or a heat-shock protein, we performed the following experiments: First, the recombinant ΔNp63α sample was treated with ATP during column purification to remove the band if it was a heat shock protein. (ATP is known to cause conformation changes in heat shock proteins.) Figure 10 showed that migration of the lower band was not affected by the addition of ATP, thus indicating that is was likely not a heat shock protein.

Immunoblot analysis was performed using three independent ΔNp63α specific antibodies (4A4, N2C1, H-129). The lower band was not detected by these antibodies, thus indicating that the lower band was not a degradation product of ΔNp63α (Figure 11). Finally, an anti-GST antibody was utilized to determine if the lower band was GST. Western blot analysis showed that lower band was detected as GST (Figure 11B). Mass spectrometry confirmed that lower band was GST (data not shown).

30
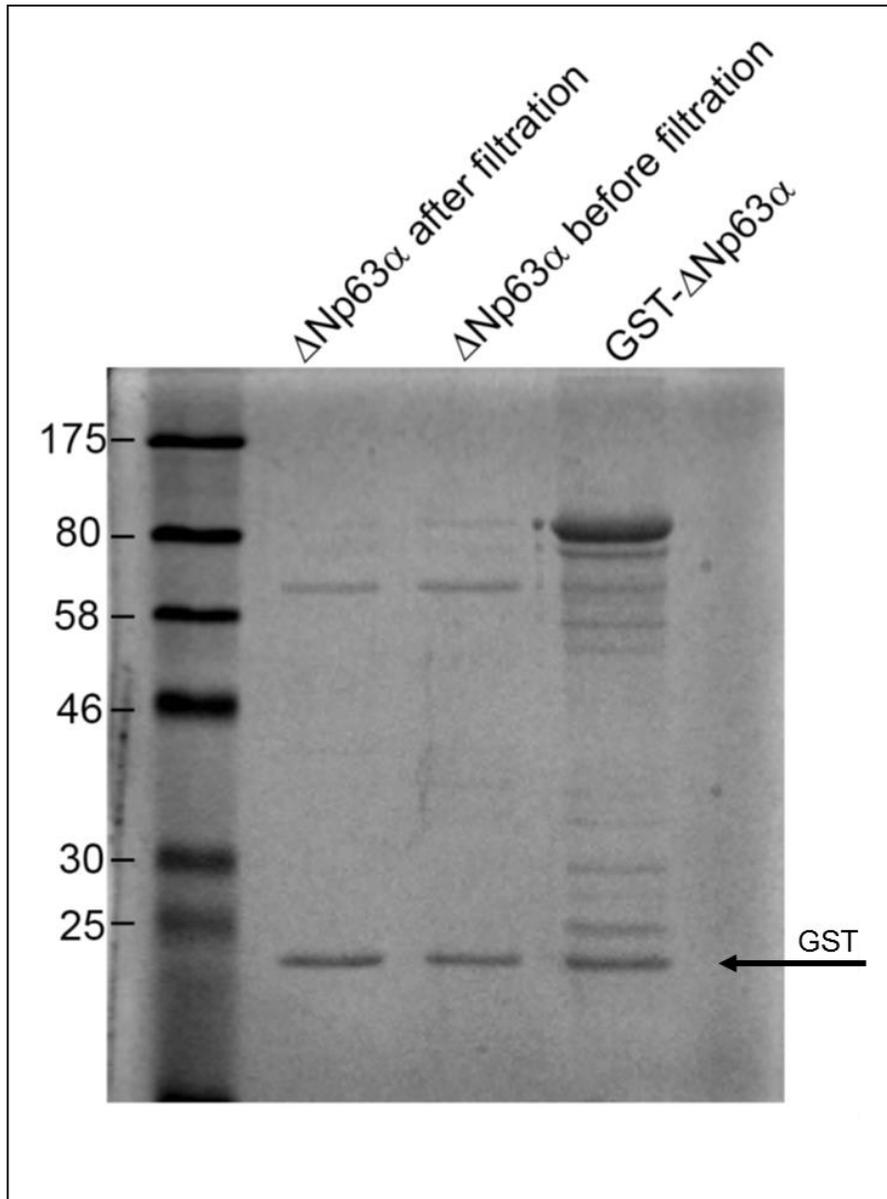
Figure 8: Size selection performed on ΔNp63α to remove nonspecific contamination.

Following the GST trap column chromatography, size selection was performed using a 50

KD cut-off Amicon filter to discard bands lower than 50 KD.

A)

B)
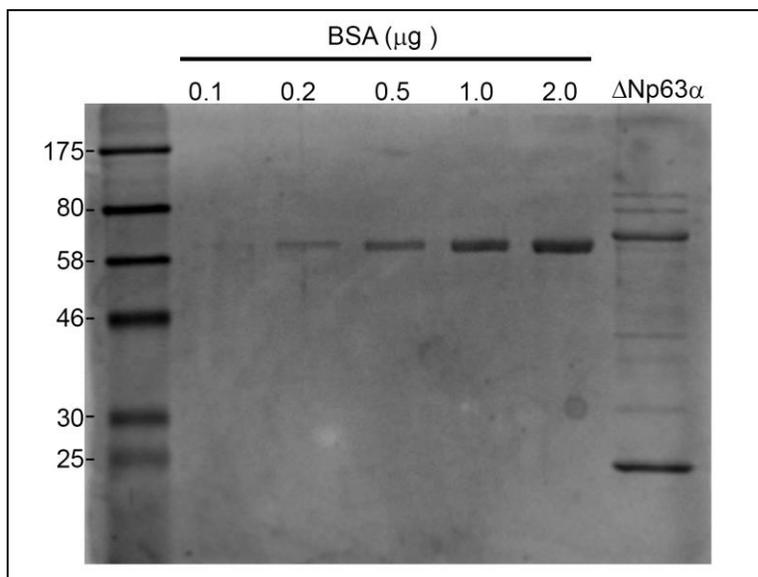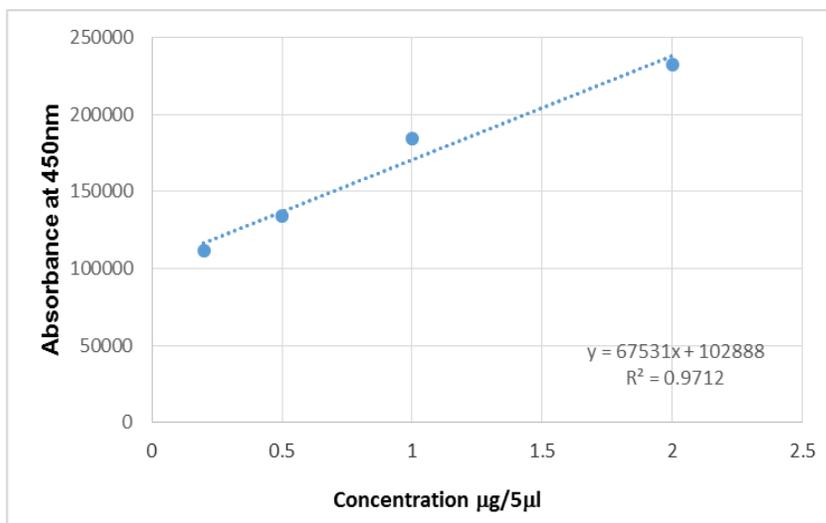
ΔNp63α concentration = 0.579 μg/μl = 116ng/μl

