Molecular Insights into p53 repressed anti-apoptotic proteins, Clusterin and Survivin in human oral cancer

By

Rupa Mishra (Nee Vishwanathraman)
LIFE09200904003

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As members of the Viva Voce Board, we recommend that the dissertation prepared by Smt. Rupa Mishra (Nee Vishwanathraman) titled 'Molecular Insights into p53 repressed anti-apoptotic proteins, Clusterin and Survivin in human oral cancer' be accepted as fulfilling the requirements for the Degree of Doctor of Philosophy.

Chairperson: Dr. Sorab Dalal, ACTREC Date: 17/6/16

S. N. Dalal

Convener: Dr. Tanuja R. Teni, ACTREC Date: 17/6/16

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V Tiraga

Member 2: Dr. Prasanna Venkatraman, ACTREC Date: 17/6/16.

Prasanna

Invitee: Dr. Shobhona Sharma, TIFR Date: 17/6/16

Shobhona

External Examiner: Dr. Moni Abraham Kuriakose, Mazumdar-Shaw Cancer Centre Date: 17/6/16

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I hereby certify that I have read this dissertation prepared under my direction and recommend that it may be accepted as fulfilling the dissertation requirement.

Date: 17.06.2016

Place: KHARGHAR, NAVI MUMBAI

TR Teni

Guide: Dr. Tanuja R. Teni.

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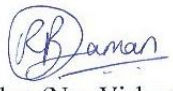

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DECLARATION

I hereby declare that the work presented in the thesis titled 'Molecular insights into p53 repressed anti-apoptotic proteins clusterin and survivin in human oral cancer' has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree/diploma at this or any other Institution or University

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- 'Expression of clusterin and its regulation by p63 in oral cancers' at the 37th All India Cell Biology Conference, 2013, Bangalore, India.
- 'Assessment of p53 family members and survivin isoforms in oral and cervical cancers' at AACR International Conference, 2011, Delhi, India.

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- 'Expression of survivin splice variants and their regulation by p63 in oral cancers' at 34th Convention of Indian Association for Cancer Research (IACR), 2013, Delhi, India.

Rupa Mishra (Nee Vishwanathraman)

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Rupa Mishra (Vishwanathraman)

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Synopsis

Homi Bhabha National Institute

Ph. D. PROGRAMME

SYNOPSIS OF Ph.D THESIS

1. Name of the Student: Rupa Mishra (Rupa Vishwanathraman)

2. Name of the Constituent Institution: TMC-ACTREC

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4. Title of the Thesis: Molecular Insights into p53 repressed anti-apoptotic proteins, Clusterin and Survivin in human oral cancer.

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1. Introduction

Oral cancers are the sixth most common cancers worldwide and among the top three cancers in India. Oral cancers account for over 30% of all cancer in India, with 60-80% of cases presenting with advanced disease [1]. A majority of oral cancers arise from premalignant lesions such as leukoplakias and submucous fibrosis. Early detection of the disease may help to increase survival and reduce the morbidity . Identification of the key molecules involved in the progression of oral cancers may facilitate early detection and better prognosis. p53, a tumor suppressor gene and a key regulator of apoptosis is mutated in various cancers, with 54% of oral squamous cell carcinomas (OSCC) showing alteration of p53 [2]. The p53 family of proteins also consists of p63 and p73. The three proteins exhibit similar structures; an acidic amino terminal transactivation domain, a DNA binding domain, and a carboxy terminal oligomerization domain. p63 and p73 are mainly involved in development while p53 is a tumor suppressor gene involved in cellular response to DNA damage. The transactivating isoforms of p63 and p73 show properties similar to p53 and are capable of transactivating p53 targets [3]. Recent studies also suggest gain of function in particular p53 mutants, affecting the regulation of downstream targets by p63 and p73 [4].

We have previously demonstrated overexpression of p53 and p16 tumor suppressor gene transcripts and nuclear accumulation of the proteins in cancer cell lines. Analysis of the molecules involved in the apoptotic pathway revealed aberrant expression of members of the Bcl2 family, survivin and clusterin in oral cancer cell lines and tissues.

Survivin, the smallest member of the inhibitor of apoptosis family of proteins, has dual functions in apoptosis and cell cycle regulation. The N-terminal BIR domain of survivin is critical for its antiapoptotic function and functions by binding to and inhibiting caspases. The long C-terminal

α-helix coiled region interacts with tubulin structures during mitosis and facilitates cell division [5]. Survivin overexpression is demonstrated in most of the cancers with undetectable expression in normal tissues [6]. The differential expression of survivin in cancers as compared to normal differentiated tissues has made it a promising therapeutic target. Overexpression of survivin has been associated with aggressive phenotype and poor prognosis [7]. Currently, six isoforms of survivin have been characterized, with three isoforms showing anti-apoptotic functions while two are implicated to be pro-apoptotic. The interaction of the various isoforms of survivin has been implicated to be essential in the role of survivin in tumor progression [8]. Studies in colorectal cancers have shown that the ratio of the isoforms to survivin wt may be an important marker for predicting disease outcomes [9]

Our initial studies had revealed a loss of the clusterin transcript in tumor tissues as compared to the normal tissues. Clusterin is a heterodimeric glycoprotein known to be involved in various physiological processes [10]. Two isoforms of clusterin have been described; the secretory form is antiapoptotic and is known to inhibit activated bax. The nuclear form on the other hand is proapoptotic and inhibits cell proliferation. However in 2012 the cds of the nuclear isoform was retracted from NCBI database. Initially identified as a chaperone protein clusterin is known to be involved in a number of processes such as lipid transportation, complement inhibition, tissue remodeling, stabilization of proteins, promotion and inhibition of apoptosis [10]. sCLU is known to be p53 repressed and is also known to be induced post radiation in the absence of p53. Repression of sCLU by p53 post radiation may thus be important for appropriate response to radiation [11]. In colorectal carcinomas overexpression of secretory clusterin (sCLU) was inversely correlated with tumor apoptotic index suggesting that overexpression of sCLU may be involved in tumorigenesis [12].

2. Rationale

Both survivin and clusterin are under the negative regulation of p53 [11, 13]. We have previously demonstrated overexpression of survivin and the loss of clusterin transcript in tumor tissues. Previous data has also shown that in oral cancers p53 is generally inactivated or mutated. In vitro studies have shown that mutant p53 is capable of binding differentially to the various isoforms of p63 and p73 and thus abrogating their function [14]. Recent studies also suggest that the presence of specific forms of mutant p53 may affect the function of p63 or p73 [4]. Also, since p63 is capable of transactivating p53 downstream targets, we hypothesized that p63 may regulate survivin and/or clusterin.

3. Objectives

1. To profile p63,p73, survivin and clusterin isoforms in oral cancer cell lines and/or tissues and assess their association if any with the patients clinicopathological parameters.
2. To examine whether p63 regulates survivin and/or clusterin expression in oral cancer cells in the background of a mutant or no p53 protein using siRNA strategies and luciferase assays.
3. To determine the role of p63 protein in oral cancer cells using different phenotypic assays, microarray analysis and radiation treatment post p63 knockdown in oral cancer cells.

4. Work Done and Results

4.1 To profile p63,p73, survivin and clusterin isoforms in oral cancer cell lines and/or tissues and assess their association if any with the patients clinicopathological parameters.

4.1.1 Expression of the p53 family proteins oral cell lines and tissues.

The expression of p53 family proteins p53, p63 and p73 was determined in four oral cancer cell lines (AW8507, AW13516, SCC040 and SCC029B), a dysplastic cell line DOK (Dysplastic oral keratinocyte) and two immortalized cell lines FBM (Fetal Buccal Mucosa) and HaCaT by reverse transcriptase PCR, real time PCR and western blotting. The transcript analysis of the isoforms of p63 and p73 showed a relative higher expression of the ΔNp63 form as compared to the TAp63 form, while TAp73 isoform expression was predominant as compared to the ΔNp73 forms in oral cell lines as well as oral cancer tissues. Protein analysis showed expression of p63 protein in four of the seven oral cell lines while p73 was detected in five of the seven oral cell lines.

4.1.2 Expression of clusterin in oral cell lines and tissues.

Real time PCR analysis of clusterin transcript in oral cell lines showed clusterin expression in all the cell lines studied. Clusterin protein was also detected in all the four oral cancer cell lines studied. On the other hand the analysis of secretory clusterin in tumor tissues showed a significant downregulation of clusterin in tumor tissues as compared to the normal tissues.

4.1.3 Expression of survivin isoforms in oral cell lines and tissues

The expression of survivin isoforms was determined by real time PCR in four oral cancer cell lines and a dysplastic cell line. The expression of the wild type isoform was found to be predominant in all the cell lines as compared to normal tissues from healthy individuals. To determine the role of the isoforms in tumorigenesis, real time PCR analysis of the six survivin isoforms was performed in 75 paired tumor and adjacent normal tissues and 12 normal tissues from healthy individuals. All the isoforms of survivin were found to be significantly overexpressed in tumor tissues as compared to normal tissues from healthy individuals. Survivin wt was found to be overexpressed in more than 80% of the oral tumor tissues in the study. The

next predominant isoform in tissues was survivin ΔEx3, overexpressed in 64% cases. Survivin 3B was found to be associated with poorly differentiated tumors, while the antiapoptotic forms of survivin were associated with TNM stage, with 64% group IV tumors showing higher expression of at least one the antiapoptotic forms of survivin.

Since the antiapoptotic forms together showed an association and since survivin is known to function as a homodimer, the ability of the isoforms to form heterodimers was studied by docking the isoforms with survivin wt. Docking analysis showed survivin 2B and survivin 3B heterodimerization with survivin wt via the L98 residue which is the core residue in the survivin wt homodimerization.

4.2 To examine whether p63 regulates survivin and/or clusterin expression in oral cancer cells in the background of a mutant or no p53 protein using siRNA strategies and luciferase assays.

Our studies showed expression of p63 protein in the two tongue cancer cell lines AW8507 and SCC040. Karyotyping analysis has shown that SCC040 has wild type p53, but the p53 protein is inactive. On the other hand previous studies from the lab have shown an R273H (DNA Binding domain) mutation in p53 in AW8507. Sequence analysis of the p63 gene in SCC040 and AW8507 did not reveal mutations in the gene for both cell lines.

4.2.1 Clusterin and Survivin expression post p63 knockdown (SCC040 and AW8507)

A pan p63 knockdown was achieved in SCC040 and AW8507 using siRNA. Post siRNA transfection, the levels of p63 were determined in both the cell lines by western blotting.

Clusterin and survivin expression was determined post p63 knockdown in both the cell lines. Clusterin was found to be upregulated post p63 knockdown in SCC040 and AW8507. On the

other hand, survivin wt was found to be significantly upregulated post knockdown in SCC040 but significantly downregulated in AW8507.

4.2.2 Luciferase assays of clusterin and survivin promoter post p63 knockdown

In silico analysis of the survivin and clusterin promoter did not reveal p63 binding sequences. However since there are reports of p63 transactivating p53 downstream targets through p53 responses elements, luciferase assays were conducted to determine difference in survivin promoter activity post knockdown. The minimal promoter of clusterin and survivin was cloned in pGL3 luciferase vector. The activity of the promoter was determined by luciferase assay post p63 knockdown in SCC040 and AW8507. Clusterin promoter activity showed a marginal increase post p63 knockdown as compared to the control siRNA in both SCC040 and AW8507. Survivin minimal promoter activity did not reflect the transcript expression levels in the two cell lines, however in SCC040 the extended promoter of survivin also showed upregulation post knockdown as seen with survivin wt transcript levels. The co-immunoprecipitation studies also determined that mutant p53 binds to p63 in AW8507.

4.3 To determine the role of p63 protein in oral cancer cells using different phenotypic assays, microarray analysis and radiation treatment post p63 knockdown in oral cancer cells

4.3.1 Microarray analysis post p63 knockdown in SCC040 and AW8507

Microarray analysis post p63 knockdown was conducted in SCC040 and AW8507. In SCC040 2939 genes were found to be differentially expressed post knockdown. Of the differentially expressed genes, 1556 genes were found to show more than 1.5 fold difference, with 781 genes being upregulated while 775 genes were downregulated post p63 knockdown. In AW8507 (which shows presence of mutant p53) 4304 genes were differentially regulated, of which 341

genes showed a higher than 1.5 fold change. Of the 341 genes, 324 genes were found to be downregulated while 17 genes were found to be upregulated. Pathway analysis of the differentially expressed genes in the two cell lines showed genes involved in TGFβ and EGFR signaling pathways, cell adhesion, cell-cell communication and angiogenesis pathways to be altered post p63 knockdown. Inhibin Beta A expression was determined by real time PCR in SCC040 and AW8507 and was found to be downregulated post knockdown as compared to vector control.

4.3.2 Effect of ΔNp63 knockdown on SCC040 and AW8507

To determine the phenotypic effect of p63 knockdown, ΔNp63 knockdown using shRNA was conducted in SCC040 and AW8507. Post ΔNp63 knockdown the number of soft agar colonies was significantly higher in AW8507 as compared to vector control, though no difference in soft agar colony formation was observed in SCC040. However a significant increase in invasion was observed post ΔNp63 knockdown in SCC040. On the other hand in AW8507 a decrease in migration and proliferation was observed in AW8507 post ΔNp63 knockdown as compared to vector control. Thus ΔNp63 affected the invasive capability of SCC040 cells while in AW8507 it resulted in a decrease in migration and proliferation.

4.3.3 To examine the time course expression of survivin and clusterin isoforms post IR in background of p63 knockdown.

Survivin and clusterin are both known to be induced post radiation. Thus to identify the specific isoforms differentially regulated post radiation the expression of survivin isoforms post p63 knockdown at different intervals post radiation was done by real time PCR in AW8507 and SCC040. As compared to the expression post knockdown or post radiation the expression of all isoforms of survivin was found to be downregulated up to 24hours post knockdown and radiation

in AW8507. In SCC040 an induction was observed post radiation, though the same was not seen in combination with the knockdown.

5. SUMMARY AND CONCLUSIONS:

The aim of the present study was to determine the isoforms of survivin and clusterin that may play a role in oral carcinogenesis and their regulation by the p53 family protein, p63. The expression of survivin and clusterin was determined in four oral cancer cell lines and in oral tumor and adjacent normal tissues. The studies suggest that besides survivin wt, the minor isoforms of survivin, survivin ΔEx3 and survivin 2B are also overexpressed in oral cancer tissues. Also the overexpression of the antiapoptotic isoforms of survivin was associated with advanced TNM stage, and survivin 3B was found to be associated with poorly differentiated tumors. The study thus suggests that the minor isoforms of survivin may also play a role in oral tumorigenesis. Secretory clusterin levels on the other hand showed significant downregulation in tumors as compared to adjacent normals. We also determined that ΔNp63 and TAp73 are predominantly expressed in oral cancer tissues and cell lines as compared to TAp63 and ΔNp73.

We further examined the effect of p63 knockdown on the expression of survivin and clusterin in the two tongue cancer cell lines, SCC040 and AW8507. Real time PCR

analysis showed a significant upregulation of survivin wt in SCC040 but significant downregulation in AW8507 which was further verified by luciferase assays. Co-immunoprecipitation studies in AW8507 also showed that mutant p53 in AW8507 may be capable of binding to p63 in the cell line. The results thus suggest that p63 may be activating survivin expression in the presence of mutant p53 (as observed in AW8507) but in the absence of p53, p63 may be repressing survivin expression (as observed in SCC040).

Further analysis on the effect of p63 knockdown on the two cell lines by microarray analysis, showed various pathways affected in the two cell lines. Genes involved in NOTCH signaling, TGFβ pathway and pathways associated with angiogenesis and cell-cell adhesion were affected post p63 knockdown. Phenotypic assays to determine the effect of ΔNp63 knockdown in the two cell lines showed an increase in invasive capability in SCC040 but a decrease in migration and proliferation in AW8507 post knockdown as compared to vector control. Survivin isoform expression was found to be downregulated in both SCC040 and AW8507 post radiation and post knockdown, though an induction is seen post radiation alone. Our studies thus suggest, that p63 may be capable of regulating survivin expression in oral cancers. Our studies also demonstrate that the function of p63 in oral cancers may be affected by the presence of mutant p53.

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Doctoral Committee:

S. No.	Name	Designation	Signature	Date
1	Dr. Sorab Dalal	Chairperson		22.07.15
2	Dr. Tanuja R. Teni	Convener		22.07.15
3	Dr. Prasanna Venkatraman	Member		22.07.15
4	Dr. Milind Vaidya	Member		22.07.15
5.	Dr. Shobhana Sharma	Invitee		22.07.15

Forwarded Through:

Dr. S.V. Chiplunkar
Director, ACTREC
Chairperson,
Academic & training Program, ACTREC

Dr. S. V. Chiplunkar
Director
Advanced Centre for Treatment, Research & Education in Cancer (ACTREC)
Tata Memorial Centre
Kharghar, Navi Mumbai 410210.

Prof. K. Sharma
Director, Academics
T.M.C.

Prof. K.S. Sharma
DIRECTOR - ACADEMICS, TMC
Mumbai - 400 012

Abbreviations

APS	Ammonium persulfate
BIR	Baculovirus Inhibitor of Apoptosis protein
BME	β-Mercapto Ethanol
BSA	Bovine Serum Albumin
BPB	Bromophenol blue
ChIP	Chromatin Immunoprecipitation
D0	Dose at which 37% population survives
DAPI	4, 6-diamidino-2-phenylindole
DBD	DNA Binding Domain
DEPC	Diethyl pyro carbonate
DMSO	Dimethyl sulfoxide
dNTP's	Deoxyribonucleotide mix
EDTA	Ethylene glycol tetra acetic acid
EtBr	Ethidium bromide
FBS	Fetal Bovine Serum
g	gram
HNSCC	Head and Neck Squamous Cell Carcinoma
IAP	Inhibitor of Apoptosis
kDa	kilo Dalton
min	Minute
ml	Milli litre
µl	micro litre
mM	milli Molar
M	Molar
nCLU	nuclear Clusterin
OD	Optical Density
OLD	Oligomerization Domain
OSCC	Oral Squamous cell carcinoma
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative Real Time PCR
sq PCR	semi quantitative Reverse Transcriptase PCR
RT	Room Temperature
SDS	Sodium dodecyl sulfate
siRNA	Small interfering RNA
TAD	Transactivation Domain
TBS	Tris buffered saline
TBST	Tris buffered saline with tween-20
TEMED	N-Tetramethylene diamine
PFA	Paraformaldehyde
wt	wild type

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1 . Introduction

In the 21st century, the burden of non-communicable diseases such as cancers is increasing not only in developed countries but in developing countries as well. The less developed countries account for 57% of cancer cases and 65% deaths due to cancers [1]. In Indian men, the most common cancers are reported to be tobacco related whereas in women, breast and cervical cancers account for majority of the cancer morbidity [2]. Cancers of the breast, uterine cervix and lip and oral cavity account for the most widely reported cancers contributing to about 34% of the total cancer burden in India [3]. Oral cancers are among the top three cancers in India, with the ninth highest incidence for oral cancers reported in the country. Squamous cell carcinomas are the most common malignancy in oral cavity and account for over 90% of oral cancer cases [4]. A majority of oral cancers arise from premalignant lesions such as leukoplakias and submucous fibrosis with transformation rates reported to be between 6.6% -36.4 %. Oral cancers are treated either by surgery or radiotherapy or chemotherapy. In advanced stages of the disease a combination of surgery and radio or chemotherapy may be utilized [5-8] (updated Sept 25, 2015). Despite the ease of accessibility, most cases of oral cancers are detected in the advanced stages of the disease as a result survival rates have remained low in the past decade [9]. Identification of the key molecules involved in the progression of oral cancers may facilitate early detection and better prognosis. Studies on the molecular changes associated with oral cancers have identified alterations in tumor suppressor genes such as p53, p16 and deletion of specific regions in chromosome 8 and 9 to be associated with oral cancer progression [10-12]. p53, a key regulator of apoptosis, is known to be mutated in several cancers. Saranath et. al. have shown that 46% of oral squamous cell carcinomas (OSCC) exhibited alterations in p53 [13]. Studies also suggest that the p53 family members p63 and p73 are rarely mutated in cancers and are capable of regulating some of the p53 downstream targets such as p21 [14, 15]. These

three proteins exhibit similar structures with greater than 60% homology in the DNA binding domain although p63 and p73 are predominantly involved in developmental processes, while p53, a tumor suppressor gene is involved in cellular response to DNA damage. Although p63 and p73 are reported to be capable of regulating p53 targets by binding to p53 response elements in their promoters, there is also evidence to suggest that a gain of function in specific p53 mutants affects the regulation of certain downstream targets by p63 and p73 proteins [16].

Previous studies from our lab have demonstrated overexpression of tumor suppressor genes, p53 and p16 transcripts and nuclear accumulation of the proteins in oral cancer cell lines. Earlier studies from the lab to assess the apoptotic molecules altered in oral cancers revealed alterations in the expression of some Bcl-2 family members, survivin and clusterin and several other genes in oral cancer cell lines and tissues. Intriguingly, both survivin and clusterin have been reported to be repressed by wild type (wt) p53 protein [17, 18].

Survivin is an inhibitor of apoptosis protein with dual functions in apoptosis and cell cycle regulation. The N-terminal BIR domain of survivin is critical for its antiapoptotic function while the C-terminal α-helix coiled region interacts with tubulin structures during mitosis and facilitates cell division [19]. Survivin is suggested to be a promising therapeutic target due to its differential expression in cancers as compared to normal tissues. Overexpression of survivin is demonstrated in most of the cancers as compared to the normal tissues. Survivin overexpression is associated with an aggressive phenotype and poor prognosis [20] however is observed to be low or undetectable in normal tissues [21]. Since survivin is differentially expressed in cancers various small molecule inhibitors targeting survivin have been tested in clinical trials. Currently,

six isoforms of survivin have been characterized, with three isoforms showing anti-apoptotic functions while two are implicated to be pro-apoptotic. The interaction of the various isoforms of survivin has been implicated to be essential in the role of survivin in tumor progression [22, 23].

The second p53 repressed anti-apoptotic protein identified in our laboratory to be altered in oral cancers was the chaperone protein clusterin also known as apolipoprotein J. Clusterin is a heterodimeric glycoprotein known to be involved in various physiological processes [24]. Two isoforms of clusterin are currently described, the secretory (sCLU) and the nuclear form (nCLU). The secretory form is antiapoptotic and is known to inhibit activated bax while the nuclear form is implied to be proapoptotic and inhibits cell proliferation. Initially identified as a chaperone protein clusterin is known to be involved in a number of processes such as lipid transportation, complement inhibition, tissue remodeling, stabilization of proteins, promotion and inhibition of apoptosis [24].

Since clusterin and survivin both are p53 repressed, the p53 status of the cell lines used in the earlier studies was determined. The analysis revealed the presence of a hot spot mutation in p53 (R273H) in the oral cancer cell lines. Thus, to understand the interplay the p53 family members and the p53 repressed anti-apoptotic proteins survivin and clusterin, the current study determined the expression these molecules in oral cancer cell lines and tissues. Two cell lines with differing p53 background were chosen on the basis of the expression analysis to delineate the role of p63 in regulating survivin and clusterin in oral cancers. The current study also determined the phenotypic effects of p63 in oral cancer cell lines with differing p53 background and the

potential pathways regulated by p63. Our studies thus identifies the isoforms of p63, survivin and clusterin that may play a role in oral tumorigenesis. Our studies also determine the effect of p53 mutant and p63 in oral cancers. The results of this study would thus not only help determine the role of p63 in oral cancers but also demonstrate that the presence of a mutant p53 might affect regulation of downstream targets by p63.

2 . Review of Literature

2.1 Oral Cancers

2.1.1 Epidemiology of Oral Cancers

Developing countries like India are currently facing a dual burden of communicable and non-communicable diseases. Among the non-communicable diseases, it is predicted that Cancer burden in India will nearly double in the next 20 years [2]. GLOBOCAN estimates show that in 2012, 14.1 million new cancer cases and 8.2 million cancer deaths were reported worldwide [25]. Although cancer incidence rates are high in developed regions, epidemiological surveys reveal that the burden is shifting towards the less developed regions [25]. Oral and pharyngeal cancers are the sixth most common cancers internationally, with 77% cancer deaths attributed to lip and oral cavity being reported in the less developed regions [25]. In India, oral cancer is among the top three cancers with 22.9% cancer deaths being attributed to oral cancer [26]. There are twice as many deaths from oral cancer as lung cancer [26]. Various factors such as delayed diagnosis, inadequate or incorrect treatment, inability to access appropriate therapies are attributed to be the chief factors for poor survival in India [2].

2.1.2 Oral Premalignant Lesions

Oral cancers are generally preceded by precancerous lesions which if detected in the early stages may help reduce oral cancer mortality [3]. Precancerous lesions are defined as morphologically altered tissue with a higher probability of oral cancer occurrence than in normal tissues [27]. Leukoplakia, erythroplakia, palatal lesion of reverse cigar smoking, oral lichen planus, oral submucous fibrosis, discoid lupus erythematosus and hereditary disorders such as dyskeratosis congenita and epidermolysis bullosa are identified as premalignant lesions by World Health Organization [28]. Although leukoplakia and erythroplakia are more common in the western

regions, in India leukoplakia and oral submucous fibrosis are the most common premalignant lesions.

Leukoplakia: Leukoplakia is defined as a keratotic white patch or plaque that cannot be scraped off and cannot be characterized clinically or pathologically as any other disease [27]. Leukoplakia is reported to have a worldwide prevalence of 2% [29] and an annual malignant transformation rate of around 1% [30]. However in India malignant transformation of leukoplakia ranges from 0.13% to 2.2% per year [27, 31, 32].

Erythroplakia: Erythroplakia is defined as a fiery red path or bright red velvety plaques that cannot be characterized as any other definable disease [27]. Erythroplakia has a higher rate of transformation among all the premalignant lesions [9] with transformation rates reported to be between 20%-60% [33]. Although less prevalent as compared to leukoplakia a majority of erythroplakia show some amount of dysplasia epithelial dysplasia. One study by Nair et. al. 2012, showed that 51% cases showed invasive squamous cell carcinoma while 40% showed severe epithelial dysplasia or carcinoma in situ [34].

Oral Submucous Fibrosis: Oral submucous fibrosis (OSMF) is characterized by inflammation and progressive fibrosis of submucosal tissues of upper digestive tract involving the oral cavity, oropharynx and upper third of the oesophagus. Clinically OSMF may present with marked rigidity of the oral mucosa, oropharynx and trismus [9, 27]. OSMF is associated with the use of areca nut and betel quid and is more common in India [35]. In India, OSMF is reported to be five times more common in men than women [10]. OSMF is reported to have a malignant transformation rate between 3-6% [36].

Oral Lichen Planus (OLP): Oral Lichen Planus is an autoimmunodisorder characterized by chronic inflammation of the oral mucosa. Clinically OLP is seen to present as white striations,

white papules or white plaques as well as erythema, erosions or blisters [9]. OLP generally involves the buccal mucosa, tongue and gingiva and although characterized as a premalignant lesion, there is currently no consensus on the conversion of OLP to undergo malignant transformation [27]

Palatal lesions in reverse cigar smokers and Nicotine stomatatis: These two conditions are associated with reverse smoking or pipe smoking. Reverse smoking wherein the lit end of the cigarette is placed inside the mouth is common in south east Asian countries and is associated with a significant risk of malignant transformation [37, 38].

Hereditary Disorders: Dyskeratosis congenital and epidermolysis bullosa are rare hereditary conditions with an increased risk of oral malignancy. Increased malignancy including oral cancers are also observed with two other hereditary conditions i.e. Xeroderma pigmentosum and Fanconi's anemia [27]

Although the gold standard for diagnosis of oral cancer is still by histological assessment of tissue biopsy other tools for early detection of oral cancers include use of tolonium chloride or toluidine blue dye, oral CDx brush biopsy kits and certain optical imaging systems [9, 27]

2.1.3 Cancer of the Oral Cavity

Oral cancers include the cancer of the dorsal, ventral and border of the anterior two thirds of tongue, the upper and lower lip mucosa, gingiva, floor of mouth, hard palate, cheek mucosa, vestibule and retromolar area.

The premalignant lesions or potentially malignant disorders (as renamed by WHO) are capable of progressing to cancer of the oral cavity [39] . The oral cavity is lined by flat squamous epithelial cells and cancers originating from these cells are termed as squamous cell carcinoma.

Squamous cell carcinomas account for more than 90% of oral cancers while cancers originating from other cell types account for around 10% of oral cancers [40]. These include malignant melanoma, salivary gland tumors and sarcomas of the soft tissues and jaw bones. Non-Hodgkin's lymphomas also show presence of primary tumors in the oral cavity.

The diagnosis of oral cancers is accompanied with clinical staging of the tumor as well. Staging of the tumors is based on primary tumor characteristics and assessment of metastases. The tumor characteristics considered during staging are tumor size and invasion of deep structures for primary tumors while advanced disease stages are defined by the invasion of bone and sinus or skin and lymphatic spread into the neck [41].

The following characteristics are defined for the TNM staging of cancer of the oral cavity

Table 2-1: TNM staging of oral cancer

T	**Primary Tumor**
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ
T1	Tumor 2 cm or less in greatest dimension
T2	Tumor more than 2 cm but not more than 4 cm in greatest dimension
T3	Tumor more than 4 cm in greatest dimension
T4a (lip)	Tumor invades through cortical bone, inferior alveolar nerve, floor of mouth, or skin (chin or nose)
T4a (oral cavity)	Tumor invades through cortical bone, into deep/extrinsic muscle of tongue (genioglossus, hyoglossus, palatoglossus, and styloglossus), maxillary sinus, or skin of face
T4b (lip and oral cavity)	Tumor invades masticator space, pterygoid plates, or skull base; or encases internal carotid artery
N	**Regional Lymph Nodes**
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest Dimension
N2	Metastasis as specified in N2a, 2b, 2c below
N2a	Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension
N2b	Metastasis in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension
N2c	Metastasis in bilateral or contralateral lymph nodes, none more than 6 cm

	in greatest dimension
N3	Metastasis in a lymph node more than 6 cm in greatest dimension
M	**Distant Metastasis**
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Oral cancers can further be categorized into different stages on the basis of the TNM classification of the tumor. The classification of oral cancers into stages is presented in Table 2-2.

Table 2-2: Staging of oral cancers based on TNM staging

Stage	**T**	**N**	**M**
0	Tis	N0	M0
I	T1	N0	M0
II	T2	N0	M0
III	T3	N0	M0
	T1	N1	M0
	T2	N1	M0
	T3	N1	M0
IVA	T4a	N0	M0
	T4a	N1	M0
	T1	N2	M0
	T2	N2	M0
	T3	N2	M0
	T4a	N2	M0
IVB	Any T	N3	M0
	T4b	Any N	M0
IVC	Any T	Any N	M1

Besides TNM staging tumor grading of the cancer is done mainly by Broder Classification wherein based on the level of differentiation the tumors are classified as well differentiated, moderately differentiated or poorly differentiated [5, 7].

2.1.4 Risk factors

The high prevalence of oral submucous fibrosis and oral cancers in the Indian sub-continent is attributed predominantly to the use of smokeless forms of tobacco in conjunction with alcohol consumption. Tobacco with/without alcohol is the primary risk factor contributing to oral cancers while biological factors such as viruses are recently also reported to be associated with oral carcinogenesis.

Tobacco

Tobacco consumption is widely reported to be the most important etiological factor contributing to oral cancer, with 25% of oral cancers worldwide attributed to tobacco usage [42-45]. Smoking of tobacco results in the formation of reactive carcinogenic intermediates when pro-carcinogens such as benzo-pyrene are metabolized by oxidizing enzymes. However in regions with high prevalence in consumption of smokeless forms of tobacco, betel quid consumption is associated with more than 50% of oral cancer cases [42]. In the Indian subcontinent smokeless forms of tobacco that are mixtures of lime, areca nut and powdered tobacco are more common . Betel quid chewing, containing areca nut and tobacco is associated with higher risk of oral cancer [44] and is also reported to be an important etiological factor in oral submucous fibrosis [46-48].

Alcohol Consumption

Although alcohol is also reported to be a major etiological factor contributing to oral cancer, there exists a certain level of ambiguity on whether alcohol is independently a risk factor for oral cancer or if its action is in synergy with smoking. Worldwide 7-19% of oral cancer cases are attributed to alcohol consumption. Alcohol is hypothesized to act in synergy with smoking, by

acting as a solvent for tobacco carcinogens and increasing the availability of these carcinogens in the cells [43, 48].

Diet

Although the association of micronutrient deficiency and oral cancers is not yet well established some studies have reported a decrease intake of fruits and vegetables and a concomitant decrease in vitamin levels to be associated with oral cancers [43, 45]

Viruses (HPV, HSV)

Role of viruses in the etiology of oral cancers is recently being implicated. HPV is reported to be a risk factor and was reported to be present in 23.5% of oral cancer cases although the HPV positivity was found to be higher in the case of oropharyngeal cancers [49-52]. Similarly HSV infection is also found predominantly in oropharyngeal cancers [4] although an association with oral cancers has also been suggested [43]. HPV E6/E7 proteins target tumor suppressor proteins p53 and Rb. HPV E6 proteins bind to and degrade the tumor suppressor p53 protein while HPV E7 binds to tumor suppressor Rb protein leading to the release of E2F transcription factor.

Infections

Although weakly associated, infectious diseases syphilis and candida are considered to be a contributing factor in some oral cancer cases [43]

2.1.5 Treatment modalities of Oral Cancers

Generally early stage cancers of the oral cavity have a better prognosis and are curable by surgery or radiotherapy while prognosis is poorer for cancers detected in the advanced stages [53, 54]. Treatment modalities for oral cancer depend on the stage of the cancer with early stage cancers being treated with surgery or radiotherapy. Cancers in the advanced stages may require a combination of surgery as well as radiation. Radiation therapy may be provided either via external beam radiation or by interstitial implantation of the radiation source. Surgery as an option is generally considered for early stage tumors, smaller size tumors and if the tumor has not invaded other local structures. Radiation therapy is for early stage tumors and may be considered for cosmetic reasons since it leaves the muscle structures intact.

2.1.6 Molecular Pathology of Oral Cancers

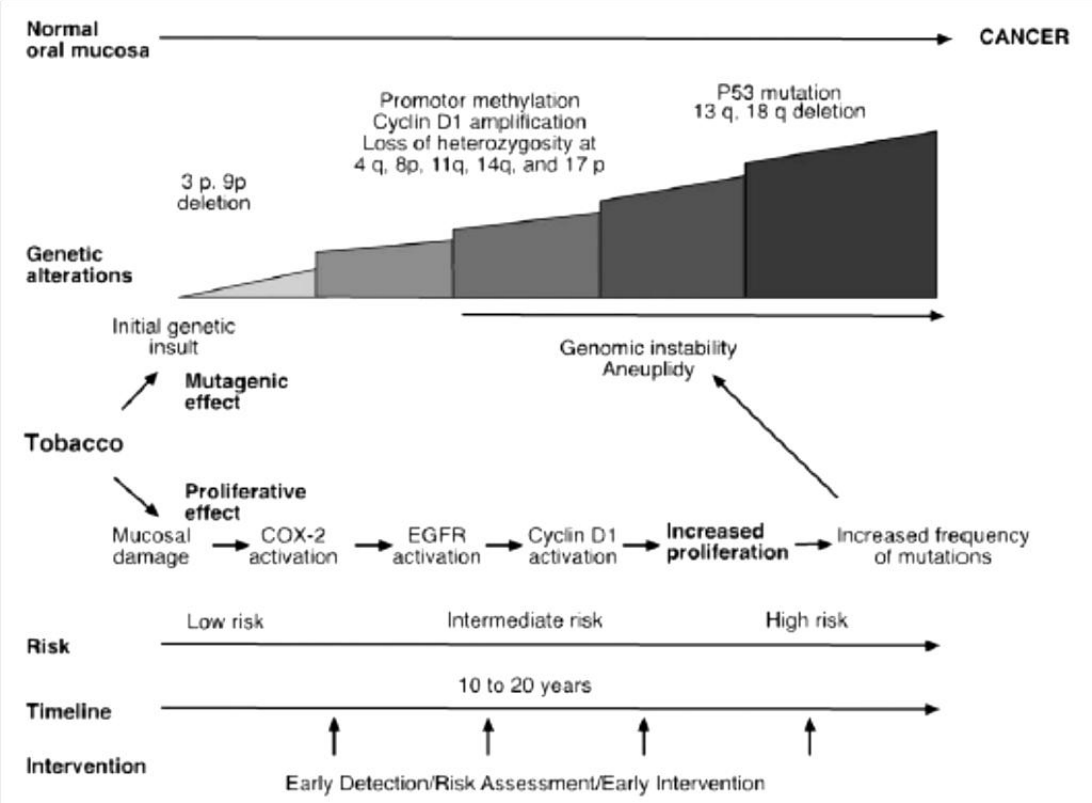
Although the precancerous or potentially malignant disorders are prone to malignant transformation, histological examination (the gold standard for diagnosis) can indicate the presence of dysplasia but not predict occurrence of malignant changes [9]. This progression of premalignant conditions to oral tumorigenesis is via a series of molecular changes that may ultimately lead to malignant transformation. Various studies have determined the molecular changes in premalignant lesions and squamous cell carcinomas to identify the tumor suppressor genes or oncogenes that may lead to oral tumorigenesis.