Figure 9: Quantitation of ΔNp63α using a bovine serum albumin standard. A) Increasing amount of BSA standard (0.5μg-2μg) and 5 μl of recombinant ΔNp63α were subjected to SDS-PAGE. B) Band density of standard BSA dilutions were used to quantify the amount recombinant ΔNp63α concentration.
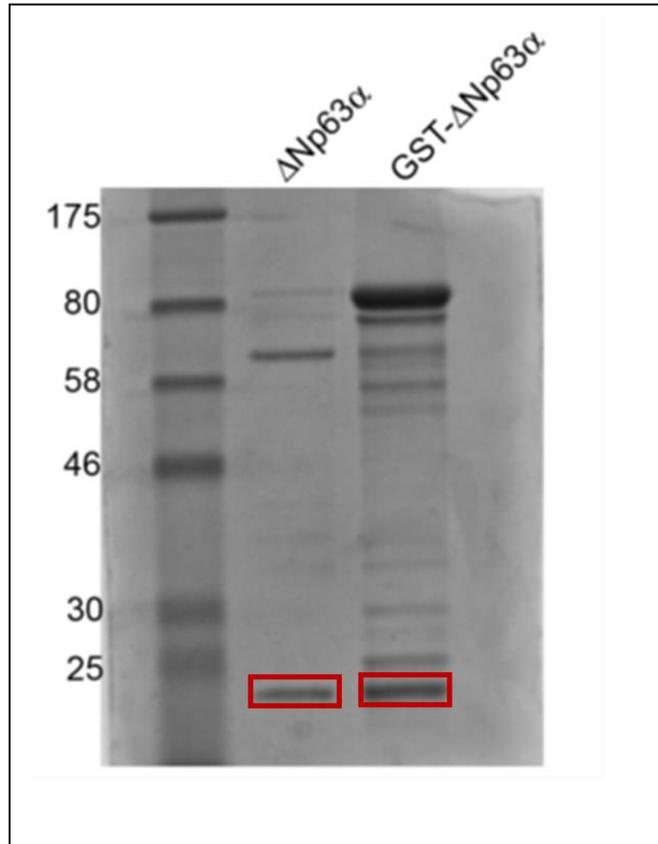
Figure 10: ATP treatment performed during the column affinity purification of ΔNp63α. The sample was applied to the column, equilibrated in wash buffer, and ATP was applied followed by sample elution. PreScission protease added and incubated overnight. This treatment would be expected to remove the lower band if it was a heat shock protein, but no effect was observed indicating the lower band was not likely a heat shock protein.

A)
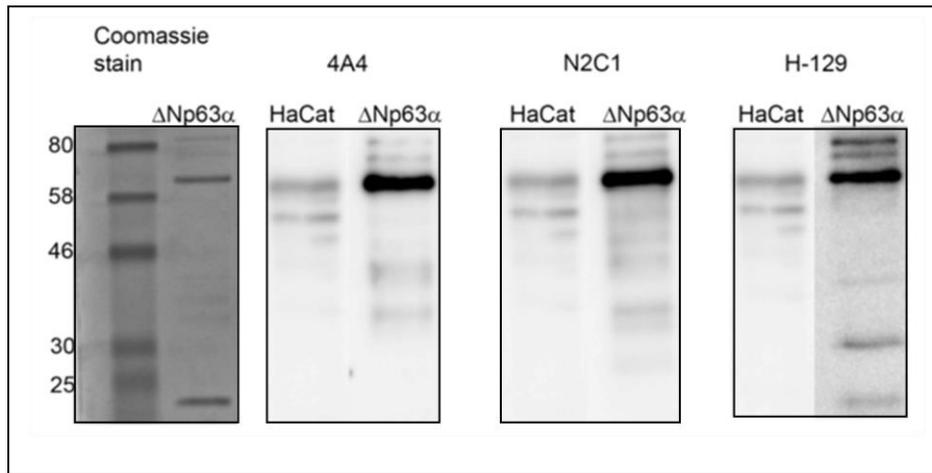
B)
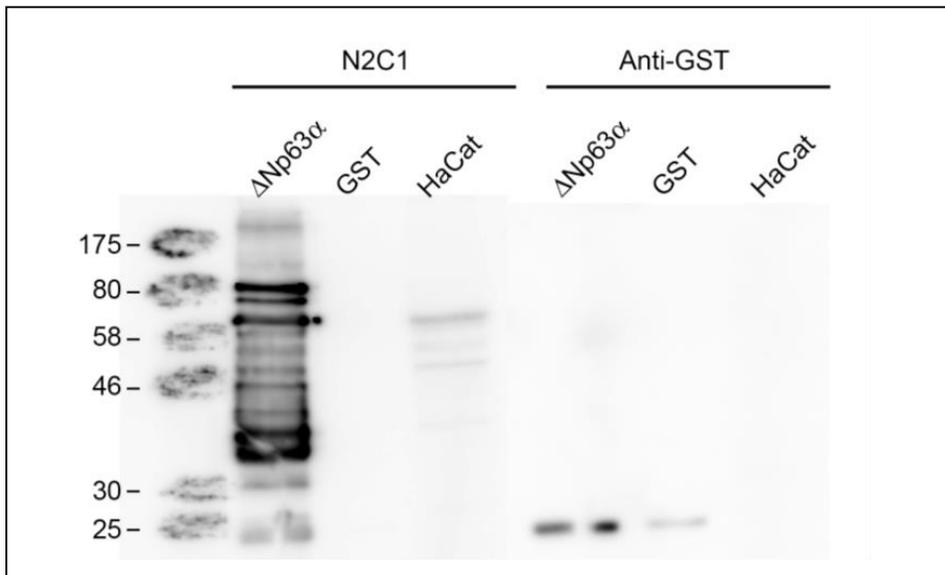
Figure 11: Detection of ΔNp63α using three specific antibodies (4A4, N2C1, H-129) and confirmation of lower band identity using an anti-GST antibody. A) An aliquot of recombinant ΔNp63α, whole-cell extract from HaCat cells (expressing endogenous ΔNp63α as a positive control, and GST were incubated with ΔNp63α specific antibodies and resolved on a 10% SDS-PAGE gel. Coomassie stained ΔNp63α was used as a control, and whole cell extract from HaCat cells were used as positive control for ΔNp63α. Lack of detection of a lower band using three anti-specific ΔNp63α antibodies indicated that the lower band is not a degradation fragment of ΔNp63α. B) Immunoblot analysis of recombinant GST, whole-cell extract from HaCaT cells and recombinant ΔNp63α using an anti-GST antibody to demonstrate that the lower band was GST.