The genetic alterations due to chronic exposure to carcinogens such as tobacco underlie the progression of potentially malignant disorders to oral cancers. Molecular alterations in oral cancers include activation of oncogenes, inactivation of tumor suppressor gene, genomic instability such as loss of heterozygosity (LOH) and microsatellite instability (MSI) as well as

epigenetic alterations. Chromosome 9p21, 3p14, 2q21-24, 2q33-35, and 2q37 which harbor tumor suppressor genes such as p16, FHIT, CASP10, BARD1, CASP8, ING5 are frequently deleted in oral cancers [55-59]. The most common epigenetic alteration observed is DNA methylation. Alterations in the Ras gene family have been reported to be associated with OSCC and RASSF2 has been shown to be methylated in 26% of OSCC tumors [60]

Allelic losses are reported most commonly in the 9p21 region in OSCC which harbors the tumor suppressor gene p16 [61]. Also, the most common gene inactivation reported in OSCC is observed in p53, a tumor suppressor gene which is inactivated in almost half of OSCCs [4, 13]. The genetic and epigenetic alterations are observed to affect the expression of the tumor suppressor and oncogenes thus facilitating tumor progression. The inactivation of tumor suppressor genes may also be accompanied by the activation of oncogenes such as EGFR, ras, c-myc [62-64]. Figure 2-1 shows the genetic changes accumulated during the progression of oral cancers [65].

Figure 2-1: Genetic and epigenetic alterations leading to oral cancers

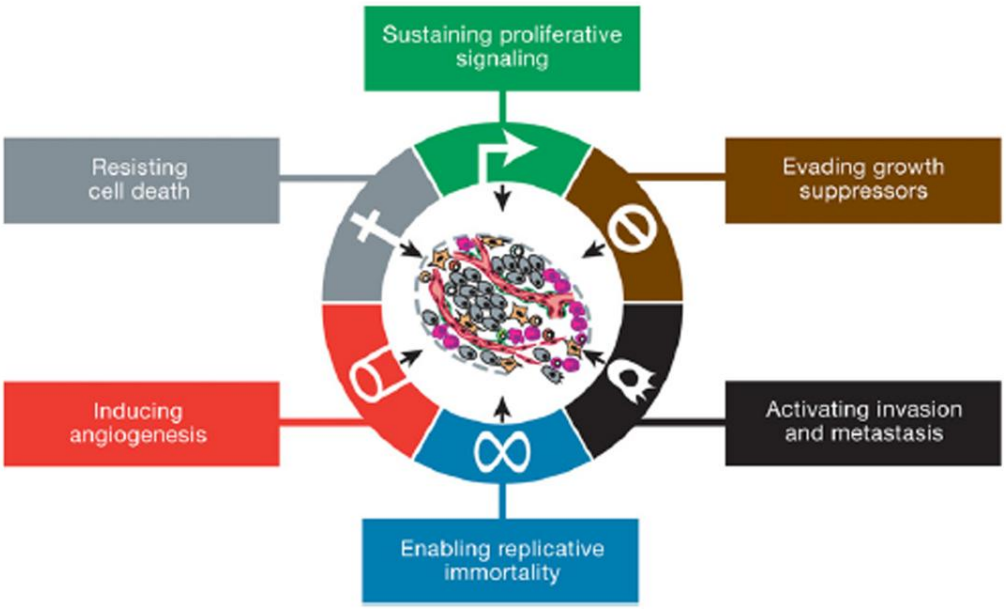


As stated previously, the most common genetic alteration observed in OSCCs is loss of function of the tumor suppressor gene p53 which may occur due to mutation, deletion or inactivation due to binding to viral or cellular proteins. p53 is a nodal protein involved in cell cycle control, DNA repair and apoptosis [4]. Besides inactivation of p53, overexpression of several anti-apoptotic proteins has also been reported. One of the hall marks of cancer is the ability of tumor cells to evade apoptosis, which is achieved predominantly by overexpressing various anti-apoptotic proteins. Numerous studies have determined the expression of the anti-apoptotic proteins in oral cancers and studies indicate that the expression of Bcl-2 family members, survivin, p16 and p21 are frequently in OSCC [4]

2.2 **Dysregulation of Apoptosis in Cancers**.

Six hallmark capabilities were described in 2000 by Hanahan and Weinberg that would enable the transformation of normal cells to neoplastic state and ultimately to tumor growth and metastasis [66]. Advances in oncology since then have identified some emerging hallmarks; deregulation of cellular energetic, evasion of immune destruction in addition to the basic capabilities described (Figure 2-2) [66]. Resisting cell death or programmed cell death, one of the key characteristics of carcinogenesis is the tightly regulated cell suicide process characterized by DNA fragmentation, shrinkage of cytoplasm and membrane changes ultimately resulting in cell death. [67]

Figure 2-2: Hallmarks of Cancer



2.2.1 **Intrinsic and Extrinsic pathways of Apoptosis.**

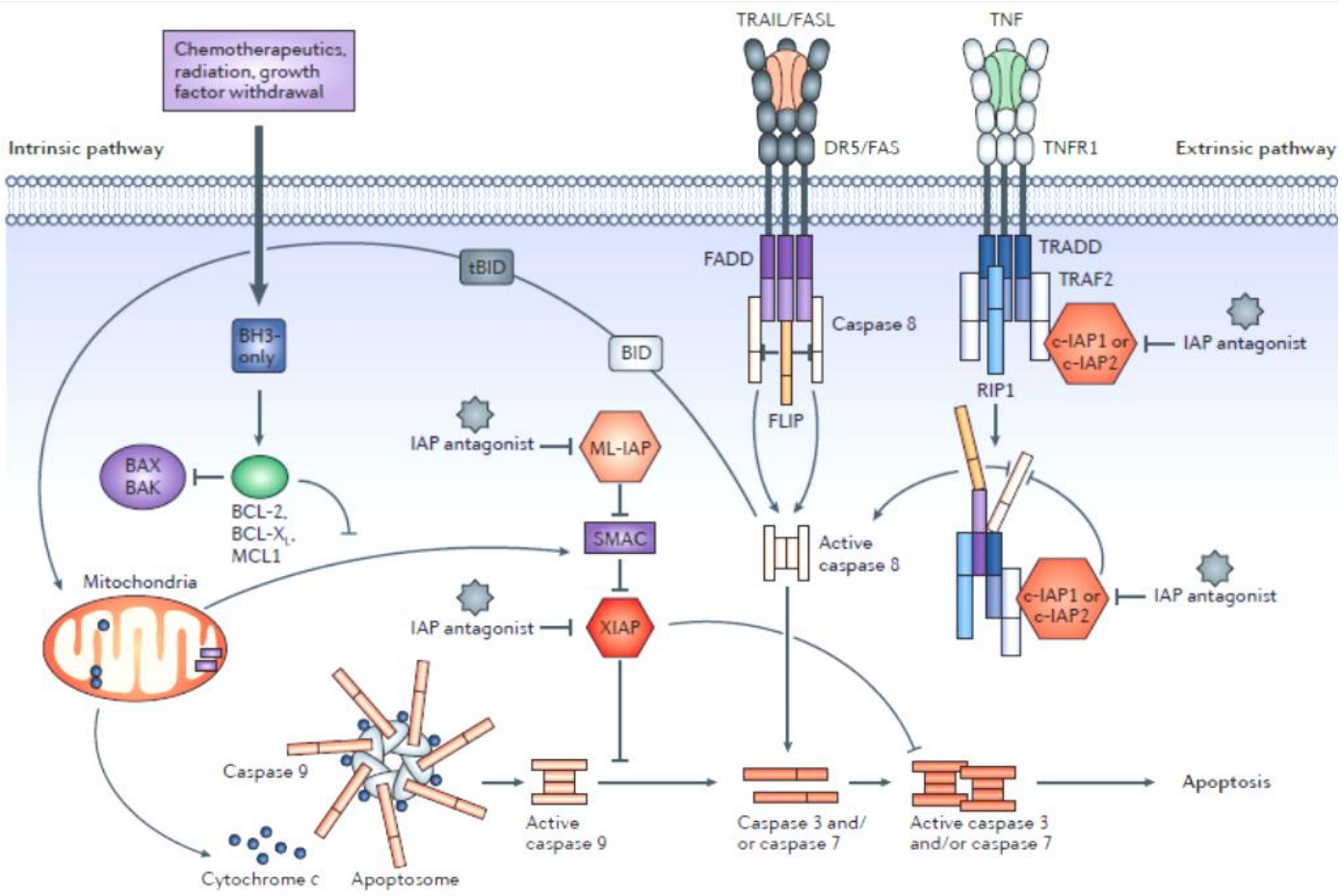
The extrinsic pathway or the death receptor pathway involves apoptosis initiation via transmembrane receptor mediated interactions. The activation of the death receptor proteins belonging to Tumor Necrosis Factor receptors (TNFR) superfamily such as TNFR, Fas (CD95,

APO-1) and TRAIL leads to the recruitment and downstream activation of initiator caspases Caspase 8 and Caspase 10 [68]. Activation of the receptors by binding of the trimeric ligand to the respective receptors i.e. FasL/FasR and TNF-α/TNFR1 recruits the death domain containing cytoplasmic adaptor proteins. For example binding of FasL to FasR results in recruitment of FADD which then associates with procaspase-8 leading to the formation of the death inducing signaling complex (DISC). The formation of DISC results in activation of procaspase-8 and further the activation of the effector caspases [69]

The intrinsic pathway or the mitochondrial pathway of apoptosis involves the loss of apoptotic suppression and activation of apoptosis, induced by stimuli such as radiation, toxins, hypoxia, hypothermia and viral infections. The outer mitochondrial membrane integrity is maintained through a delicate balance between pro-apoptotic and anti-apoptotic members of the Bcl-2 family proteins. The apoptotic stimuli result in the activation of the proapoptotic Bcl-2 proteins, BAX and BAK which cause changes in outer membrane permeabilisation resulting in loss of mitochondrial transmembrane potential and thus release of cytochrome c into the cytosol [70, 71]. Cytochrome c further activates Apaf-1 and pro-caspase 9 leading to the formation of apoptosome [69]. Additionally Smac/DABLO and HtrA2/Omi that are released into the cytosol promote apoptosis by inhibiting the inhibitor of apoptosis proteins [69].

Each of the pathways ultimately results in the activation of the effector caspase procaspase 3. Caspase 3 further cleaves key substrates thus resulting in the molecular and morphological changes associated with apoptosis [72]. The extrinsic and intrinsic pathways are depicted in Figure 2.3 [71] .

Figure 2-3: Mechanisms of programmed cell death

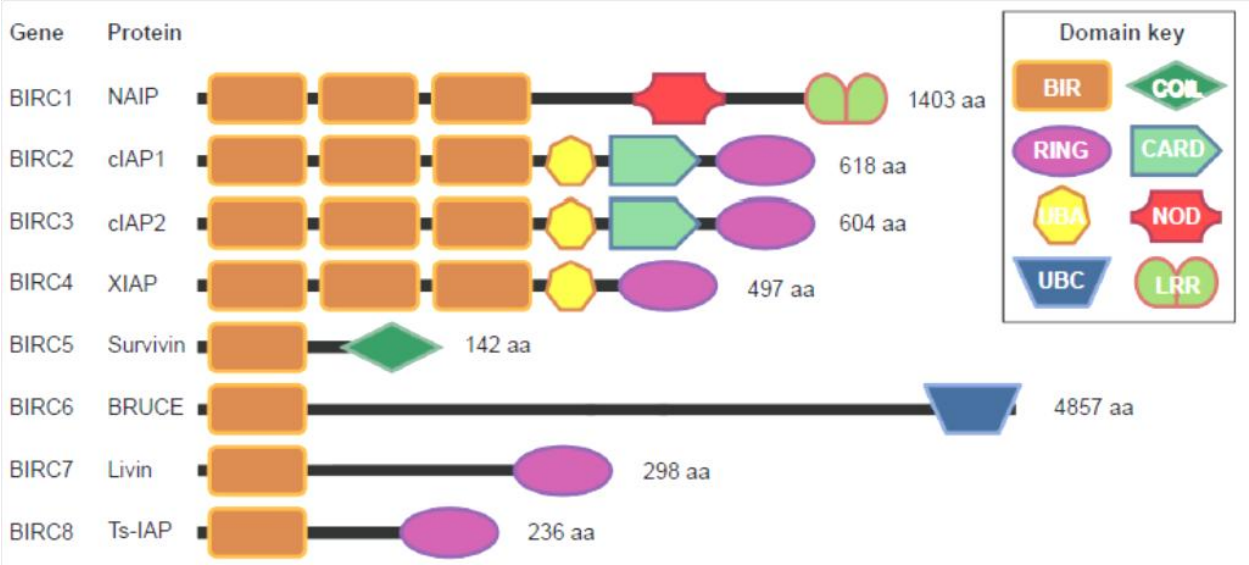


2.2.2 Inhibitor of Apoptosis Proteins

The inhibitor of apoptosis proteins or IAPs consist of eight proteins, namely NAIP (BIRC1), c-IAP1 (BIRC2), c-IAP2 (BIRC3), XIAP (BIRC4), survivin (BIRC5), Bruce (BIRC6), Livin/ML-IAP (BIRC7) and ILP-2/Ts-IAP (BIRC8) that are involved in regulation of cell death, cell cycle and inflammation [73]. The IAPs consist of at least one BIR (Baculovirus inhibitor of apoptosis protein) domain which is a 80 residue domain characterized by three Cystine and one Histidine residues that coordinate with a Zn^{2+} ion. Structurally the domain consists of 3 β strands and 4-5 α helices. Besides the BIR domain the IAPs may also possess Really Interesting New Gene

(RING) domain, Caspase Activation recruitment (CARD) domain or a Ubiquitin associated domain [74, 75]. Figure 2-4 is a cartoon depicting the structures of the eight IAP proteins in the humans [76].

Figure 2-4: Representation of the domain structure of the eight inhibitor of apoptosis proteins in humans



NAIP: Neuronal Apoptosis Inhibitory Protein (NAIP) consists of three BIR domain, one nucleotide-binding oligomerization domain (NOD) and a leucine-rich repeat (LRR) domain which are considered to be important for pathogen recognition [75]. NAIP expression has been reported in liver, placenta and central nervous system. Due to the presence of the NOD and LLR domains, NAIP may be classified in the NOD-like receptor (NLR) family which are involved in mediation of innate immune response. NAIP is capable of inhibiting apoptosis by binding to and inhibiting the effector caspases Caspase 3 and Caspase 7 and may also be capable of inhibiting procaspase 9 [77, 78].

cIAP1 and cIAP2: Cellular IAP proteins, c-IAP1 and c-IAP2 contain three repeats of the BIR domain, one CARD domain and one RING domain. Both are reported to be expressed in most

human tissues and inhibit caspase 3 and 7 [79, 80]. The proteins also show an E3 ligase activity via the RING domain and are known to ubiquitinate RIP1 and NIK. RIP1 when nonubiquitinated forms a complex with FADD and caspase 8 leading to apoptosis [81, 82] while polyubiquitination of NIK blocks the non canonical NF-κB activity [83, 84]. The function of the CARD domain however is not yet completely understood.

XIAP: X-linked IAP also known as hILP also has three BIR domains and a RING finger domain and binds and inhibits caspases 3 7 and 9 [80, 85]. The Ubiquitin associated domain (UBA) present in XIAP as well as cIAP1, cIAP2 and ILP2 binds to ubiquitinylated proteins and modulates cell signaling. XIAP is reported to interact with focal adhesion kinase (FAK) activating ERK-dependent endothelial cell migration or can promote invasion by regulating actin polymerization by binding to RhoGDI [86-88].

Survivin: Survivin is the smallest member of the IAP proteins and contains a single BIR domain. Although survivin expression is observed in fetal tissues, it is negligibly expressed in adult tissues but overexpressed in tumor tissues [19, 89]. When present in the cytoplasm survivin is anti-apoptotic while nuclear survivin is involved in cell cycle regulation. Although survivin is not shown to directly bind to Caspase 3 and 7, it is reported to inhibit Caspase 9 activity by binding to Caspase 9 with the cofactor HBXIP [90]. Survivin plays a pivotal role in mitosis as part of the chromosomal passenger complex with INCENP, Borealin and Aurora Kinase B [91]. Survivin and XIAP together are also reported to upregulate several ECM proteins [92]

Bruce/Apollon: BRUCE consists of an N-terminal BIR domain and a C-terminal ubiquitin-conjugating domain that characterizes the E2 ubiquitin conjugating enzymes [75]. Apollon is reported to catalyze the ubiquitinylation of SMAC/DIABLO, Caspase 9 and HtrA2 [93-95]. Bruce, like survivin is also known to be involved in cytokinesis [96].

Livin: Livin also known as Melanoma IAP (ML-IAP) shows the presence of a RING finger and one BIR domain that is homologous to the BIR3 domain of XIAP, cIAP1 and cIAP2. It is known to be expressed in normal fetal liver and kidney as well as adult testis and thymus [75, 97]. Livin, similar to cIAP1 is capable of binding and inhibiting the function of Caspase 3 and Caspase 7 [98]

Ts-IAP: Ts-IAP (Testis specific IAP) also known as ILP-2 also shows one BIR domain and one RING finger domain. ILP2 is capable of inhibiting Caspase 9 but does not inhibit the functions of Caspases 3 and 7 [99].

2.3 Survivin

Survivin is the smallest member of the Inhibitor of apoptosis proteins with dual functions in cell survival as well as cell cycle. Since its discovery in 1999 survivin has been touted to be a potential therapeutic target due to its differential expression in cancer tissues as compared to adult normal tissues [100]

Survivin gene is located at Chromosome 17q25. The survivin promoter does not contain a TATA box, and transcription of survivin utilizes the three Sp1 sites and CDE/CHR boxes [101, 102]. Alternative splicing of the survivin gene results in the formation of six splice variants with opposing functions; survivin wt, survivin 2B, survivin 3B, survivin ΔEx3, survivin 2α and survivin 3α.

Splice Variants of Survivin

Survivin wt: Survivin wt is the predominantly expressed isoform of survivin, transcribed from all the four exons. It is a 142 amino acid protein with a molecular weight of 16.5kDa, an N-terminal BIR domain which is linked to an amphipathic α helix at the C terminal [103]. Survivin wt is involved in cell cycle regulation as part of the chromosomal passenger complex with

Borealin, INCENP and Aurora Kinase B [104]. Survivin is exported into the cytoplasm via by CRM1 on recognition of the Nuclear Export Signal (NES). Survivin wt inhibits caspase 9 as well as caspase 3 and caspase 7 and apoptosis on binding to cofactors HBXIP or XIAP [105, 106]. Survivin wt is considered to be primarily localized in the cytoplasm although its expression is also observed in the nucleus and mitochondria [107, 108].

Survivin ΔEx3: Survivin ΔEx3 is formed by the deletion of Exon 3 and is also implied to be anti-apoptotic in function and is reported to show nuclear localization [108]. Survivin ΔEx3 contains a BH2 domain which may be important for its anti-apoptotic function as well as a mitochondrial localization signal. Caldas et. al. 2005 have shown that survivin ΔEx3 may be capable of forming a complex with survivin wt and that it is this heterodimer that localizes to the mitochondria and inhibits mitochondrial dependent apoptosis [22].

Survivin 3B: Survivin 3B is formed due to the insertion of a 165 bp region of intron 3 termed as Exon 3B. The insertion results in an in-frame stop codon resulting in a truncated protein of 120 amino acids [109]. Survivin 3B has been reported to have a cytoprotective function and is also the only isoform besides survivin wt to be shown to bind to CRM1 and also with Borealin and INCENP as part of the chromosomal passenger complex [110]. Survivin 3B is also reported to inhibit the death receptor pathway mediated apoptosis by interacting with procaspase 8 [111].

Survivin 2B: Survivin 2B is formed by the transcription of an 69 bp region from intron 2 termed as intron 2B [100]. The insertion of the 23 amino acid sequence is implied to affect the folding of the BIR domain of the protein resulting in a decrease in the antiapoptotic activity of this isoform

[100]. Besides survivin ΔEx3, only survivin 2B has been shown to immunoprecipitate with survivin wt. In contrast to the other three isoforms survivin 2B is considered to be proapoptotic and is reported to promote autophagy [112, 113]. The ability of survivin 2B to heterodimerize with survivin wt may result in an attenuation of the antiapoptotic function of survivin wt.

Survivin 2α: Survivin 2α protein is coded by exons 1 and 2 and is a small protein of 74 amino acid. with a predicted molecular weight of 8.5 kDa. Due to the deletion of exons 3 and 4 survivin 2α lacks the C terminal α helical coiled coil domain and has a truncated BIR domain [114]. Survivin 2α is implied to have a pro-apoptotic function and has also been shown to be capable of interacting with survivin wt to form a heterodimer [22, 114]. Similar to survivin 2B, heterodimerization of survivin 2α with survivin wt may result in an attenuation of the antiapoptotic function of survivin wt.

Survivin 3α: Survivin 3α is a 78 amino acid protein with a predicted molecular weight of 8.9 kDa and is formed by the translation of the first three exons of survivin gene. Very few studies have reported the expression of survivin 3α and the function of this isoform of survivin is not yet elucidated although one study has implied a role for the protein in breast cancer [115]

Survivin in Cancer:

Survivin has been considered to be a potential therapeutic target in cancer due to its negligible expression in normal differentiated tissues but overexpression in cancers of various origins. Since its discovery survivin has been shown to be overexpressed in a variety of cancers including breast, prostate, lung, colorectal and gastric cancers [116-123]. Nuclear expression of survivin is

reported to be a poor prognostic marker for breast cancer [116] but in gastric cancers cytoplasmic survivin was associated with poor prognosis [121].

Overexpression of survivin has also been related to resistance to chemotherapy. It has been shown that survivin overexpression results in resistance to commonly used chemotherapeutic agents such as cisplatin, tamoxifen, vincristin. [124-131]. Survivin overexpression in B cell lymphoma is also reported to result in resistance to etoposide and oxaliplatin [132].

Survivin is known to be induced post radiation and has been associated with radioresistance in various cancers such as rectal, pancreatic, as well as Head and neck cancers [133-136].

Although various reports demonstrate the role of survivin in tumorigenesis, the association of survivin overexpression with survival is ambiguous. This has been largely attributed to the presence of the alternatively spliced variants with opposing functions, since the splice variants cannot be distinguished at the immunohistochemical level. Various studies have determined the expression of survivin and its isoforms in cancers and some of these are summarized in Table 2-3.

Table 2-3: Summary of survivin isoform studies in cancers between 2009-2015

Isoform	**Function**	**Cancer Type**	**Up/ Downregulation**	**Association**	**Ref**
Survivin wt	Antiapoptotic, Cytoplasmic Localization	GBM	+		[137]
		Breast	+		[138, 139]
		Colorectal	+	T stage	[23]
		Bladder	+	In Metastatic stages	[140, 141]
		AML	+	poor clinical outcome	[142]
		Oral	**+**		**[20]**
Survivin 3B	Antiapoptotic	Breast	+	shorter overall survival	[143, 144]
		Colorectal	+	Survivin/Survivin 3B higher	[145]

				in neoplastic compared to normal tissues	
Survivin 2B	Proapoptotic/ Antiapoptotic	Breast	+ +	Tumor grade high grade ER negative and invasive tumors Reduction after chemotherapy associated with responsive tumors	[146] [143] [144]
		Colorectal	+	2B/Survivin wt with ER positivity Morphologic type, Tumor grade	[138] [23, 145, 147]
		Oral	**+**	**tumor grade**	[20]
		Renal	+	progressive T stage	[140]
		AML	+	poor clinical outcome	[142]
		Ovarian	+	overall survival	[148]
Survivin ΔEx3	Antiapoptotic	GBM	+	tumor progression and reduced DFS	[137]
		Thyroid	+		[149]
		Breast	+	histological grade	[143, 146]
		Oral	**+ (Second highest expression)**	**decreased in advanced stages and in metastasis cases**	[20]
		Colorectal	+	T stage	[23]
		Bladder	+		[141]
Survivin 2α	Proapoptotic	Thyroid	+		[150]
		Breast	+ (Most dominant isoform)	low grade and non-invasive tumors	[143] [151]
		Renal	-		[140]
Survivin 3α	-	Astrocytoma	-		[152]
		Breast	+		[151]

As is evident from the above table the expression of survivin isoforms has been shown to be upregulated in various cancers however only one study till date has determined the expression of four of the isoforms of survivin in oral cancers [20]

Regulation of Survivin:

Survivin expression is known to be tightly regulated in cells due to its bifunctional role in apoptosis as well as cell cycle regulation. Survivin expression is known to be upregulated in the G2/M phase of cell cycle. Survivin is transcriptionally repressed by the tumor suppressor genes PTEN, BRCA1 and p53 [18, 153-156]

2.4 Clusterin/Apolipoprotein J

Clusterin is an 80 kDa glycoprotein and is also known as Apolipoprotein J, Complement cytolysis inhibitor and sulphated glycoprotein 2. It is normally expressed in the Blood, Retina and Liver and is involved in complement modulation and lipid transport in blood. It is also known to have an important role in many biological functions such as tissue differentiation and remodeling, membrane recycling, lipid transportation, cell–cell or cell–substratum interaction, cell motility, cell proliferation, and cell death [157, 158].

In humans, *CLU* gene is located on chromosome 8, in a region that is frequently deleted in prostate cancer (8p21-p12) [159]. *CLU* is organized in 9 exons and 8 introns of variable size, ranging from 126 to 412 bp and spanning a region of 17,877 bp with a single promoter [160, 161]. It is considered to have a central role in homeostasis. Two isoforms of clusterin are currently characterized, the secreted form, present in the cytoplasm (sCLU) is pro-survival in nature while the other splice variant is present in the nucleus (nCLU) and is pro-apoptotic [161]. Figure 2-5 represents the formation of the clusterin transcript variants [162] . However there is

an ambiguity in the mechanism of nCLU formation. Although one group reports the loss of exon 2 to be responsible for formation of nuclear clusterin, the other suggests the use of an alternative promoter in exon 1 to result in the formation of nuclear clusterin [163, 164].

Figure 2-5: Clusterin transcript variants

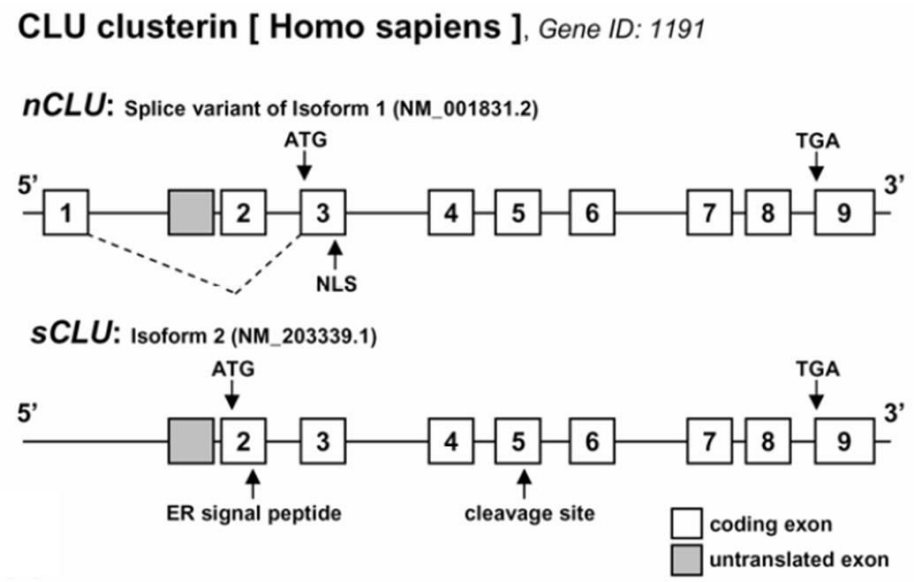


Figure 2-5: Clusterin transcripts: nCLU formed by the skipping of exon two and containing the nuclear localization signal. The sCLU is formed by the usage of a second promoter on exon two.

The cytoplasmic clusterin or secretory clusterin, a 60 kDa protein (449 amino acids) , contains a leader peptide sequence which transports it to the endoplasmic reticulum where it is glycosylated and cleaved to form α and β chains held by five disulfide bonds. This mature clusterin is the 80 kDa heterodimeric protein secreted outside the cell. The monomeric α and β chains are predicted to have a molecular weight of around 36kDa [165]. The secreted protein is known to have anti-proliferative effect on SV40-immortalized human prostrate cells [166]. The nCLU is a 49 kDa protein and is reported to be induced by ionizing radiation and contains a Ku70 binding domain

at the C-terminal. The Ku70-nCLU interaction reported to result in apoptosis [163]. nCLU has an anti-proliferative effect on CLU negative PC-3 prostate cancer cells [167]. Zhang et. al. 2006 have demonstrated that in presence of full length secretory protein cells are capable of resisting apoptosis, but the truncated 49 kDa protein results in apoptosis by TNF-α mediated pathway [168]. Over expression of clusterin has been associated with tumorigenesis and severity of disease [161]. High sCLU levels are associated with poor prognosis in colorectal cancer [169] whereas in patients with non-small cell lung cancer it is associated with increased survival of the patients [170]. Clusterin is overexpressed in pancreatic cancers but not in normal pancreatic cells and is associated with longer survival of the patient [171]. Clusterin is also found to be over expressed in Human hepatocellular carcinoma (HCC) and is associated with increased tumor proliferation. It is being considered as a potential biomarker for HCC [172]. In colorectal carcinomas sCLU overexpression was inversely correlated with apoptotic index [173]. sCLU is negatively regulated by p53 but is reported to be induced in response to radiation in the absence of a functional p53 [17]. sCLU expression has also been associated with radioresistance and chemoresistance in cancers [24]

2.5 The p53 family members

TP53 gene is a 20 kb tumor suppressor gene present on Chromosome 17p13, that encodes the 53 kDa transcription factor p53 [174, 175]. It is activated in response to various stimuli and plays an important role in regulation of apoptosis, cell cycle arrest and DNA repair [176]. Under normal conditions p53 is inhibited by MDM2 either by binding to its transactivation domain or by functioning as a ubiquitin ligase and promoting degradation of p53 [177].The transcriptional activity of p53 is sequence specific and is activated in response to various stress signals to

maintain genomic and cellular integrity. The p53 consensus sequence has been identified to be a 10 base pair region 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3' present in two copies separated by 0=13 bases [178]. p53 function as a tetramer and binds to the promoter of target genes as a dimer of dimers and modulates RNAPII activity at the target by recruiting coregulators such as histone modifying enzymes, chromatin remodeling factors [179] . DNA damage may result in cell cycle arrest at G1 or G2 phase of the cell cycle which is for the most part p53 dependent. p21, GADD45 and 14-3-3σ are some of the targets of p53 that are involved in cell cycle regulation [180]

Beside regulating cell cycle checkpoint, p53 also has a major role in apoptosis and regulates various genes involved in induction of apoptosis such as the Fas/Apo1 and DR5 of the TNFR superfamily, p53 apoptosis effector related to PMP- 22 (PERP) in the extrinsic pathway. Besides these, p53 also activates genes involved in the intrinsic pathway such as the proapoptotic Bcl-2 family protein Bax, PUMA, Noxa as well as p53 regulated apoptosis inducing factor [180]. Microarray studies have identified more than 100 genes that are activated by p53 that have varied functions in apoptosis, cell cycle arrest, extracellular matrix and adhesion genes [181].

Besides transcriptional regulation the wt p53 protein also demonstrates non-transcriptional activities. Studies have shown that mitochondrial or cytoplasmic localization of p53 is associated with induction of apoptosis by p53 by behaving like a BH3 only protein. p53 has been shown to induce apoptosis by inducing permeabilization of the outer mitochondrial membrane and triggering the release of proapoptotic factors. It has also been shown to be co-immunoprecipitated with Bcl-2, Bcl-XL and Bak under pro-apoptotic conditions [182-185].

Besides transcriptional activation, p53 is also capable of repressing a number of genes and various mechanisms of p53 mediated repression have been reported. p53 can mediate repression

by recruitment of corepressors to the specific promoter, or by transactivation of the repressor protein, for example transactivation of p21 by p53 would inhibit E2F regulated genes and activation of Slug would repress Puma, it can bind to the p53 response element and prevent the binding and activity of the transcriptional activator [179, 186-188].

The p53 protein family also consists of p63 and p73 proteins. p53, p63 and p73 encompass three main domains, an N-terminal transactivation domain (TAD), a core DNA binding domain (DBD) and a C- terminal oligomerization domain (OLD). p63 and p73 share a significant homology with p53, with close to 60% homology in the DNA binding domain of the proteins [189, 190].

Phylogenetic analysis shows that the p53 family originated from a p63/73-like ancestral gene early in metazoan evolution [191]. One of the ancestral functions is maintenance of genetic stability in germ cells [192]. The p53 family regulates many vital biological processes like cell differentiation, proliferation and apoptosis [193]. The expression of the three proteins are regulated by a similar mechanism and may be transcribed by using two alternative promoters P1 and P2, where P2 is an alternative intragenic promoter. Depending on the promoter used the products formed are categorized as either the TA or transactivating isoforms or the ΔN or the N terminal deleted forms. Further diversity in the isoforms is achieved by C terminal splicing which can yield the α, β, γ, δ forms in p63 and also additionally includes the ε, η and ζ forms for p73. The α isoforms of p63 and p73 also show the presence of a Sterile Alpha Motif (SAM) domain which is reported to be important for protein protein interaction and is also considered important for transcriptional repression [194] The formation of the isoforms and the protein structure of the three p53 family proteins is depicted in Figure 2-6.

Figure 2-6: Structure of the p53 family proteins

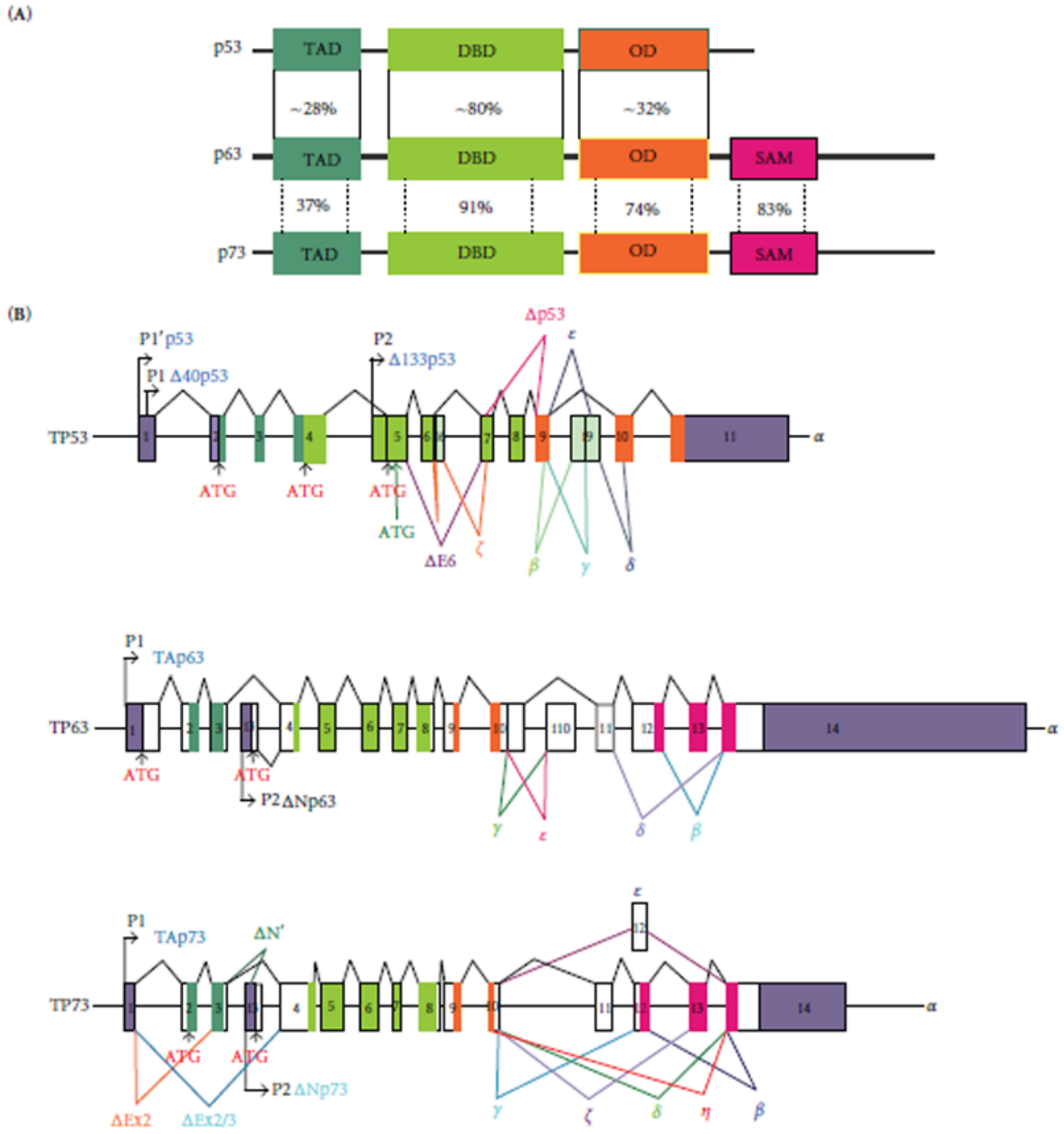


Figure 2-6: Structure of the p53 family proteins. (A). Depicts the homology between the TAD, DBD and OLD domains of the p53 family. (B) Depicts the formation of the TA and ΔN isoforms using the alternative promoters P1 and P2 and the C terminal spliced isoforms of p63 and p73 [191].

p63 is generally known to be involved in differentiation and development specifically in limb formation and epidermal morphogenesis [189]. Indeed, p63 null mice show severe developmental abnormalities such as absence of limbs and craniofacial malformations and do not survive more than a few days [189]. Similarly p73 also plays an important role in development and is reported to be involved in neurogenesis, pheromonal signaling and normal fluid dynamics of cerebrospinal fluid. p73 null mice are reported to exhibit loss of large bipolar neurons in the cortex and hippocampus and thus exhibit hippocampal dysgenesis and suffer from hydrocephalus [189].