D. Confirmation of ΔNp63α specific binding to peptides to ΔNp63α by Dynabead assay:

The ability of six biotinylated peptides to bind to ΔNp63α was first examined using a Dynabead assay system, where the streptavidin beads bind biotinylated peptides. Immunoblot analysis using p63-specific antibody indicated that binding of ΔNp63α to the peptide. Figures 12A and 12B show a western blot probed with H-129 antibody for the six biotinylated peptides bound to ΔNp63α using streptavidin M-280 Dynabeads. Two different concentrations of each of the selected peptides were incubated with recombinant ΔNp63α. In Figure 12A, loading the beads and ΔNp63α protein as a negative control resulted in appearance of Np63 band in absence of peptide, indicating nonspecific binding of ΔNp63α to M-280. In another experiment, no ΔNp63α band was observed in the negative control (Figure 12B), and biotinylated P5 and P6 showed specific binding to ΔNp63α compared to M-280 and ΔNp63α alone (Figure 12B). Concerns about non-specific binding of the M-280 Dynabeads to ΔNp63α prompted further analysis with different Dynabead styles. The manufacturer stated binding capacities are listed in Table 2.

Various streptavidin Dynabeads with low-level non-specific binding to ΔNp63α were tested; M-270, Myone C1 and Myone T1. The six specific biotinylated peptides and four non-specific biotinylated peptides to ΔNp63α (NE1, NE2, L4, L5) were tested using M-270. As shown in Figure 13, western blot analysis showed non-specific binding of ΔNp63α to M270 Dynabeads. Furthermore, three of biotinylated non-specific peptides to ΔNp63α (NE1, NE2, L5) showed positive binding.

A)

B)

Figure 12: Binding of biotinylated peptides to recombinant ΔNp63α using streptavidin M-280 Dynabeads. A) Either 50 or 100 pmol concentrations of each of four biotinylated peptides (P1, P2, P3, P4) were incubated with streptavidin M-280 Dynabeads followed by the addition of recombinant ΔNp63α. B) Either 50 or 100 pmol concentrations of biotinylated peptides (P5, P6) were incubated with streptavidin M-280 Dynabeads followed by addition of ΔNp63α. ΔNp63α alone was used as a positive control. Streptavidin M-280 Dynabeads alone and in the presence of ΔNp63α were used as negative controls.

Table 2: Binding Capacity of four different streptavidin Dynabeads

BEADS	Binding Capacity/ 25 µl beads	P1 pmol	P2 pmol	P3 pmol	P4 pmol	P5 pmol	P6 pmol
M280	50 pmol	50.57	53.8	49.4	54.9	50.9	48.4
M270	50 pmol	50.57	53.8	49.4	54.9	50.9	48.4
Myone C1	100 pmol	101.2	108	98.9	110	102	96.8
Myone T1	100 pmol	101.2	108	98.9	110	102	96.8

			+									P1 50 pmol
				+								P2 50 pmol
					+							P3 50 pmol
						+						P4 50 pmol
							+					P5 50 pmol
								+				P6 50 pmol
									+			NE1 50 pmol
										+		NE250 pmol
											+	L4 50 pmol
												+	L5 50 pmol
	+	+	+	+	+	+	+	+	+	+	+	+	ΔNp63α14.38pmol
+	+	+	+	+	+	+	+		+	+	+	+	M270 beads

ΔNp63α 73KDa

43
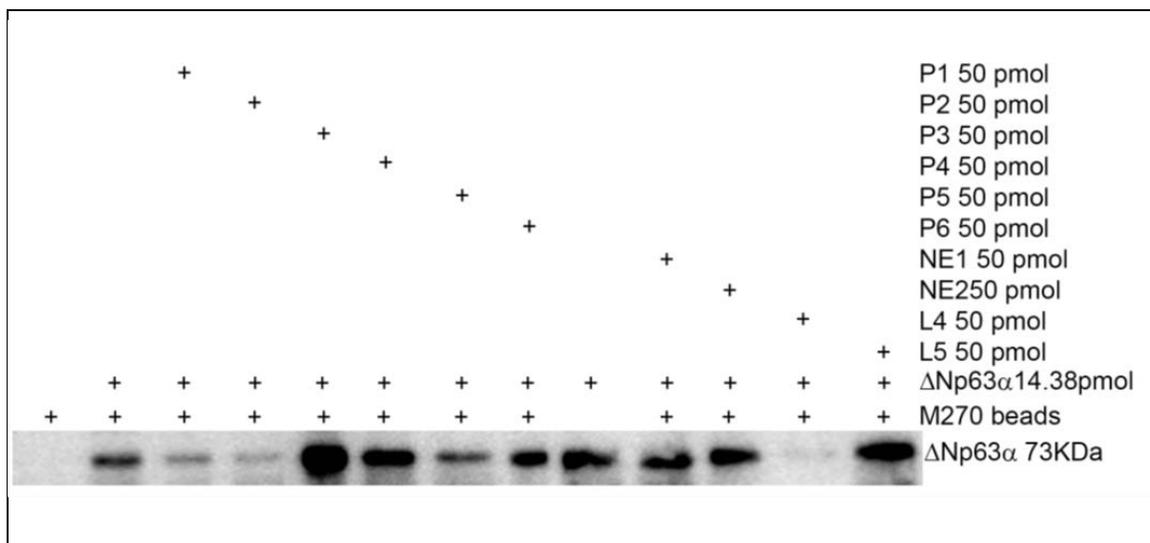
Figure 13: Binding of different concentrations of biotinylated peptides to recombinant ΔNp63α using streptavidin M-270 Dynabeads. Each biotinylated peptide (P1, P2, P3, P4, P5, P6) was incubated with streptavidin M-270 Dynabeads followed by the addition of recombinant ΔNp63α. ΔNp63α was used as positive control. ΔNp63α non-specific biotinylated peptides (NE1, NE2, L4, L5) were used as negative controls. Streptavidin M-270 Dynabeads alone and in the presence of ΔNp63α were also used as negative controls.

The third streptavidin Dynabeads utilized in the binding study was Myone C1. Western blot analysis using H-129 specific ΔNp63α antibody revealed that there is non-specific binding of ΔNp63α to Myone C1 Dynabeads (Figure 14A, 14B).