The existence of two classes of p53-family target genes is proposed. One class in which p53 regulates the genes in the presence or absence of p63 and p73, for example, *MDM2* and *p21*. The other class in which p63 and p73 are required for recruiting p53 and for it to function properly, for example, *PERP* and *bax* [195] . p63α and ΔNp63α can also induce p21 which is a p53 target and inhibit cell proliferation by arresting the cells in G_1 phase of the cell cycle [196].

The TA forms of both p63 and p73 due to the presence of the transactivation domain can bind to and induce p53 target genes while the ΔN isoforms inhibit both wtp53 and TAp63 [197]. p63 is expressed mostly is basal cells of various epithelial tissues with ΔNp63α being the most predominantly expressed isoform. TA isoforms of p63 are overexpressed post DNA damage [198] while the ΔN forms are downregulated [199]. ΔNp63α is over expressed in 80% of HNSCC (Head and Neck Squamous Cell Carcinoma) and is reported to be critical for cell survival [200]. The *TP63* gene is rarely mutated in tumors while over expression is demonstrated in several cancers including breast cancer, lung cancer, bladder cancer and HNSCC [201].

p73 transcripts have been identified in human thymus, prostate, heart, liver, skeletal muscle, and pancreas [202]. Although p73 mutations are rare in cancer, like p63, higher levels of p73 have been reported in neuroblastoma, lung cancer, colorectal cancer, breast cancer and bladder cancer [203]. p73 is also known to induce apoptosis via a E2F-1 dependent pathway by T-Cell Receptor activation-induced cell death (TCR-AICD) [204]. MDM2 inhibits the transcriptional function of p73 (but has no effect on its stability) [205] but has no effect on p63 [206].

However unlike p63 and p73, mutations and inactivation of p53 are reported in more than 50% of cancers [207]. Inactivation of p53 has also been widely reported in oral cancers with earlier studies from our laboratory showing inactivation of p53 in 46% oral cancer cases [100, 208-210]. Although the p53 mutants detected in cancers were earlier thought to be inactive, recent evidence suggests that some of the p53 mutants specific in cancers may be gain of function mutations. More than 80% of somatic or germline mutations in TP53 are missense mutations that stabilize the protein and result in nuclear accumulation of the protein in tumor cells [211]. Mutant p53 has also been reported to modify gene expression by using p63 as a chaperone for DNA binding [212]. In HaCaT cells it has been demonstrated that p63 in the presence of mutant p53 functions as an activator of KLF4 instead of repressing the function of the protein as is observed in primary keratinocytes [213]. Gain of function of p53 is now reported to be associated with increased tumorigenesis, metastasis as well as chemoresistance [211]

Earlier studies from the lab demonstrated alterations in the expression of survivin and clusterin in oral tumor tissues as compared to normal tissues and in oral cancer cell lines. In literature only one study has determined the expression of survivin isoforms in oral cancers. Currently among the six reported isoforms of survivin, two are pro-apoptotic while three including the wild type

are anti-apoptotic. The proapoptotic isoforms are also hypothesized to be capable of binding to and attenuating the function of the anti-apoptotic forms, thus the balance of the pro apoptotic to anti-apoptotic forms may determine cell fate. The present study thus determined the expression of the six survivin isoforms, clusterin and the isoforms of p63 and p73 in oral cancer cell lines as well as tissues. Also, although two of the isoforms of survivin are reported to bind to survivin wt, the binding pockets and the residues involved in the dimerization have not been identified. In the current study we performed docking analysis to determine the ability of the isoforms to heterodimerize with survivin wt and the residues involved in the dimerization.

Survivin and clusterin are both repressed by wild type p53. Earlier studies from the lab had identified nuclear accumulation of p53 in oral tumor tissues as well as oral cancer cell lines. Mutational analysis of p53 in the oral cancer cell lines identified R273H hot spot mutation in two of the tongue cancer cell lines used in those studies. R273H p53 mutant is a DNA binding mutation in the p53 protein and is associated with gain of function in lung cancer, osteosarcoma and colon adenocarcinoma cell lines [214]. Although the mutant p53 proteins are associated with increased tumorigenic properties, the mechanism of mutant p53 function is not yet completely elucidated. Mutant p53 may directly transactivate target genes, or may be capable of binding to specific transcription factors to regulated genes involved in invasion, migration and proliferation. However the gain of function for mutant p53 R273H is as yet not well established in oral cancers. Figure 2.6 illustrates the probable mechanisms of mutant p53 functions.

Figure 2-7: Mutant p53 gain of function, mechanisms of action

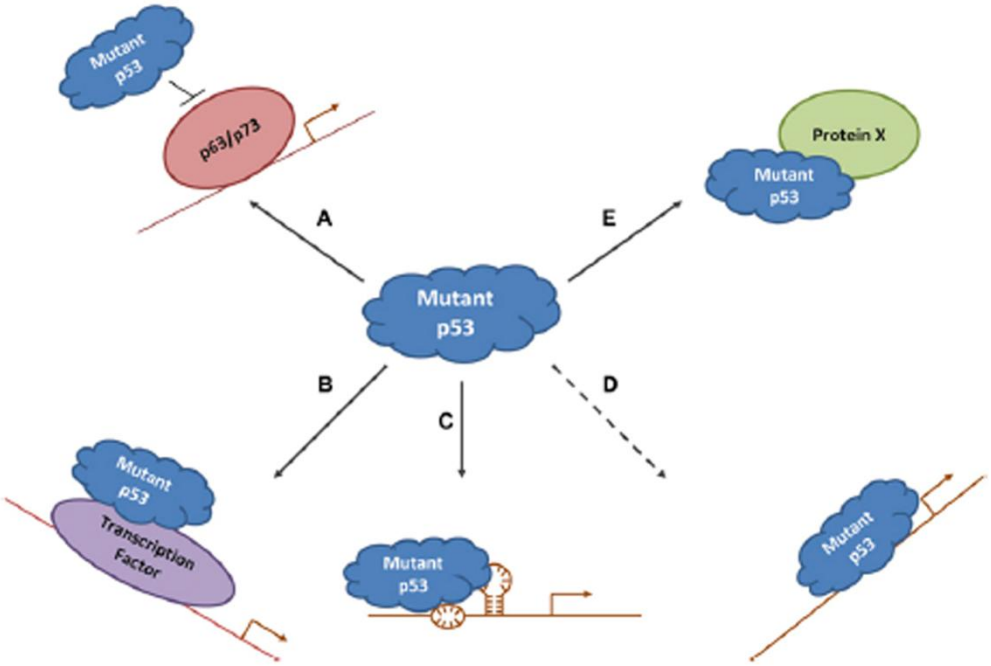


Figure 2-6: Mutant p53 gain of function, mechanisms of action. Mutant p53 gain of function may be via binding to transcription factors, or inhibiting the function of p63/73. Although no direct evidence is as yet reported mutant p53 may also function by direct binding to unique sequences and activating transcription.

We thus hypothesized that p63 and /or p73 may regulate the expression of survivin and /or clusterin in the absence of a functioal p53 protein, contributing to their aberrant expression in oral cancers. We also hypothesized that the regulation of the p53 downstream targets by p63 may be affected by the presence of a mutant p53 protein.

3 . Aims and Objectives

Aims and Objectives

In light of the literature, the aim of the present study was to determine the role of p63 in regulation of the p53 repressed anti-apoptotic proteins survivin and clusterin in oral cancers. The objectives outlined to address the same are as follows

1. To profile p63,p73, survivin and clusterin isoforms in oral cancer cell lines and/or tissues and assess their association if any with the patients clinicopathological parameters.
2. To examine whether p63 regulates survivin and/or clusterin expression in oral cancer cells in the background of a mutant or no p53 protein using siRNA strategies and luciferase assays.
3. To determine the role of p63 protein in oral cancer cells using different phenotypic assays, microarray analysis and radiation treatment post p63 knockdown in oral cancer cells.

4 . Materials and Methods

4.1 Materials

The following chemicals/materials were obtained from:

Abcam: p63 antibody (ab111449)

Amersham Lifesciences: Enhanced Chemiluminescence Kit.

Applied Biosystems: 2X Power SYBR Green PCR Master Mix (Part No. 4367659), and MicroAmp Optical Adhesive Film Kit (Part No. 4360954).

Axygen and Tarson: Disposable tips and DNase, RNase free eppendorf tubes, MicroAmp optical 384-well Reaction Plate with Barcode (CAT: PCR-384M2-C)

BD Biosciences: Cell culture inserts, culture plates with notch, BD Matrigel matrix.

Dharmacon: siGLO RISC-Free Control siRNA, SMARTpool:siGENOME HUMAN TP63 siRNA

Gibco: Fetal Bovine Serum, DMEM, IMDM, Ampicillin.

Invitrogen: LMP (low melting point) Agarose, Trypsin, L-Glutamine, TRIzol, Lipofectamine 2000.

Kodak: X-ray films.

MBI Fermentas: First strand cDNA kit, 6X loading dye, 25mM MgCl2, Protein ladder, 10X PCR buffer, Taq polymerase, Proteo-block protease Inhibitor, Proteo-jet Mammalian cell lysis reagent.

Merck Millipore: PVDF membrane, Chromatin Immunoprecipitation (ChIP) Assay kit (17-295)

Nunc and BD-Falcon: Tissue culture petridish and flasks.

Qualigens: Boric acid, Disodium hydrogen hydrophosphate, $MgCl_2$, NaOH pellets, Sodium dihydrogen orthophosphate, Glacial acetic acid, Glycine, Xylene, DPX Mountant.

Roche: dNTPs, Protease inhibitors, Taq polymerase, X-treme Gene HP transfection reagent

Santacruz Biotechnology: Survivin antibody (sc-10811), Clusterin antibody (sc-8354), anti-rabbit HRP (sc-2005), anti-mouse HRP (sc-2004)

Sigma: Paraformaldehyde, Acrylamide, β-mercapethanol, Bovine Serum Albumin, EDTA, Glycine, N-N Methylene Bis-Acrylamide, Sodium Dodecyl Sulphate, Tween-20, Triton X-100, Tris, TEMED, DTT, Bromophenol Blue, Coomassie brilliant blue, Di-amino benzidine, DMSO, Ethidium Bromide, Fast green, Oligonucleotides, Proteinase K, Insulin, Glutaraldehyde, Low melting agarose, Insulin, Erythrosine B, Ponceau stain, DAPI, Tri-Sodium citrate, Crystal Violet.

SISCO Research Laboratories: Chloroform, Folin Ciocalteu reagent, Isopropanol, Isoamyl alcohol, Methanol, Phenol, Sodium chloride

USB: DEPC (Diethylpyrocarbonate)

Water: The water used for the preparation of all solutions and reagents was Ultrapure water (Resistivity = 18 MΩ cm) obtained from a Milli-Q water plant (Millipore, Billerica, MA, USA).

Primers: All the primers (3 O.D) used in the study were obtained from Sigma or Eurofinns in lyophilized form and reconstituted using Milli-Q.

4.2 Methods

4.2.1 Cell Culture Techniques

Freezing: Freezing of cells was done at 70-80% confluence. The cells were washed twice with 1X PBS followed by incubation with 1ml trypsin at 37^0C. Once the cells detached, 2ml of complete medium was added and the detached cells collected in a centrifuge tube. The cells were then centrifuged at 1200rpm for 50mins at room temperature. The supernatant was then discarded and freezing solution (maintained at 4^0) was added to the cells to achieve a final concentration of 1 x 10^6 cells/ml. The freezing solution was prepared in serum with 10% DMSO. The cells were then frozen in liquid nitrogen with step wise cooling.

Revival: The frozen ampoules were thawed at 37^0C water bath. The cells were then resuspended in 3ml complete medium and centrifuged at 1200 rpm for 5mins at room temperature. The supernatant was discarded and the cells were washed twice in complete medium. The cell pellet was then resuspended in 1ml medium and seeded.

Passaging of Cells: Cells were passaged once 80-90% confluence was achieved. The cells were washed twice with 1X PBS and incubated with 1ml trypsin at 37^0C till the cells detached from the surface. The cells were collected in a centrifuge tube after addition of 2ml of complete medium. The cells were then centrifuged at 1200 rpm for 5mins at room temperature. The supernatant was discarded and the cell pellet was resuspended in 1ml medium. For counting, 10µl of cell suspension was diluted in 10µl of Erythrosin B and loaded onto Neuber's hemocytometer for cell count. The cells were then plated according to requirement.

Cell lines and medium used for the study:

Table 4-1: List of cell lines used in the study

Cell Line	Medium	Reference
FBM (Fetal Buccal Mucosa)	IMDM + 10% FBS	[215]
HaCaT	DMEM + 10% FBS + HEPES	[216]
DOK (Dysplastic Oral Keratinocyte)	DMEM + 10% FBS + HEPES + Hydrocortisone	[217]
AW8507 (Tongue cancer)	IMDM + 10% FBS	[218]
AW13516 (Tongue cancer)	IMDM + 10% FBS	[218]
UPCI - SCC040 (Tongue cancer)	DMEM + 10% FBS + HEPES	[219]
UPCI - SCC029B (Buccal Mucosa cancer)	DMEM + 10% FBS + HEPES	[219]

4.2.2 Tissue Samples used in the study and clinicopathological parameters:

75 oral tumor and adjacent normal tissues were obtained from the Tumor Tissue Repository of Tata Memorial Centre. The study was IRB approved and samples were obtained with informed consent of the patient. In addition to the oral tumor tissues, 12 normal tissues were obtained from Sharad Pawar Dental College after informed consent from healthy individuals. These tissues were obtained from patients undergoing minor or major surgical procedure such as removal of impacted third molar, cleft lip/palate surgery. The tissue samples obtained were snap frozen and stored at -80^0C until further processing. H & E analysis was conducted to determine the pathology of the samples and only representative tissues with >80% tumor content were included in the study.

The clinicopathological characteristics of the samples used in the study are given below. The seventh edition of TNM staging (2010) was used for staging the oral tumors and all pathological reports were used for TNM staging.

Table 4-2: Clinicopathological characteristics of the patient samples used in survivin isoform expression analysis by real time PCR (75 samples)

Characteristics	No (%)
Age	
< 45	24/75 (32)
> 45	51/75 (68)
Sex	
Male	52/75 (69.3)
Female	23/75 (30.7)
Site of Lesion	
Alveolus	25/75 (34.3)
Buccal Mucosa	30/75 (40.0)
Mandible	3/75 (4.0)
Tongue	16/75 (21.3)
Missing	1/75 (1.3)
Tumor (T) Status	
T1-T2	9/75 (12.0)
T3-T4	63/75 (84.0)
Missing	3/80 (4.0)
Nodal (N) Status	
N0-N1	49/75 (65.3)
N2-N3	25/75 (34.3)
Missing	1/75 (1.3)
Differentiation Status	
Poorly differentiated	11/75 (14.7)
Moderately differentiated	43/75 (57.3)

Well differentiated	13/75 (17.3)
Missing	8/75 (10.7)

4.2.3 RNA Extraction

Cell Lines: The cell pellet was washed twice with 1X PBS and resuspended in appropriate amount of Trizol (Invitrogen).

Tissues: Thin 4µ section of the tissues were obtained using the cryotome. The sections were collected in Trizol and homogenized. For extraction 50 sections of each tissue was utilized.

Extraction: After homogenization of the tissues/cells in Trizol, 200µl chloroform was added per 1ml of Trizol and shaken vigorously by hand for 15secs. The samples were then centrifuged at 15000rpm for 15mins at 4^0C. The aqueous phase was then transferred to a new 1.5ml eppendorf tube and 500µl of isopropanol per ml of Trizol was added to precipitate the RNA. The samples were then incubated at room temperature for 10mins and centrifuged as stated previously. The supernatant was discarded and the precipitated RNA was washed twice with 200µl 70% ethanol prepared in DEPC treated distilled water and centrifuged at 15000rpm for 10mins at 4^0C. The RNA precipitate was then dissolved in 20µl DEPC and incubated at 55^0C for 5mins. The extracted RNA was quantified and stored at -80^0C until further use.

4.2.4 cDNA synthesis and semi-quantitative PCR

cDNA was synthesized using Revert Aid cDNA synthesis kit. 1µg (cell lines)/ 500ng (tissues) was mixed with 1µl of Random hexamer primer in a total volume of 12µl and incubated at 65^0C for 5mins to denature secondary structures of the RNA . The tubes were immediately place on ice and the following components were added in the sequence.

5X Reaction Buffer	4µl

10mM dNTPs	2 µl
RiboLock RNAse Inhibitor	1 µl
Mu-MLV (Reverse Transcriptase Enzyme)	1 µl

The tubes were then incubated under the following conditions

25^0C	5 mins
42^0C	60 mins
70^0C	5 mins

4.2.5 Semi-quantitative RT-PCR.

The sqRT-PCR reactions were performed with 500ng cDNA extracted as described in Section 4.2.3 and 4.2.4. The expression of the TA and ΔN isoforms of p63 and p73 were determined by semi-quantitative PCR. The list of primers used is elaborated along with the respective annealing temperatures in Table 4-3.