Finally, Myone T1 streptavidin Dynabeads were utilized to examine the binding of biotinylated peptides to ΔNp63α. However, there was also nonspecific binding of ΔNp63α to Myone T1 Dynabeads as shown with other streptavidin Dynabeads (Figure 15).

E. ELISA confirmation of ΔNp63α specific binding to peptides to ΔNp63α by streptavidin:

Binding affinity of biotinylated peptides to recombinant ΔNp63α was examined using a streptavidin coated plate approach. An indirect ELISA was performed to identify the most specific peptide to bind to ΔNp63α (Figure 16). The indirect ELISA utilizes both a primary antibody (e.g. rabbit anti-p63) and a secondary conjugated antibody (e.g. goat-anti-rabbit-HRP) to deliver enhanced sensitivity over the single antibody detection used in direct ELISAs. In the indirect ELISA, the use of multiple antibodies results in signal amplification and improved overall assay sensitivity.

Two different ΔNp63α specific antibodies were used. First, polyclonal H-129 antibody was used against ΔNp63α. As shown in Figure 17, two concentration of ΔNp63α were incubated with fixed concentration of peptides. The fold increase over ΔNp63α alone (Y-axis) in presence and absence of peptides is shown. P2 showed a higher fold change compares to ΔNp63α alone. P3, P5 and P6 showed the highest fold change in presence of ΔNp63α, suggesting that they have the highest affinity to bind to ΔNp63α. However, P1 and P4 fold change did not change in presence of both P4 and ΔNp63α, suggesting that P4

had the weakest affinity to bind to ΔNp63α. The nonspecific peptide L4 was used as a

negative control which showed no change in fold change in presence of ΔNp63α.

A)
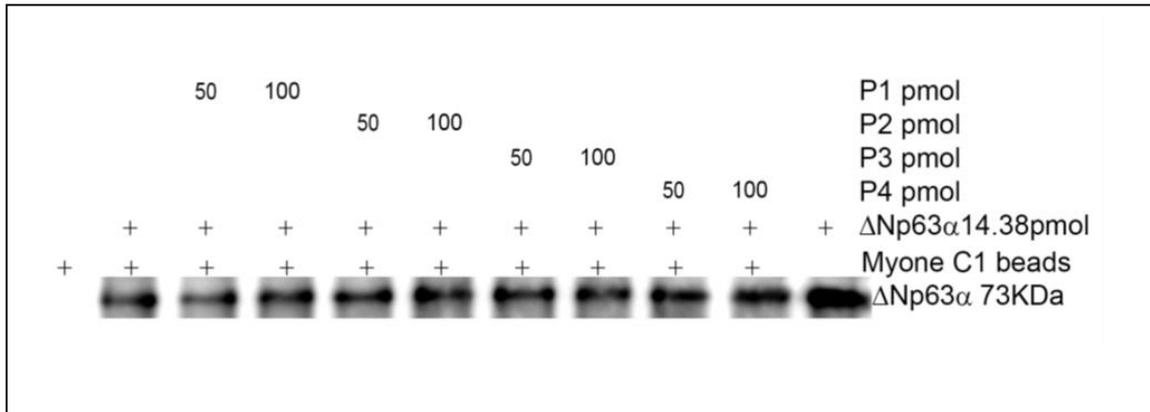
B)
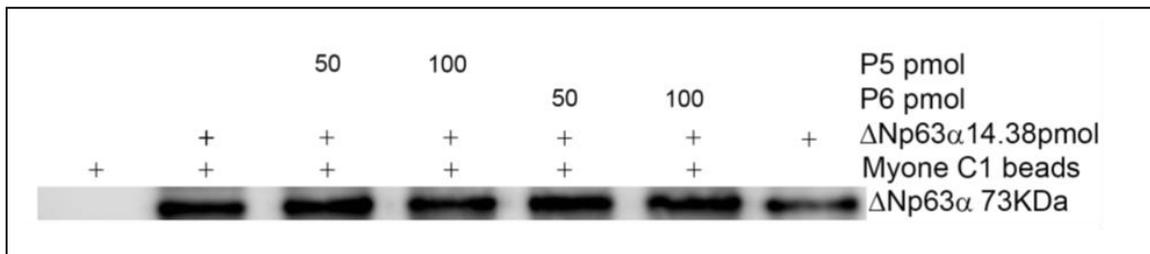
Figure 14: Binding of biotinylated peptides to recombinant ΔNp63α using streptavidin Myone C1 Dynabeads. A) Two concentrations of each biotinylated peptides (P1, P2, P3, P4) were incubated with streptavidin Myone C1 Dynabeads followed by the addition of recombinant ΔNp63α. B) Two concentrations of biotinylated peptides (P5, P6) were incubated with streptavidin Myone C1 Dynabeads followed by addition of ΔNp63α. ΔNp63α was used as positive control. Streptavidin Myone C1 Dynabeads alone and in the presence of ΔNp63α were used as negative controls.

												P1 50 pmol
												P2 50 pmol
												P3 50 pmol
												P4 50 pmol
												P5 50 pmol
												P6 50 pmol
												NE1 50 pmol
												NE250 pmol
												L4 50 pmol
												L5 50 pmol
	+	+	+	+	+	+	+	+	+	+	+	ΔNp63α14.38pmol
+	+	+	+	+	+	+	+	+	+	+	+	Myone T1 beads
												ΔNp63α 73KDa

Figure 15: Binding of biotinylated peptides to recombinant ΔNp63α using streptavidin Myone T1 Dynabeads. Each biotinylated peptide (P1, P2, P3, P4, P5, P6) was incubated with streptavidin Myone T1 Dynabeads followed by the addition of recombinant ΔNp63α. ΔNp63α was used as positive control. ΔNp63α non-specific biotinylated peptides (NE1, NE2, L4, L5) were used as negative controls in addition to Streptavidin Myone T1 Dynabeads alone and in the presence of ΔNp63α.

ELISA Assay

Figure 16: Schematic diagram showing the principle of the indirect ELISA assay.
Biotinylated peptides were incubated in 96 well streptavidin-coated plate overnight. Excess peptides were washed away, followed by incubation with ΔNp63α. ΔNp63α specific primary antibodies were then added and allowed to incubate for 45 minutes. Next, an HRP conjugated secondary antibody was added and allowed to incubate for 45 minutes. The color was developed using TMB and the intensity was read at wavelength 450nm.
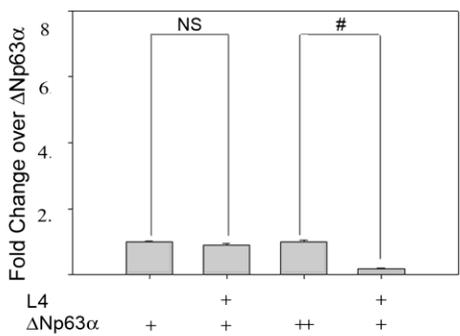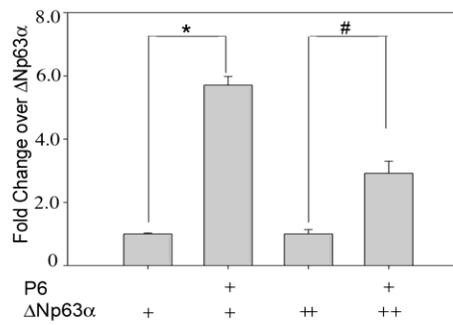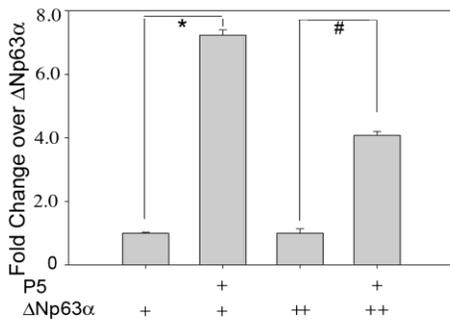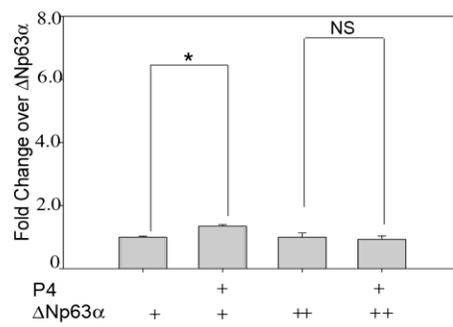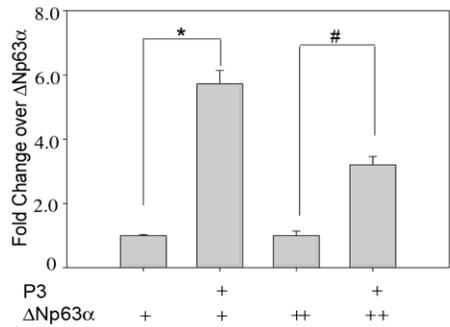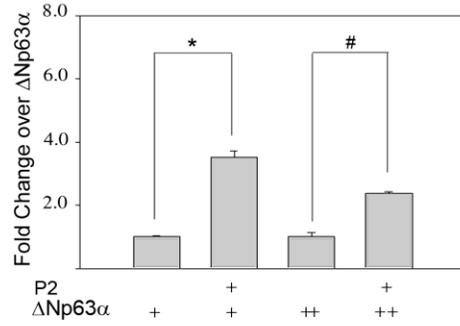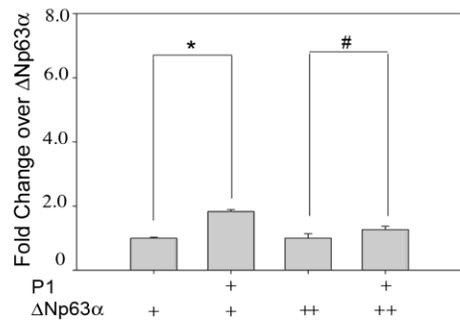
Figure 17: Binding of biotinylated peptides (P1, P2, P3, P4, P5, P6) to ΔNp63α using a polyclonal antibody (H-129). Graphs display fold-change over ΔNp63α as determined using ELISA assays with two concentrations (+ = 1.73pmol, ++ = 4.32pmol) of ΔNp63α alone and with 200 pmol biotinylated peptides. The non-specific biotinylated peptide (L4) was used as negative control. ELISA measurements were performed in triplicate. Error bars indicate standard deviations. Error bars represent standard deviation between triplicates. *=p≤0.05 compare to ΔNp63α at 1.73pmol and # =p≤0.05 compare to ΔNp63α at 4.32pmol. NS= non-significant compare to ΔNp63α.

Finally, we examined the binding of peptides to ΔNp63α utilizing monoclonal antibody (N2C1). The results (Figure 18) were similar to those obtained using the polyclonal antibody. P3, P5, P6 and P2 showed high fold change over ΔNp63α. Repeated experiments also indicated that P3, P5 and P6 have the highest affinity to bind to ΔNp63α (data not shown).

Since the recombinant ΔNp63α sample had residual GST contamination, we tested if the GST interfered with binding to the biotinylated peptides using an indirect ELISA. Testing indicated that there was no binding interference from residual GST (Figure 19). The fold change of GST is shown on the Y-axis, and the data is plotted relative to GST alone and in presence of peptides (Figure 19). Overall, the ELISA experiments and those described previously showed that P5 has the highest affinity to bind to ΔNp63α. Thus, further experimentation was completed using biotinylated peptide P5.

A dose dependent study was performed using an ELISA approach to identify the binding capacity of P5 to bind to ΔNp63α. A graph of increasing concentrations of P5 and constant concentration of ΔNp63α was drawn to identify the binding capacity of P5 to ΔNp63α as shown in Figure 20A. Biotinylated peptide P5 binding to ΔNp63α was increased as its concentration increased until reached the saturation at 125 pmol of B-P5. A dose-dependent assay of ΔNp63α (Figure 20B) indicated that the saturation reached at 2.4 pmol ΔNp63α. The dose dependent assay was repeated for each new ΔNp63α preparation. P-value and the fold changes for both antibodies are shown in table 3.