Table 4-3: Primers used for p63 and p73 isoform expression analysis

Transcript	Sequence 5' to 3'	Amplicon Size
TAp63 Forward	GTCCCAGAGCACACAGACAA	267 bp
TAp63 Reverse	GAGGAGCCGTTCTGAATCTG	
ΔNp63 Forward	CTGGAAAACAATGCCCAGAC	198 bp
ΔNp63 Forward	GGGTGATGGAGAGAGAGCAT	
Pan p63 Forward	GACAGGAAGGCGGATGAAGATAG	299 bp
Pan p63 Reverse	TGTTTCTGAAGTAAGTGCTGGTGC	
TAp73 Forward	CCGGCGTGGGGAAGATGG	257 bp
TA p73 Reverse	TTGAACTGGGCCATGACAGATG	
ΔNp73 Forward	CAAACGGCCCGCATGTTCCC	256 bp
ΔNp73 Reverse	TGGTCCATGGTGCTGCTCAGC	

GAPDH Forward	TGCTTTTAACTCTGGTAAAGT	703 bp
GAPDH Reverse	ATATTTGGCAGGTTTTTCTAGA	

The cycling conditions used for the primers are elaborated in Table 4-4.

Table 4-4: Cycling conditions for primers for p63 and p73 isoform expression analysis.

Transcript	**Denaturation**	**Initial Denaturation**	**Annealing**	**Extension**	**Cycles**	**Final Extension**
TAp63	94^0C for 5mins	94^0C for 30s	57^0C for 40s	72^0C for 30s	40	72^0C for 5'
ΔNp63	94^0C for 5mins	94^0C for 1'	57^0C for 40s	72^0C for 30s	40	72^0C for 5'
Panp63	94^0C for 5mins	94^0C for 1'	60^0C for 1'	72^0C for 1'	35	72^0C for 5'
TAp73	94^0C for 5mins	94^0C for 1'	70^0C for 1'	72^0C for 1'	35	72^0C for 5'
ΔNp73	94^0C for 5mins	94^0C for 1'	68^0C for 1'	72^0C for 1'	35	72^0C for 5'
GAPDH	94^0C for 5mins	94^0C for 1'	52^0C for 1'	72^0C for 1'	30	72^0C for 5'

Cycling conditions for primers for p63 and p73 isoform expression analysis (' indicates minutes, s indicates seconds)

The standard set up for the semi-quantitative PCR reaction is provided in Table 4-5

Table 4-5: Reaction setup for semi-quantitative PCR

Reagent	**Stock Concentration**	**Working Concentration**	**Volume**
PCR buffer	10X	1X	2.5µl
$MgCl_2$	50mM	1.5mM	0.75 µl
dNTPs	2mM	0.2mM	2.5 µl
Forward Primer	100ng/ µl	100ng/ µl	1 µl
Reverse Primer	100ng/ µl	100ng/ µl	1 µl
Taq Polymerase	5 Units	1 Unit	0.2 µl
cDNA (100-200ng)			1 µl
Distilled Water			16.05 µl

4.2.6 Agarose Gel Electrophoresis

The PCR products were visualized on 2% agarose gel and were separated on the basis of their charge. EtBr (1mg/ml), DNA intercalating agent was used for visualization of the products under UV gel documentation system. 5µl of EtBr was added to 100ml of 1X TBE containing 2gm agarose. PCR products were loaded using 6X loading dye (10µl product + 2µl dye). Amplicon sizes were determined by comparison with standard DNA marker (Mass Ruler 100bp DNA ladder SM0383).

4.2.7 Real Time PCR

The survivin and clusterin isoform expression was determined by real time PCR using SYBR Green chemistry. The expression of TA and ΔNp63 isoforms post knockdown of p63 was also determined by real time PCR. Table 4-6 lists the real time PCR primers used for the detection of the six survivin isoforms and clusterin isoforms. The real time PCR reactions were set up in Quant Studio 12K Flex system in 384 well plates. 5µl reactions were set up as described in Table 4-7. The reactions were set up in duplicates for cell lines as well as tissue samples. Analysis of the real time data was determined by the ΔCt method as described by Livak et. al. 2001 [220]. Fold change of expression was determined by $2^{-\Delta\Delta Ct}$ method.

Table 4-6: Primers used for real time PCR

Survivin wt Forward	AAGGACCACCGCATCTCTAC
Survivin wt Reverse	TGTTCCTCTATGGGGTCGTCA
Survivin 3B Forward	CAGATTCAGGGAGGGACTGG
Survivin 3B Reverse	CAAACATCAGGCTCTTCCTCG
Survivin 2B Forward	GCACGGTGGCTTACGCCTG
Survivin 2B Reverse	AACCGGACGAATGCTTTTTATGTTCC
Survivin 2α Forward	GCTTTGTTTTGAACTGAGTTGTCAA

Survivin 2α Reverse	GCAATGAGGGTGGAAAGCA
Survivin ΔEx3 Forward	GATGACGACCCCATGCAAA
Survivin ΔEx3 Reverse	AGGCCTCAATCCATGGCAG
Survivin 3α Forward	ACTGAGAACGAGCCAGACTTG
Survivin 3α Reverse	TCTTTTGCTTCCAGTCCCTCC
TAp63 Forward	GCCCTGACCCTTACATCCAGCG
TAp63 Reverse	GTGTGCTCTGGGACATGGTGGA
ΔNp63 Forward	CTGGAAAACAATGCCCAGAC
ΔNp63 Reverse	GGGTGATGGAGAGAGAGCAT
GAPDH Forward	GAAGGTCGGAGTCAACGGATTT
GAPDH Reverse	GATGACAAGCTTCCCGTTCTCA
Keratin 14 Forward	CCAGTTCTCCTCTGGATCGCAG
Keratin 14 Reverse	GATCTTCCAGTGGGATCTGTGTCCA
βA Forward	AAGCTTACCATGGACGGGCTG
βA Reverse	GAATTCTCAGGCGCAGCCGCA

Table 4-7: Reaction set up for Real Time PCR using SYBR Green Chemistry

Reagent	**Volume**
5X SYBR Green Mix	2.5 µl
Forward Primer 10pm	0.25 µl
Reverse Primer 10pm	0.25 µl
cDNA (5ng/ µl)	2 µl

4.2.8 SDS-PAGE and Western Blotting

Protein Extraction

Cells were trypsinized, collected in 1.5ml Eppendorf tubes and the cell pellets were washed twice with 1X PBS. The cell lysates were obtained using ProteoJET Mammalian Cell lysis reagent (Fermentas) containing 1% Protease inhibitor cocktail. The cell pellets were solubilized in the lysis reagent, centrifuged at 15,000 rpm for 15mins at 4^0C. The supernatant fraction

containing the lysed proteins was collected in fresh tubes and estimated using Folin -Lowry method for protein estimation.

Estimation of Protein Concentration

The extracted protein was quantified using Folin Lowry method. BSA standards were diluted from 1mg/ml stock while the unknown protein samples were diluted such that the concentration falls within the range of the standard protein concentration. 500µl of Folin reagent was added to the above standards and unknown protein samples. The absorbance was measured at 750nM on a spectrophotometer. The standard curve was then plotted and R^2 value for the curve was determined. The concentration of the unknown samples was determined using the standard curve. The preparation of the standards and unknown protein samples is elaborated in Table 4-8.

Table 4-8: Preparation of standard BSA for Folin-Lowry protein estimation

Concentration (µg/µl)	BSA (1mg/ml)	Distilled Water (µl)	CTC (µl)		Folin (1:6 diluted) (µl)	
Blank	0	1000	1000	Incubate for 10mins in Dark at RT	500	Incubate For 20mins in Dark at RT. O.D measured at 750nm
5	5	995	1000		500	
10	10	990	1000		500	
15	15	985	1000		500	
20	20	980	1000		500	
25	25	975	1000		500	
30	30	970	1000		500	

The protein estimations for luciferase assays were done using Bradford Protein Estimation. The preparation of standards for Bradford estimation is presented in Table 4-9. All the samples were run in duplicates.

Table 4-9: Preparation of BSA standards for Bradford Protein estimation

BSA concentration(µg/µl)	BSA volume(µl)	MilliQ water volume(µl)	Bradford reagent volume(µl)
0	0	5	200
0.5	0.5	4.5	200
1	1	4	200
1.5	1.5	3.5	200
2	2	3	200
2.5	2.5	2.5	200
3	3	2	200
4	4	1	200

SDS-PAGE

20µg of protein was loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). The percentage of the gel signifies the pore size, formed by the polymerization of Acrylamide and Bis-acrylamide. 4cm space was used above the resolving gel wherein 5% of the stacking gel was poured. The stacking gel and resolving gels differ in the pH of the Tris Buffer used to prepare the gels. The composition of the resolving gels and stacking gels is presented in Table 4-10

Table 4-10: Composition of resolving and stacking gel for SDS-PAGE

Resolving Gel	**12%**	**15%**		**Stacking Gel**	**5%**
Distilled Water	4.3 ml	2.3 ml		Distilled Water	6.8 ml
30% Acrylamide	4.3 ml	5 ml		30% Acrylamide	1.7 ml
1.5 M Tris pH (8.8)	2.5 ml	2.5 ml		1.0 M Tris (pH 6.8)	1.25 ml
10% SDS	0.1 ml	0.1 ml		10% SDS	0.1 ml
10%APS	0.1 ml	0.1 ml		10%APS	0.1 ml
TEMED	0.008ml	0.008ml		TEMED	0.008ml

To assess the expression of p53 family proteins and clusterin protein, whole cell protein lysates were resolved on 12% polyacrylamide gel (15% for survivin). The proteins were loaded onto the gel with 6X Laemmli Dye diluted to final concentration of 1X. The mixture was boiled for 10mins in a water bath and loaded onto the gel along with pre-stained protein ladder.

Transfer of proteins onto PVDF membrane (Western Blotting)

Once the proteins are sufficiently resolved, the proteins are blotted onto a PVDF membrane. The PVDF membrane was place on the gel and setup in the transfer apparatus. Proteins were transferred for 16 hours at a constant voltage of 16V. The transfer of the proteins was determined by Ponceau staining. The blots were then blocked in either 3% BSA or 5% Milk for 2 hours at RT. After 3 TBS washes for 5mins, the blots were incubated with the primary antibody overnight at 4^0C. The blots were given 6 TBST washes, 10mins each and incubated with the respective secondary antibody for 1 hour at RT. The blots were again given 6 TBST washes and developed using ECl Prime Chemiluminescence reagent (Amersham). The blots were developed on X-ray film using an automated developing machine. The primary antibodies and their corresponding dilutions and blocking conditions used in the study are listed in Table 4-11

Table 4-11: Dilutions and blocking conditions of the antibodies used for western blotting and immunofluorescence studies.

Antibody (Catalogue No)	Clone and Dilution	Blocking Conditions	Dilution for IF
p53(sc6243)	Rabbit Polyclonal; 1:1000	5%Milk	1:100
p63 (ab111449)	Mouse monoclonal; 1:300	5%Milk	1:50
p63 (ab735)	Mouse monoclonal; 1:200	5%Milk	
Survivin (sc10811)	Rabbit Polyclonal; 1:1000	5%Milk	
Clusterin (sc8354)	Rabbit Polyclonal; 1:1000	5%Milk	

p21 (BD 554228)	Mouse ; 1:1000	3% BSA	
Actin (sc 1616)	Rabbit Polyclonal; 1:2500	5%Milk	
Secondary Antibody for Western Blotting			
Anti Rabbit IgG (sc2004)	Secondary Ab; 1:2500		
Anti Mouse IgG (sc2005)	Secondary Ab; 1:2500		
Secondary Antibody for IF			
Alexa Fluor 568 (Invitrogen: 11011)	Goat Anti-Rabbit		1:100
Alexa Fluor 488 (Invitrogen: 11059)	Goat Anti-Mouse		1:100

4.2.9 IF staining of p53 and p63 proteins in AW8507 and SCC040 cells

Beside western blotting, p53 and p63 proteins were also detected in AW8507 and SCC040 by immuno-fluorescence. Cells were grown on coverslips till a confluence of 50-70% was achieved. The cells were then immediately fixed in chilled absolute methanol for 5mins by placing the coverslips in methanol. The coverslips were then washed carefully twice with 1X PBS without Phenol Red. The cells were then permeabilised using chilled methanol containing 0.3% Triton-X 100 for 90secs. The coverslips were then washed with 1X PBS thrice and placed in a humid chamber. The cells were then blocked using 50µl serum for 1 hour at RT. The serum was carefully placed on parafilm and the coverslips were inverted onto the serum for blocking. After blocking the cells were again washed twice with PBS and incubated with 40µl primary antibody as described earlier, overnight at 4^0C. The next day the coverslips were washed thrice with PBS and incubated with the appropriate secondary antibody for 1hour in dark. The coverslips were then washed, and the cells were stained with DAPI (0.5µg/ml in PBS) for 5mins. The coverslips were mounted on acid washed slides using Vecta shield antiquenching mounting medium (Vector Laboratories) and sealed. Z stack images were then obtained in LSM 510 Zeiss Confocal System.

4.2.10 Sequencing of p63 gene in AW8507 and SCC040

Primers targeted to amplify exons 1 to 10 of p63 were designed using Primer 3. The primer sequences are listed in the Table 4-12. The PCR products resolved on an agarose gel, gel extracted and sequenced using an automated sequencer. 2µl (50 ng) of the amplicon and 1 µl of 1.6pmoles/ µl forward/reverse primer were subjected to sequencing. The sequences were then identified using BioEdit and compared to the genomic TP63 sequence in the NCBI database. Comparison with NCBI sequence identified all wild type p63 sequences, indicating primer efficiency to be a hundred percent.

Table 4-12: TP63 exon sequencing primers

Primer	Sequence 5' to 3'	Amplicon Size	Annealing Temp
p63 Ex1 F	GACCCTATTGCTTTTAGCCTC	235	56
p63 Ex1 R	CATTCATAATACACAAGGCACTTC		
p63 Ex2 F	CCTGCATGGTTTTATAGATTCACTTG	305	58
p63 Ex2 R	GACCACCCACATTTACCTATTTAG		
p63 Ex3 F	CCCTTTCCATGCCTAACTCACT	209	60
p63 Ex3 R	CCAAAGACTGAAGAGAAAGCCTG		
p63 Ex5 F	GGCTTCAGCGGCTAATATTGGG	353	61
p63 Ex5 R	GTGAAGCCCATCCTTGGACTTG		
p63 Ex6 F	TCTCCTTCCTTTCTCCACTGGC	284	61
p63 Ex6 R	TGCCCACAGAATCTTGACCTTC		
p63 Ex7 F	GCCACCAACATCCTGTTCATGC	258	61
p63 Ex7 R	GTCTACTCAGTCCATAGAGGTGTTG		
p63 Ex8 F	GAAGGAACAACGTCAGTTTAAACCC	245	63
p63 Ex8 R	AAAGCAGCCACGATTTCACTTTGCC		
p63 Ex9 F	GTGGTAGATCTTCAGGGGACTTTC	263	60
p63 Ex9 R	CCAACATCTGGAGAAGATTCAACC		
p63 Ex10 F	GTGTTCCCAGGATGAAACTTGC	153	61

p63 Ex10 R	GAAGCAACCATGAACACCCAAGG		
p63 Ex11 F	CCACACTTCTAACAGTTCTACAGC	278	60
p63 Ex11 R	TCATCAATCACCCTATTGCTGATCC		

Gel Elution

The PCR products were run on a 2% agarose gel and visualized on the UV gel documentation system. The specific bands were excised using a scalpel such that minimum amount of gel volume is extracted. The gel slice was kept in a pre-weighed 2 ml tube and weight of the gel slice was determined. Binding buffer was added in a ratio of 1:1 (volume : weight) to the gel slice. This mixture was then incubated at 50-60°C or till the gel slice was completely dissolved. The tubes were inverted every few minutes to facilitate the dissolution. After dissolution of the gel fragment, 1:2 volume of isopropanol was added to the solubilised gel and mixed thoroughly. Upto 800 µl of the solubilised gel was transferred to GeneJET purification column. The column was centrifuged for 1 minute, flow-through discarded and column was placed back in the collection tube. Since the elutes were to be processed for sequencing, 100 µl of binding buffer was added to the column and centrifuged for 1 minute. The flow-through was discarded and the column placed in the same collection tube. 700 µl of wash solution was added to the column and centrifuged for 1 minute. Flow-through was discarded and column was placed in same collection tube. One dry spin was given for 1 minute to remove residual wash solution. The column was transferred to a 1.5 ml centrifuge tube and 20 µl elution buffer (warmed at 65^0C) was added to the centre (on the membrane) of the column and centrifuged for 1 minute. The DNA was stored at -20° C.

4.2.11 siRNA and shRNA transfection

p63 knockdown was achieved by transfection of siGENOME HUMAN TP63 siRNA using Lipofectamine 2000 (Invitrogen). The transfection of the siRNA was standardized using siGLO RISC-Free Control. The cells were seeded in antibiotic free medium such that a confluence of 50-60% confluence was achieved in 24 hours. For AW8507 and SCC040 2 x 10^5 cells were seeded in 24 well plates. 2µl of Lipofectamine 2000 was mixed with 50µl of incomplete medium and incubated for 5 mins at RT. The Lipofectamine mixture was then mixed with 50µl of incomplete medium containing the siRNA and incubated at RT for 20mins. 100µl of this mixture was then added to the cells and incubated for 24 hours at 37^0C. The medium was changed after 24 hours and cell lysates were collected at 24 and 48 hours and tested for p63 protein by western blotting.

Stable knockdown of ΔNp63 was achieved in AW8507 and SCC040 cells using shRNA cloned in pEGFP vector (Srikanta Basu, Unpublished data). The ΔNp63 shRNA and the empty vector were transfected in the cells using X-TREME Gene HP transfection reagent (Roche). 2µg of DNA was mixed with the transfection reagent (1:6) in 200µl of incomplete medium and incubated at RT for 30 mins. The mixture was then added to the cells and incubated at 37^0C and media was changed after 24 hours. When the cells achieved confluence the cells were trypsinized and seeded in a 100mm dish. The selection agent puromycin was added to the cells after 24 hours and maintained in the selection medium till colonies of individual clones were visible. The puromycin concentration was standardized for the two cell lines. The individual clones were then spot trypsinized, checked for GFP expression and p63 knockdown was determined using western blotting.