55
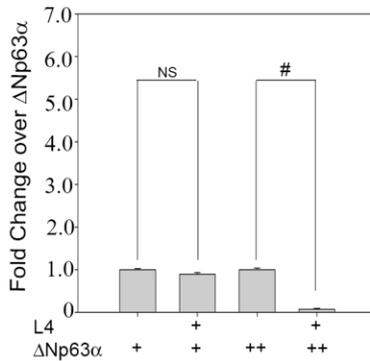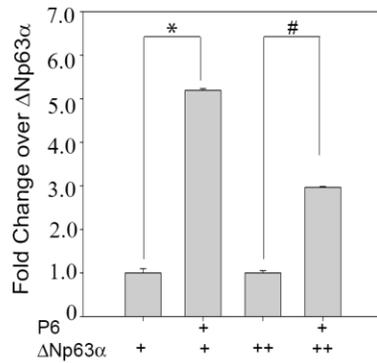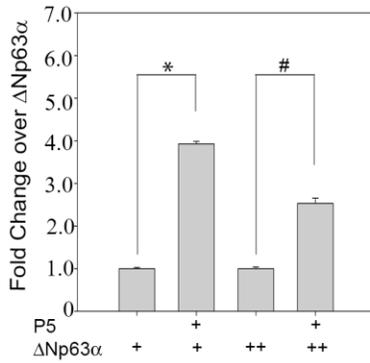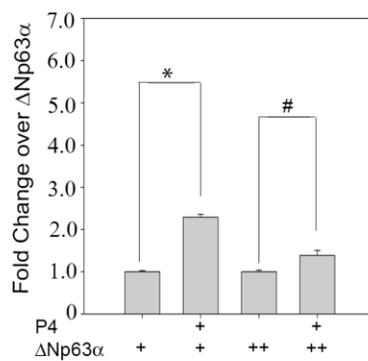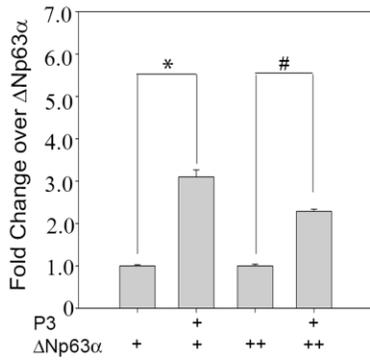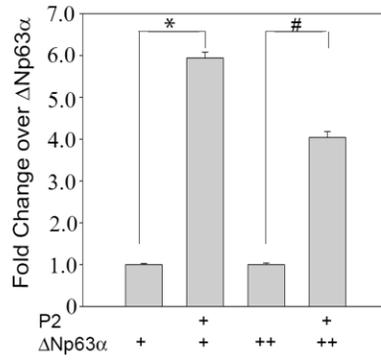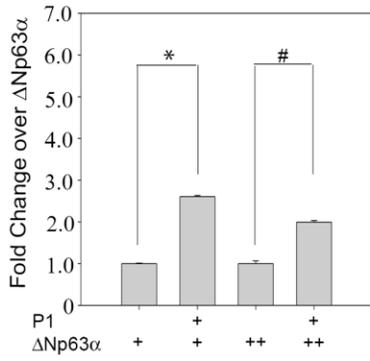
Figure 18: Binding of biotinylated peptides (P1, P2, P3, P4, P5, P6) to ΔNp63α using a monoclonal antibody (N2C1). The fold-change over ΔNp63α indicated was determined by ELISA assay using with two concentrations (+ = 1.73pmol, ++ = 4.32pmol) of ΔNp63α alone and with 200 pmol biotinylated peptides. Non-specific biotinylated peptide (L4) was used as negative control. Error bars represent standard deviation between triplicates. *=p≤0.05 compare to ΔNp63α at 1.73pmol and # =p≤0.05 compare to ΔNp63α at 4.32pmol. NS= non-significant compare to ΔNp63α.

Peptide	H-129		N2C1	
	Fold Change	**P.value**	**fold Change**	**P.value**
P1	1.82	0.001	2.6	0.0003
P2	3.53	0.0003	3.92	2.07E-06
P3	5.72	0.0006	3.13	0.001
P4	1.34	0.004	2.29	0.0004
P5	7.22	7.08E-06	6.83	9.62E-06
P6	5.7	0.0001	5.19	1.68E-05

Table3: fold changes and P.values calculation for six biotinylated peptides.

GST	+	+	+	+	+	+	+
P1		+					
P2			+				
P3				+			
P4					+		
P5						+	
P6							+
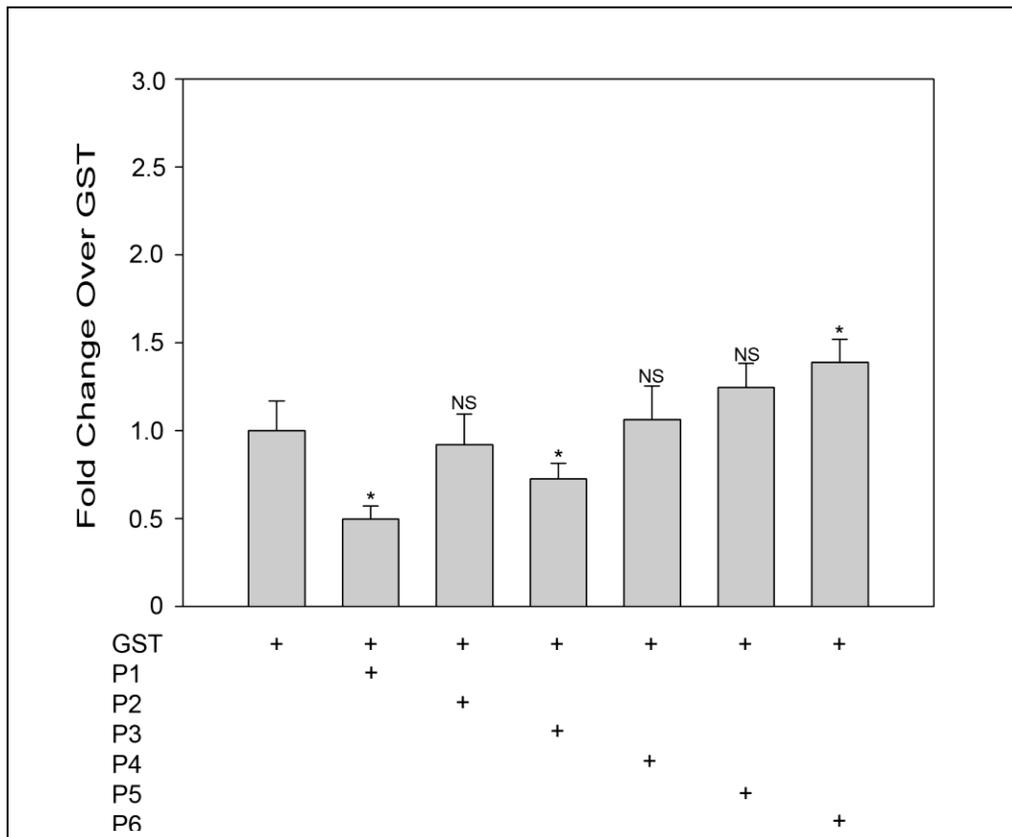
Figure 19: Detection of binding of biotinylated peptides (P1, P2, P3, P4, P5, P6) to GST. The fold-change over GST was determined using an ELISA assay with GST alone and after incubation with biotinylated peptides. Error bars represent standard deviation between triplicates. *=p≤0.05 compare to GST at 1.75 pmol. NS= non-significant compare to GST at 1.75pmol.

A)
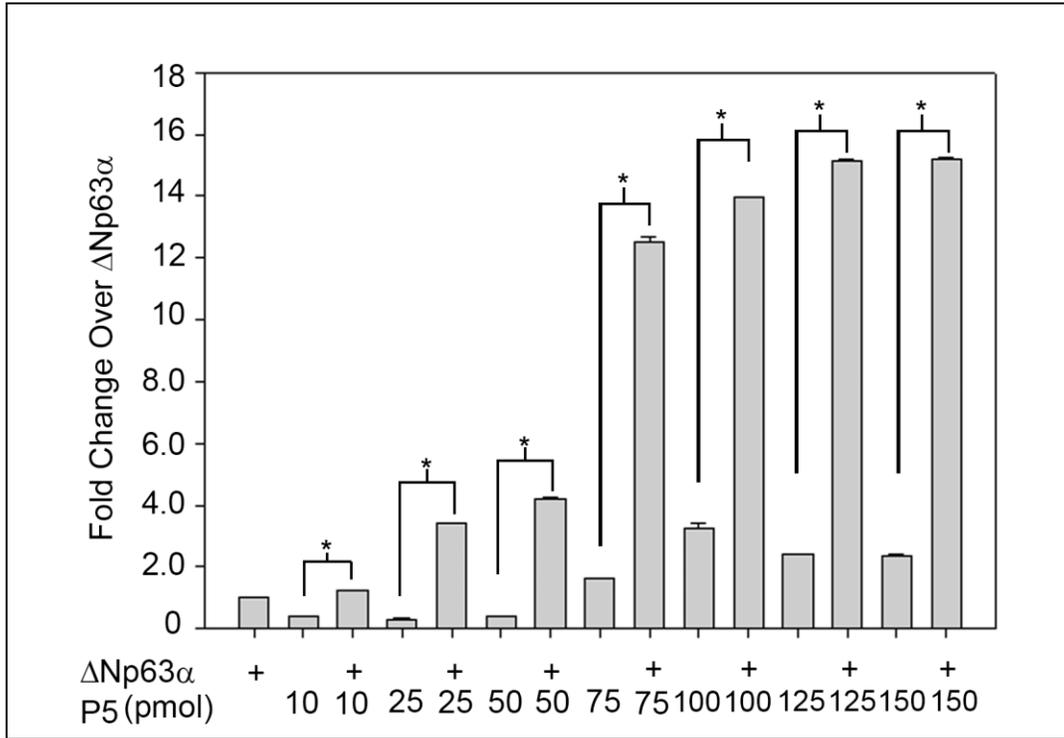
B)
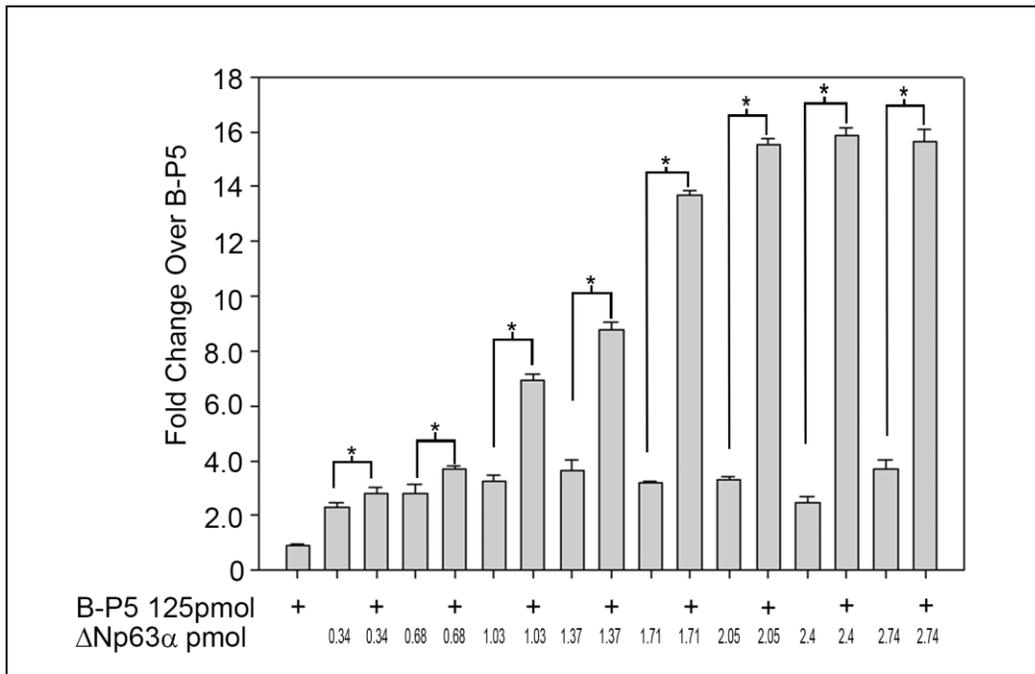
Figure 20: Dose dependent assay of B-P5 and ΔNp63α. A) Fold-change over ΔNp63α alone and in the presence of increasing concentrations of B-P5 display the saturation concentration of B-P5 to bind to ΔNp63α. Error bars represent standard deviation between triplicates. *=p≤0.05 compare to P5. B) Fold-change over B-P5 alone and in the presence of increasing concentrations of ΔNp63α showing the saturation concenration of ΔNp63α. Error bars represent standard deviation between triplicates. *=p≤0.05 compare to ΔNp63α.

Finally, we examined the binding of biotinylated P5 to transfected ΔNp63α in H1299 cells using streptavidin coated plate using ELISA. Figure 21 shows the fold change over biotinylated peptide P5 (Y-axis) relative to vector transfected cells and ΔNp63α transfected cells in the presence of biotinylated peptide P5. As expected, an increase in the fold change of ΔNp63α was observed in the presence of B-P5 but not for empty vector.