4.2.12 Luciferase Reporter Assays

The promoter activity of survivin and clusterin post p63 knockdown in AW8507 and SCC040 was determined by luciferase reporter assays. The minimal promoter and the extended promoter of survivin , and the promoter of clusterin was cloned in pGL3 Basic vector. Cells were seeded into 24 well plates as described earlier and p63 was knockdown using siRNA. The cells were also simultaneously transfected with Renilla Luciferase vector using XTREME Gene HP (Roche) After 48 hours the cells were washed with PBS and lysed using 100µl of Lysis Buffer (Dual Luciferase Reporter Assay System, Promega). The cells were incubated with the lysis buffer for 15mins at RT on rocker. The lysate was then centrifuged at 13,000 rpm for 30s at 4^0C. The protein supernatant was collected in a fresh tube and the protein was estimated using Bradford Assay.

For the assay, 50µl of LARII reagent was added into white bottom 96 well plate. 10µl of lysates were added to the LARII reagent, and for Blank 10µl of Lysis reagent was added. The luminescence was then measured using the Berthold Luminometer and designated as the FL intensity, the firefly luciferase (FL) intensity. 50µl of the Stop Glow reagent was then added to the wells and the Renilla Intensity (RL) was then measured. The respective blank values were used for normalization of the FL and RL values and the ratio of the FL to RL values were calculated. The ratio was then divided by the protein concentration and plotted to determine the luciferase activity of the promoters.

4.2.13 Soft agar Assay

The anchorage independent growth of the ΔNp63 knockdown clones of AW8507 and SCC040 was determined by Soft agar colony formation assay. 2% soft agar (1.6% for SCC040) was prepared using low melting agarose in distilled water, autoclaved and maintained at 42^0C during the assay . The lower layer was made by diluting the agarose with 2X DMEM/IMDM and 1ml of the mixture was coated onto 35mm dishes and incubated at RT for 30mins. The upper layer was prepared by adding 400µl cells with 2ml 1X IMDM and 800µl each of 2X IMDM and 2% Agarose and coating 1ml of the upper layer onto the lower layer. For SCC040 400µl cells were added to 2100 ml and 750µl each of 2X DMEM and 1.6% agarose and coated onto the upper layer (1ml/dish). The plates were again incubated at RT for 30mins after addition of the upper layer. The plates were then incubated for 14 days at 37^0C with addition of complete medium every alternate day. The experiment was performed in triplicates and three independent assays were used for analysis. For AW8507 1000 cells were plated per dish (4000cells in 400µl) and for SCC040 50,000 cells were plated per dish (2,00,000 in 400µl). The colonies were photographed on an inverted microscope and analyzed using Image J software.

4.2.14 Cell Invasion Assay

Invasive ability of the cells was determined by analyzing number of cells invading through the matrigel using Bowden Chambers. 100µl of matrigel was added onto the inner side of the chambers and incubated at 37^0C for 1hour. 600µl of complete medium was then added onto 24well notch plates and the chambers were placed over it such that no bubbles were present. 20,000 cells in complete medium were then seeded in the chamber and incubated at 37^0C for 20 hours.

Staining of the Invasion Chambers:

After 20 hours the cells were fixed in 4% Paraformaldehyde by incubating at RT for 15mins. The inner lining of the chambers were then gently wiped to remove uninvaded cells and the chambers were then washed twice with 1X PBS. The invaded cells in the membrane were then stained with DAPI for 5mins and visualized using LSM 780 Zeiss Confocal Microscope. The number of cells invaded cells were calculated by counting cells in 10 random fields.

4.2.15 Wound Healing Assay for Migration

Cells were grown in 6 well dishes to a confluence of 90%. and serum starved for 16 hours. Serum starvation would result in the absence of growth factors available to the cells and thus inhibit proliferation, allowing the migration of the cells to be calculated on the basis of the rate of wound healing. At 90% confluence three wounds each were made per well with 200µl tip and the cells were then washed with 1X PBS. The plate was then incubated in a 37^0C chamber and observed under time lapse in the Inverted Microscope (Axiovert 200M). Images were taken every 30mins for 21 hours at 10X using the AxioCam Camera (Zeiss). The cell migration was measured using Image J as percent wound healed.

4.2.16 MTT Cell Proliferation Assay

1000 cells each were seeded in 96 well microtitre plate in triplicates in 100 µl complete medium. 20µl of MTT was added to each well and the plates were incubated at 37^0C for 4hours. The metabolism of MTT by the cells results in the formation of formazan crystals which were solubilized by the addition of 100µl of 10% Acidified SDS. The absorbance was measured at 540nM in an ELISA plate reader after 24 hours. The absorbance of the cells was determined at 0 hour, 24 hours, 48 hour and 72 hours and growth curve was plotted using three independent experiments, to determine the cell proliferation.

4.2.17 Radiation Treatment

Cells were seeded onto 35mm dishes and radiated once 60% confluence was achieved. The D0 dose of radiation was delivered using Co-60-γ-radiator, Bhabhatron 2 and the cells were then incubated at 37^0C. The cells were then harvested at various time points and RNA was extracted as described previously.

4.2.18 Immunoprecipitation

To determine if mutant p53 binds to p63 in AW8507, p53 and p63 were co-immunoprecipitated in AW8507. On the first day 15µl of beads were washed thrice with 600µl of NETN Buffer (100mM NaCl) and centrifuged at 4^0C for 1min. The beads were then incubated with the 2µg of primary antibody (p53 mouse IgG2) in 600 µl of NETN buffer. The beads were then incubated overnight in rotating centrifuge at 4^0C.

Cells were trypsinized from two 100mm confluent dishes and given 2 washes with 1X PBS without phenol red. Cells were lysed with EBC lysis buffer containing 10% protease inhibitor cocktail and incubated on tube rotator for 30mins at 4^0C. The lysates were then centrifuged at 4^0C at 13,000 rpm for 15mins and the supernatant transferred to fresh tube. The protein extracted was estimated by Folin Lowry method. The lysates were then precleared by incubating with 10µl of beads equilibrated in NETN. 600 µl of protein lysate was added to each tube (at least 1mg of protein used) while 40µl of lysate was kept separately and loaded as input. The tubes were incubated with beads on the tube rotator for 1hour at 4^0C. The beads were then centrifuged at 2000 rpm for 1min at 4^0C. After preclearing the lysates were aspirated and added to the control and test beads that had been incubated previously with the primary antibody. The control used was the isotype control for the primary antibody. The lysates were incubated with the primary antibody for 4hours and then centrifuged at 200rpm for 1min at 4^0C. The beads were then

washed thrice with NETN buffer (300mM). 15µl of 3X sample loading buffer while 10 µl 3X loading buffer was added to the 40µl input. The samples were boiled for 2 mins at 100^0C and loaded onto a 12% gel. The proteins were transferred onto a PVDF membrane as described earlier and probed with p63 antibody (ab111449).

4.2.19 Chromatin Immunoprecipitation Assay (ChIP)

DNA Shearing

AW8507 cells were grown to 90-100% confluence, and cross linked with 1% formaldehyde for 10mins at 37^0C (270µl 37% formaldehyde to 10ml of growth medium). The medium was decanted and cells were washed twice with ice cold PBS containing 10% Protease inhibitors. Cells were scraped in 1ml PBS and collected in conical tube. The cells were then pelleted at 2000rpm at 4^0C for 4mins. 200µl of SDS buffer was added per $1*10^6$ and incubated on ice for 10mins. The lysates were sonicated in Bioruptor to shear DNA such that the sheared DNA length was between 200-1000bp. For AW8507, the sonication was standardized at 12 cycles, 30s on and 30s off.

Immunoprecipitation

The sonicated samples were centrifuged for 10mins at 13000 rpm at 4^0C and the supernatant was transferred to a new 2ml microcentrifuge tube. The sonicated cell supernatant was diluted 10 fold in ChIP Dilution buffer with protease inhibitor (1800 µl ChIP dilution buffer to 200 µl sonicated cell supernatant). The 2ml diluted cell supernatant was precleared with 75 µl of Protein A Agarose beads for 30mins at 4^0C with agitation. The agarose was pelleted by centrifugation at 1000 rpm for 1min at 4^0C. 2% of the supernatant was separated to be loaded as input. The immunoprecipitating antibody (p53 mouse antibody) was added to the 2ml supernatant fraction and incubated overnight at 4^0C with rotation. For the isotype control, IgG2 antibody was added

to the 2ml supernatant fraction and incubated overnight. The next day the supernatant containing the immunoprecipitating antibody was incubated with 75 µl of agarose beads and incubated in rotating centrifuge for 2hours. The agarose was then pelleted by centrifugation at 1000 rpm for 1min at 4^0C. The agarose beads were then given one wash each of Low Salt Immune Complex Wash Buffer, High Salt Immune Complex Wash Buffer, LiCl Immune Complex Wash Buffer and two washes of 1X TE. Briefly 1ml of the buffer was added to the beads and incubated at 4^0C on rotating platform for 4mins, then centrifuged as described previously. One fraction each of the p53 containing antibody and the isotype control was processed for western blotting, while a second fraction of each was utilized for DNA extraction.

The fractions to be processed for immunoblotting were centrifuged after the last wash for 1min at 1000 rpm at 4^0C. 25µl of 1X Laemmli buffer was added to each sample and boiled for 10mins. The samples were then loaded onto 12% PAGE gel and probed for p63 as described in the immunoprecipitation protocol.

DNA Extraction and PCR:

The elution buffer was prepared freshly and 250µl of elution buffer was added to the pelleted beads, vortexed briefly and incubated on a rotating platform for 15mins at RT. The agarose beads were then centrifuged and supernatant transferred to fresh tube and the elution was again repeated. To the combined 500µl eluate 20µl 5M NaCl was added and incubated at 65^0C overnight to reverse the crosslinks. The input fraction kept earlier was also reverse crosslinked at this step. After reversing the cross links 10µl 0.5M EDTA, 20 µl1M Tris-HCl and 2µl 10m/ml Proteinase K was added to the eluates and incubated at 45^0C for 1 hour. DNA was then extracted using the Qiagen DNA extraction Kit. 200 µl of Buffer AL was added and incubated at 56^0C for 10mins. 200 µl ethanol was added and placed on the 2ml collection tube. The tubes were then

centrifuged at 8000rpm for 1min, the flow through was discarded and the spin columns were transferred to a fresh collection tube. 500 µl of buffer AW1 was applied to the spin tube, the tubes were then centrifuged for 1min. The spin column was again transferred to a new collection tube and 500 µl of Buffer AW2 was applied, tubes were centrifuged for 3min at 14000 rpm. The flow through was discarded and the spin tubes were transferred to a new microcentrifuge tube. The DNA was eluted using 20 µl of Buffer AE applied to the centre of the spin column, incubated for 1min at RT and then centrifuged for 1min at 8000rpm. The DNA was again eluted using 20 µl Buffer AE. The DNA was quantitated using Nano drop.

PCR for Survivin Promoter Binding sites

PCR for the p21 control gene was then run with the Input, control and the p53 immunoprecipitated DNA. The p53 consensus sites on the survivin promoter were identified using ALGEN Promoter Sequence [221, 222]. The transcription factor binding sites were predicted using the TRANSFAC database.

The primers were designed such that the p53 binding sites on the survivin extended promoter were amplified. The list of the primers and their annealing temperatures are listed in Table 4-13.

Table 4-13: List of primers for the p53 binding sites on survivin promoter.

Primer	**Sequence (5' to 3')**
SurChS1 Forward	GGTGGGAGAAGAGAGGGCAA
SurChS1 Reverse	AAGGCAGGAGTCAGGATGGT
SurChS2 Forward	TGCGTCTAGACATGCGGATA
SurChS2 Reverse	AACCGAGTTCCAGGAGCTTC
SurChS3 Forward	TGAAGCTCCTGGAACTCGGT
SurChS3 Reverse	CAGGCACAGACCAAGGACGC

SurChS4 Forward	GTGGTGCGTCCTTGGTCTGT
SurChS4 Reverse	CTCTCTGCTTCTTCCTCCCT
SurChS5 Forward	ATTCTTTGTACTATTCTTGC
SurChS5 Reverse	ACTGTCTTTTTCATTTTGGG
SurChS6 Forward	TGAAAAAGACAGTGGAGGCA
SurChS6 Reverse	ACAAATGAACAGGGGAGGGA
SurChS7 Forward	CACTCCATCCCTCCCCTGTT
SurChS7 Reverse	AATCTGGCGGTTAATGGCGC
p21 (control) Forward	CACCACTGAGCCTTCCTCAC
p21 (control) Reverse	CTGACTCCCAGCACACACTC

4.2.20 cDNA Microarray

Global gene expression analysis post p63 knockdown was determined by cDNA microarray using the Affymetrix GeneChip Human Primeview Array on a 64 midi array format (iLife Discoveries, Gurgaon India). The RNA was extracted from the provided cell pellets using RNEasy Mini Kit, Qiagen. The probes and template were hybridized for 16 hours at 60 rpm at 45^0C. The arrays were then scanned using the Affymetrix GeneChip Scanner 3000 7G.

Data Analysis: Preprocessing and Normalization

The raw data sets were obtained from the raw intensity file (Cel files) obtained after scanning and were analyzed using GeneSpring GX12.6 software. The cel files were preprocessed using the RMA (Robus Multichip Average) algorithm consisting of background adjustment, normalization and summarization. Genes of low intensity information were then filtered by excluding probe of intensities less than 20 percentile in the raw data. The raw values indicate the linear data obtained after thresholding and summarization which is performed by computing the geometric mean. The normalized value is generated by log transformation and normalization and baseline transformation. For normalization control probes were included in the array and the

probe values were utilized only if an exact match was obtained between the technology and sample for the probes. The average intensities of the values among the replicates were then taken for further analysis.

In AW8507 from a total of 49,395 probesets; 44,296 probesets were usable after data preprocessing and normalization. Baseline transformation was performed by taking a median of all the probesets. Box and whisker plots were created using the normalized intensity values of the probe. The median in the box and whisker is shown in the middle of the box and depicts the 25th and 75th quartile.

Similarly in SCC040 for data preprocessing probes of intensity less than 10.0 percentile in the raw data were excluded from the analysis. For SCC040 data preprocessing and normalization yielded 44,205 probesets from a total of 49,395 probesets. Here again transformation was shifted 20-75% for raw intensity data in the GeneSpring tool on the basis of the median value.

Figure 4-1 and Figure 4-2 show the box and whisker plots of the normalized data for AW8507 and SCC040 control and p63 knockdown samples

Figure 4-1: Box Whisker plot depicting the RMA normalized expression values for AW8507 control and AW8507 knockdown in duplicates.

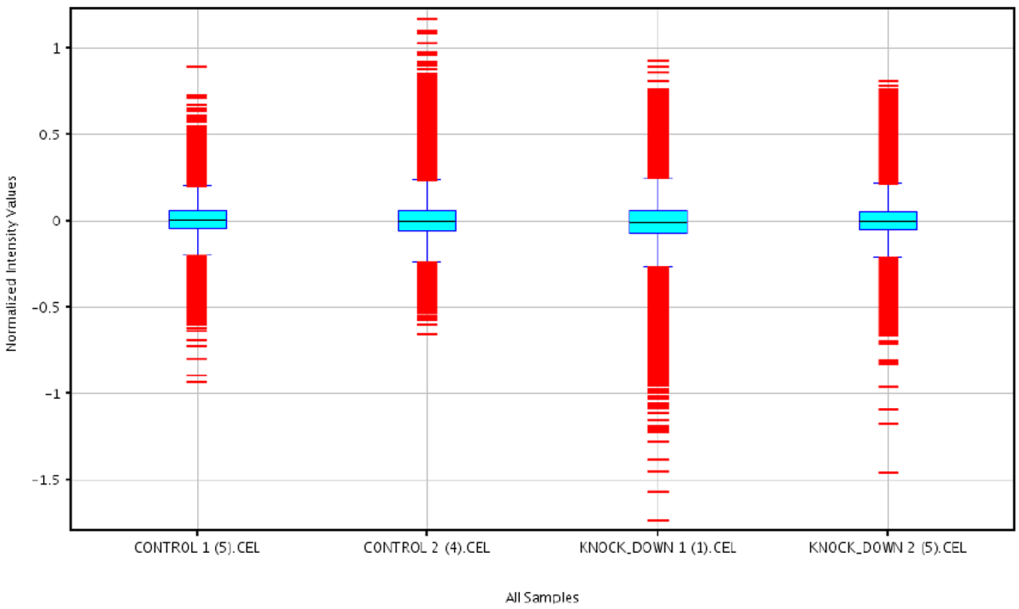
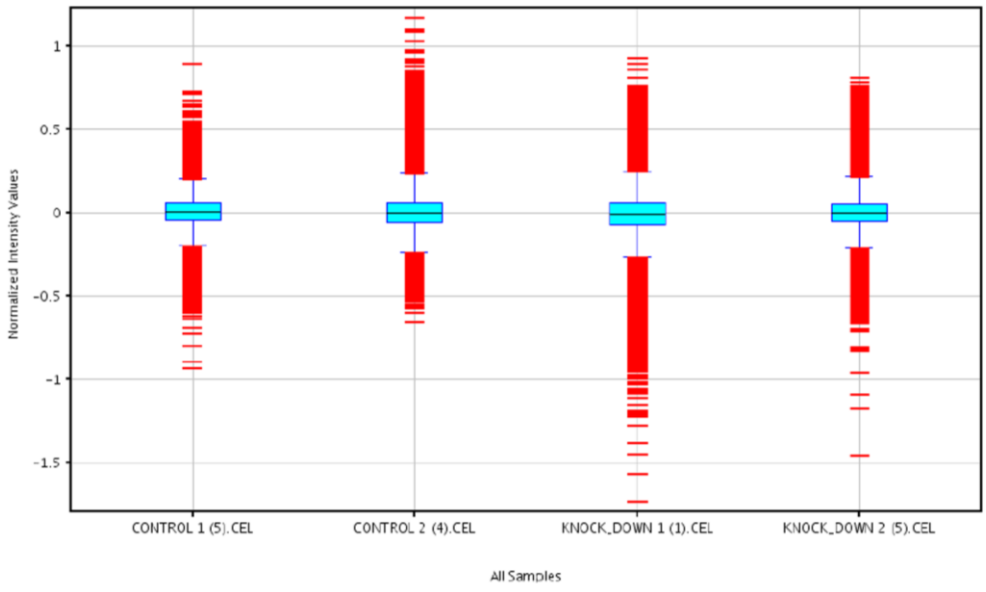


Figure 4-2: : Box whisker plot of the RMA normalized expression values of SCC040 control and p63 knockdown.