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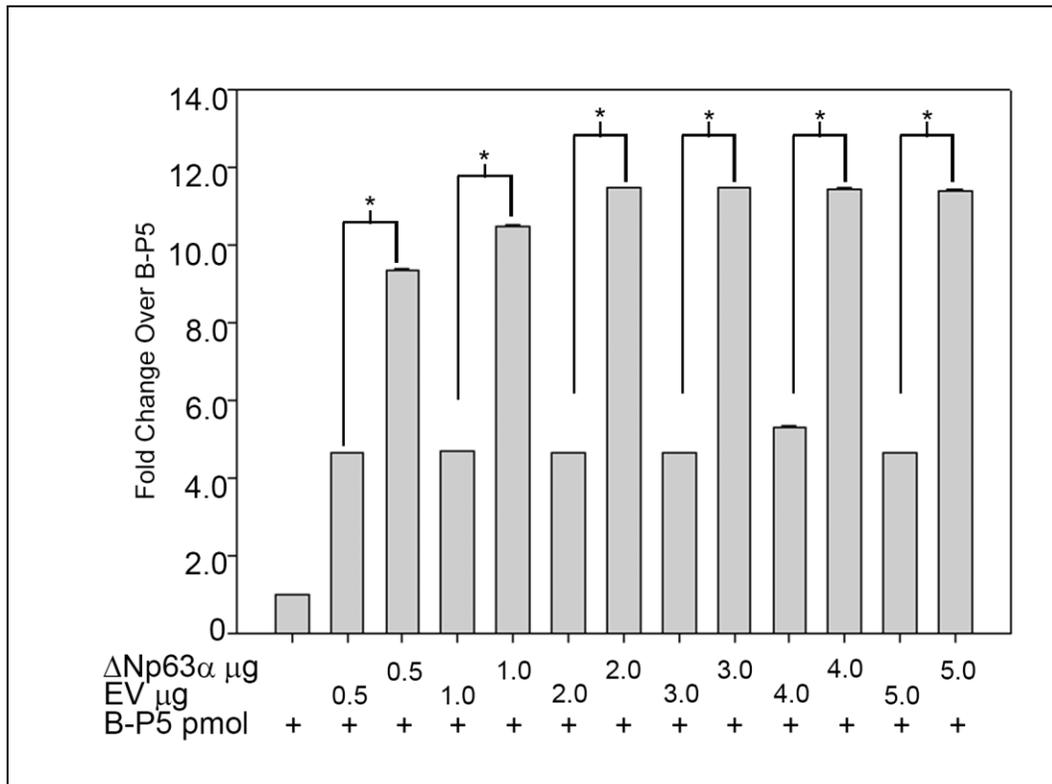
Figure 21: Binding of biotinylated peptides P5 to ΔNp63α compare to empty vector.
Y-axis represents fold change over B-P5 related to ΔNp63α and empty vector in presence
of biotinylated peptides in all cases. Error bars represent standard deviation between
triplicates. *=p≤0.05 compare to empty vector.

IV.   DISCUSSION

It is well recognized that peptides can serve as receptor agonists, antagonists or modulators of protein biological function (Smith & Petrenko, 1997). In cancer, many genes are involved in the tumor growth, making it difficult to identify upstream regulators for a given gene of interest. Here we utilized phage display technology (Wilson & Finlay, 1998) to identify upstream modifiers of oncogenic ΔNp63α function. This approach was performed to focus on the peptide-protein interactions to examine the affinity of specific peptide(s) which bind to the protein and affect its function. The foundation of this approach was to prepare pure recombinant ΔNp63α and to utilize peptide phage display technique to find specific peptide binders.

Many studies have shown the effectiveness of peptides, identified by phage display, to modulate or regulate protein function. (Shen et al, 2013) used phage display to identify a peptide which serves as a ligand for prostate-specific membrane antigen (PMSA) in cancer cells. Another group using phage display to identify a novel peptide called AP8 which is expressed in breast cancer which binds to the acidic fibroblast growth factor (aFGF) and correlates with disease grade via a direct interaction with fibroblast growth factor receptors (FGFRs). Functional analysis indicated that AP8 functions as an aFGF antagonist by preventing aFGF binding to its receptor which inhibits aFGF-stimulated cell proliferation (Dai et al, 2014).

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In this study, we designed a purification strategy which facilitated the successful purification of recombinant ΔNp63α sufficient for use in subsequent binding studies. The method yielded abundant amount of soluble protein, but retained residual GST contamination.

Our lab previously identified a selection of phage-peptides that bind to the ΔNp63α protein. This project demonstrated the functional binding of these peptides to ΔNp63α and recombinant ΔNp63α. The candidate peptides were synthesized as biotinylated peptides and *in vitro* testing using several types of Dynabeads with different binding capacity to validate binding of these peptides to ΔNp63α. However, we were unable to confirm the binding of the peptides to ΔNp63α due to non-specific binding between ΔNp63α and all styles of Dynabeads tested. Manufacturer guidelines suggested that Dynabead size or concentration may correlate with the degree of non-specific binding (i.e. using smaller diameter or fewer beads would help to reduce non-specific binding). Accordingly, different diameter Dynabeads used in the assay this study, but our data did not support the contention that smaller beads resulted in lower levels of nonspecific binding. However, a fixed amount of Dynabeads were used regarding their diameter differences, and it remains possible that improved results could be obtained by reducing the number of beads used in the assay. Reduction of nonspecific binding is frequently achieved by adding BSA or Tween 20 to the reaction mixture per manufacturer recommendations, but both were ineffective at reducing the non-specific binding (data no shown). It is also possible that the observed non-specific binding could be overcome by pre-blocking the beads before ΔNp63α addition. This would serve to reduce nonspecific hydrophobic interactions.

The Dynabead assay suggested that some of the candidate peptides bound specifically to ΔNp63α, particularly P5 and P6. An alternate streptavidin coated plate ELISA strategy was performed to clarify the specificity of binding of these peptides to ΔNp63α. The ELISA showed that the P3, P5 and P6 peptides bind to ΔNp63α with higher affinity than the other peptides. P5 bound to ΔNp63α with the highest affinity, and was thus selected for further evaluation to test the specificity and the affinity of P5 binding to ΔNp63α.

If the peptide binding affinity to ΔNp63α is determined, a recognition site may be identified which may reveal the specific function of the P5 peptide. It is possible the P5 peptide is involved in targeting ΔNp63α for degradation or may blunt its signaling by altering the functional confirmation of ΔNp63α. The peptide could inhibit protein oligomerization and retain the protein in an inactive form (Cardinale et al, 2011) in a manner similar to that observed by the interaction with the oncogenic Head Shock Protein (gp96). Gp96 binding causes a confirmation change in ΔNp63α which increases binding to the HER2 receptor and prevents ΔNp63α dimerization; thus, the signaling pathway is terminated leading to the degradation of HER2, decreased cell growth and increased apoptosis (Li et al, 2015).

Future work will focus on the targeting and internalization of the specific peptide and thorough an evaluation of the ability of the peptide to modulate the oncogenic effect of ΔNp63α such as examination of the peptide binding affinity to ΔNp63α and an analysis of the effect of peptide binding to ΔNp63α Further, the activity and affinity of P5 peptide will be determined using kinetic assays. Finally, the *in vivo* efficacy of P5 in modulating

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ΔNp63α could be investigated, but testing would require modification of the peptides to produce cell permeable peptides inhibitors. This study would first reqire that the candidate peptides be modified to allow cell membrane penetration. One possible modification includes conjugating the peptides with adapters effective in penetratint the cell membrane such as those used in the cell penetrating peptides system (CPP). The CPP is proven to successfully penetrate the cell membrane; however, the ability of the CPP to a target a linked peptide is not fully elucidated. In addition, another possible modification to allow cell membrane penetration is by modifying the peptides with membrane-translocating moieties that increase their hydrophobicity and facilitate cellular entry.

In conclusion, the data presented herein illustrates the effectiveness of phage display libraries in the search for new peptide based lead structures designed to mimic or inhibit therapeutically important protein-protein interactions or targeting a specific protein for degradation.

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V.   BIBLIOGRAPHY

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