After normalization the duplicates were grouped according to the experiment conditions. Replicates were found to be placed in the same group after which the normalized intensities were

taken over the replicate sets. Figure 4-3 shows the profile plots summarizing the overall expression pattern of the data sets for AW8507 and SCC040. The probesets and genes are represented separately in the form a line graph. The X axis represents the experiment name while Y axis shows the normalized intensity value for the experiment.

Figure 4-3: Profile plots of control and p63 knockdown in (A) AW8507 and (B) SCC040.

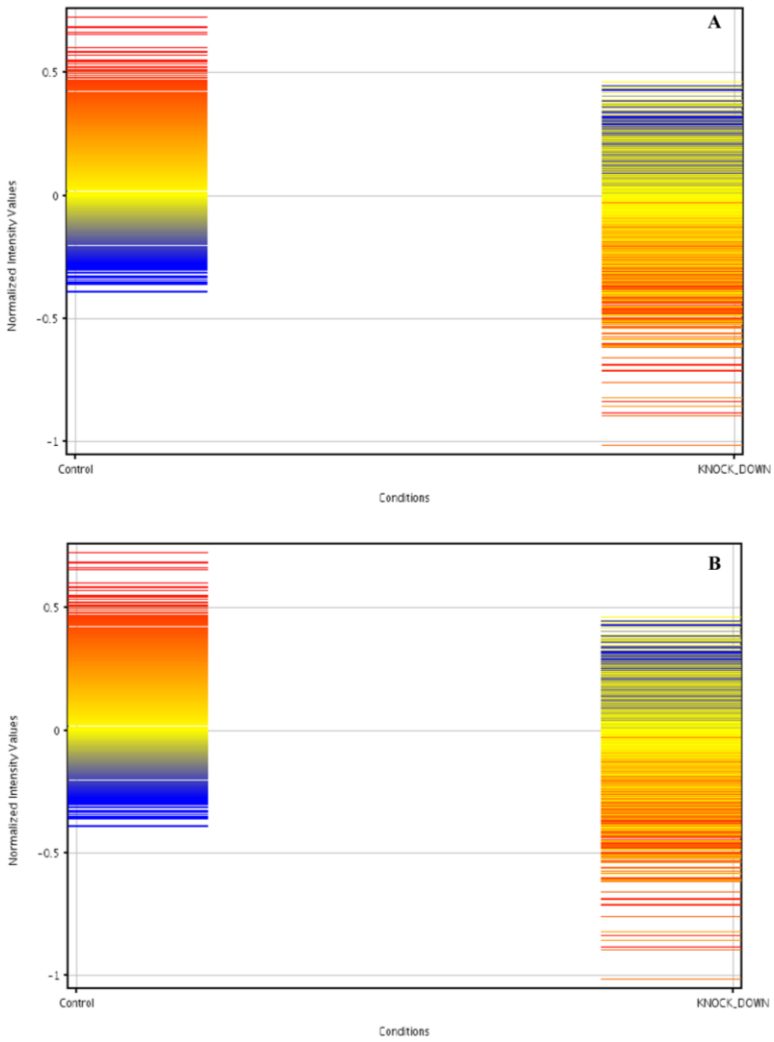


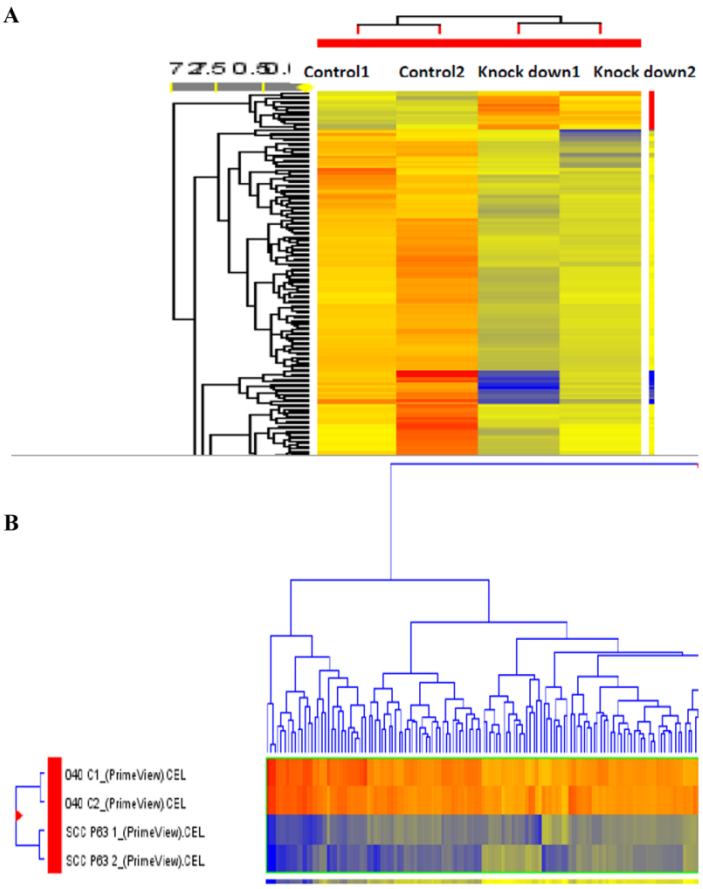
Figure 4-3: Profile plots of control and p63 knockdown in (A) AW8507 and (B) SCC040.The plot shows the expression patterns of genes, with the Y axis showing the normalized ratio and X axis representing the

experiment set name. The normalized expression value >0 indicates overexpressed genes and <0 indicates under expressed genes.

Cluster Analysis

Cluster analysis was performed to identify and group the genes having similar expression pattern. Here hierarchical clustering was performed for analysis of gene expression data. Hierarchical clustering is an unsupervised clustering technique that follows and agglomerative approach wherein similar expression profiles are joined to form a group. The profiles are joined further in a tree structure and are represented in the form of a dendogram. The hierarchical clustering was performed on conditions and genes to explore the coexpression or co-regulation of genes in the control and knockdown groups. the cluster image is represented in the form of a dendogram and the expression is color coded. Overexpression is indicated by red color, blue color indicates underexpression while yellow color indicates normal expression of the genes. The genes having similar expression profile are clustered under adjacent or same tree node. Figure 4.4 is a representative image of the hcl dendogram obtained in AW8507 and SCC040

Figure 4-4: Representative view of the hierarchical cluster tree (A) AW8507 and (B) SCC040.



Red color shows overexpressed genes, blue color denotes underexpressed genes.

Pathway Analysis and Functional Annotation

After the analysis and identification of the differentially expressed genes (DEGs), genes which were altered more than 1.5 fold in the p63 knockdown compared to control were functionally annotated using PANTHER [223], REACTOME [224] and KEGG databases [225].

4.2.21 Statistical analysis

Statistical analysis was performed on SPSS 15.0. Normality distribution was determined using the Shapiro Wilk test. Associations were determined using Chi squares for categorical variables,

and using the Students t test for numerical variables. Correlation between the expression of the various isoforms was determined using Pearson correlation for normally distributed samples and Spearman correlation for non Gaussian distribution.

4.3 Composition of Buffers and Chemicals used in the study.

Folin Lowry Protein Estimation:

a. Copper tartarate carbonate (CTC) Solution

0.4% hydrated copper sulphate ($CuSO_4$. $5H_2O$) (0.1g in 25ml distilled water)

0.4% potassium sodium tartarate (0.1g in 25ml distilled water)

20% sodium carbonate. (10g in 50ml distilled water)

20% Na2CO3: 0.2% CuSO4.5H2O : 0.2% Sodium Potassium Tartarate

2 : 1 : 1

b. CTC Working solution

CTC : 0.8N NaOH : 10% SDS : Distilled water

1 : 1 : 1 : 1

c. Folin-Ciocalteau working solution was freshly prepared. Working solution was (1:6) diluted (v/v) in distilled water.

SDS PAGE and Western Blotting

a. 30 % Acryl amide Mix (100 ml)

Acryl Amide : 29.2 g

Bis-Acryl Amide : 0.8 g

Make up volume to 100ml

b. 5X Electrode Buffer (1000 ml)

Tris base	: 15.1 g
Glycine	: 72.06 g
SDS	: 5 g

Make up volume to 1000 ml

c. Destainer (For 100 ml)

Methanol	: 45 ml
Distilled Water	: 45 ml
Glacial Acetic Acid	: 10 ml

d. 0.1% Fast Green

Fast Green Dye	: 100 mg
Destainer	: 100 ml

e. 0.1% Ponceau

Ponceau dye	: 0.1gm
Destainer	: 100ml

f. 10X TBS**;** pH 7.6 (For 1000 ml)

Tris Base	: 24.2 g
NaCl	: 80.0 g

Make up volume to 1000ml

g. 0.1% TBST

1ml Tween 20 added to 1000ml of 1X TBS

h. Transfer Buffer (3 Liters)

Tris Base	: 9.0 g

Glycine : 39.5 g

Methanol : 600 ml

Make up volume to 100ml

i. Stripping Buffer (50 ml)

100 mM Beta mercapto ethanol : 0.815 g

10% SDS : 10 ml

1M Tris-Cl (pH 6.8) : 3.125 g

Distilled Water : 36.06

j. Sample Loading Dye

Components	Concentration	Stock	For 100 ml
Tris pH 6.8	50 Mm	1 M	5 ml
Glycerol	10%		10 ml
SDS	2%	10%	20 ml
BPB	0.1%		0.1 gm

Make up volume to 100ml

For 950 µl of the sample buffer, 50 µl of the BME was added

Mammalian Cell Culture

a. Incomplete IMDM

IMDM (Iscoves Modified Dulbecco's medium)

$NaHCO_3$ - 3.024 g/lit

b. Incomplete DMEM

DMEM (Dulbecco's Minimal Essential Medium)

$NaHCO_3$ – 3.7 g/lit

c. Complete Medium

Incomplete IMDM/DMEM : 90 ml

Fetal Bovine Serum : 10 ml

Antibiotic solution : 1 ml

d. Antibiotic solution

Amphotericin B (2.5 mg/ml) : 0.2 ml

Penicillin (50000 units/ ml) : 2.5 ml

Streptomycin (100000 units/ ml) : 0.4 ml

Distilled water : 6.9 ml

e. Trypsin –EDTA (1000 ml)

EDTA Sodium salt : 0.1 g

D-glucose : 1.0 g

KCl : 0.4 g

NaCl : 8.0 g

Phenol red : 10.0 g

$NaHCO_3$: 0.58 g

Trypsin : 0.25 g

f. 0.4% Erythrosin B

0.4 g of Erythrosin B powder in 100 ml of 1X PBS

h. 10X Phosphate Buffer (PBS) (1000 ml)

NaCl : 80 g

KCl : 2.0 g

Na_2HPO_4 : 11.5 g

KH_2PO_4 : 2.0 g

Make up volume to 1000 ml

Co-Immunoprecipitation

a. EBC lysis buffer (100ml)

1M Tris pH 8.0 : 5ml

2.5M NaCl : 5ml

NP-40 (Sigma) 0.5% : 0.5ml

Distilled Water : 89.5ml

b. NETN 100mM NaCl (100ml)

1M Tris pH 8.0 : 2ml

2.5M NaCl : 4ml

0.5M EDTA pH 8.0 : 0.2ml

NP-40 (Sigma) : 0.5ml

Distilled Water : 93.3ml

c. NETN 100mM NaCl (100ml)

1M Tris pH 8.0 : 2ml

2.5M NaCl : 12ml

0.5M EDTA pH 8.0 : 0.2ml

NP-40 (Sigma) : 0.5ml

Distilled Water : 85.3ml

d. 3X Laemmli's buffer (100ml)

1M Tris pH 6.8 : 15ml

Glycerol : 30ml

SDS : 6g

Bromophenol blue : 0.3g

Make up volume to 100 ml

Agarose Gel Electrophoresis

5X Tris Borate EDTA buffer (TBE) (100 ml)

Tris Base : 54

Boric Acid : 27.5

0.5M EDTA pH 8 : 20ml

Make up volume to 100 ml

MTT Proliferation Assay

MTT powder : 5mg

1X PBS : 1ml

Incubate in dark.

Bacterial Culture

Plasmid Preparation

a. Solution I

1 M Glucose	5.0 ml
1 M Tris (pH 8)	2.5 ml
0.5 M EDTA; pH 8	2.0 ml
Distilled Water	90.5 ml

b. Solution II (Prepared fresh)

2 M NaOH	1.0 ml

10%SDS 1.0 ml

Distilled Water 8.0 ml

c. Solution III (Prepared fresh)

5 M Potassium acetate 30.0 ml

Glacial acetic acid 5.75 ml

Distilled Water 14.25 ml

d. Phenol : Chloroform : Isoamylalcohol = 25:24:1

5 . Results

5.1 To profile p63, p73, survivin and clusterin isoforms in oral cancer cell lines and/or tissues and assess their association with clinicopathological parameters.

5.1.1 Expression profile of p53 family proteins, p53, p63 and p73 in oral cell lines and tissues.

The expression of p53, p63 and p73 transcript and protein was determined in four oral cancer cell lines, one dysplastic oral keratinocyte and two immortalized cell lines (FBM and HaCaT). The transcript expression of the ΔN and TA isoforms of p63 and p73 in the cell lines was determined by semi quantitative PCR as shown in Figure 5-1. The ΔNp63 and TAp73 isoforms are predominantly expressed in the oral cell lines as compared to TAp63 and ΔNp73 isoforms respectively.

Figure 5-1: Expression of ΔN and TA isoforms of p63 and p73 in oral cell lines

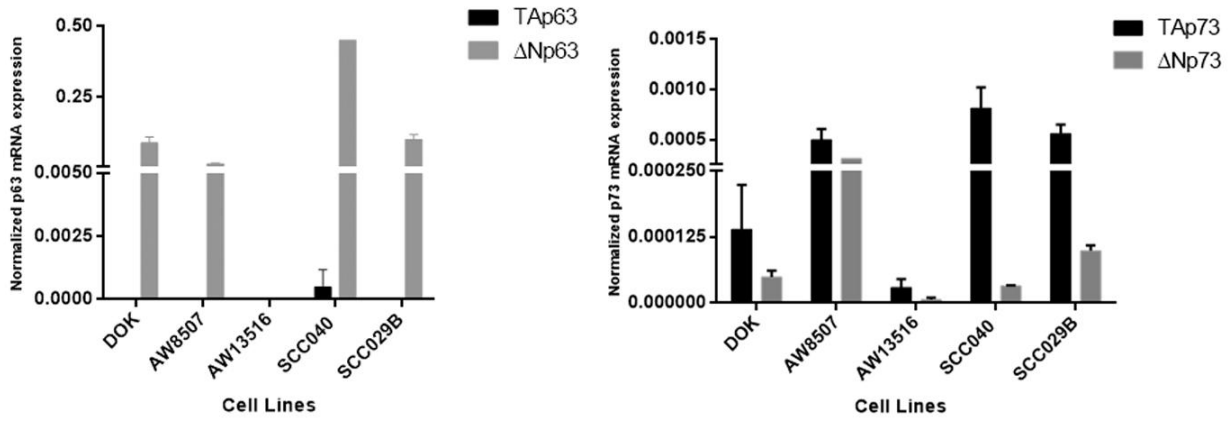


Figure 5-1: Expression of ΔN and TA isoforms of p63 and p73 in oral cell lines. Y axis denotes the relative expression of (A) p63 and (B) p73 in oral cell lines determined by real time PCR analysis. Expression was normalized using GAPDH and RPS as the internal control. In cell lines ΔNp63 was found to be overexpressed as compared to the TAp63, while TAp73 was predominantly expressed as compared to ΔNp73.

The expression of the p53 family proteins was also determined by western blotting. Figure 5-2 shows the protein profile for the p53 family proteins in the oral cell lines used in the study. p53 was detected in all the oral cell lines except UPCI-SCC040. p63 however was detected only in DOK and HaCaT and two oral cancer cell lines; AW8507 and UPCI-SCC040. p73 was found to be expressed in five of the seven oral cell lines studied. Interestingly, AW8507 which shows presence of mutant p53 and UPCI-SCC040 which does not show the presence of p53 protein both showed the expression of p63 and hence were used for further studies to investigate the role of p63 in oral cancers.

Figure 5-2: Expression of p53, p63 and p73 proteins by western blotting in oral cell lines

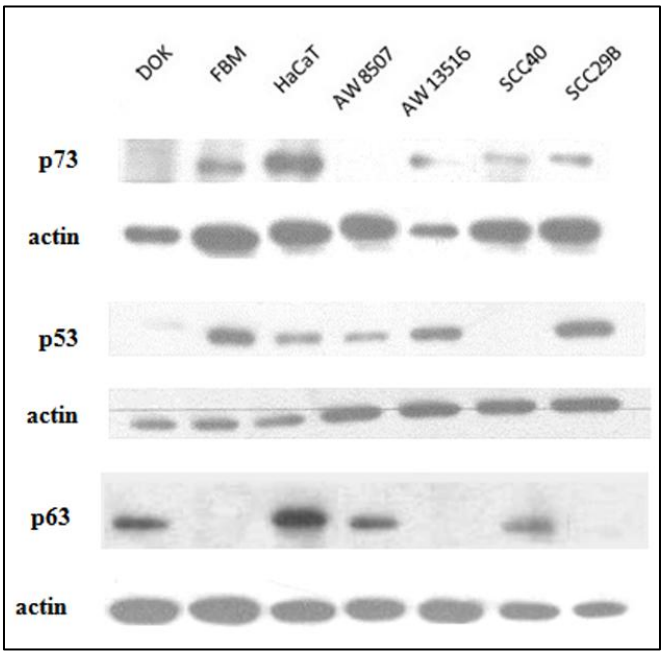


Figure 5-2**:** Expression of p53, p63 and p73 proteins by western blotting in oral cell lines. p53 was expressed in six of the seven cell lines while p63 was detected in four and p73 in five of the seven oral cell lines studied. Actin was used as loading control.

The expression of the ΔN and TA isoforms of p63 and p73 was also determined in 20 oral tumor and adjacent normal tissues. As observed in cell lines, ΔNp63 was predominantly expressed as compared to TAp63 in oral tumor tissues as well. Interestingly ΔNp63 was upregulated while TAp63 was downregulated in oral tumor tissues as compared to adjacent normal tissues. p73 expression on the other hand was found to be very low in comparison to p63 expression and did not show a difference in oral tumor tissues as compared to adjacent normal tissues (Figure 5-3).

Figure 5-3: Expression of TA and ΔN isoforms of p63 and p73 in oral tissues

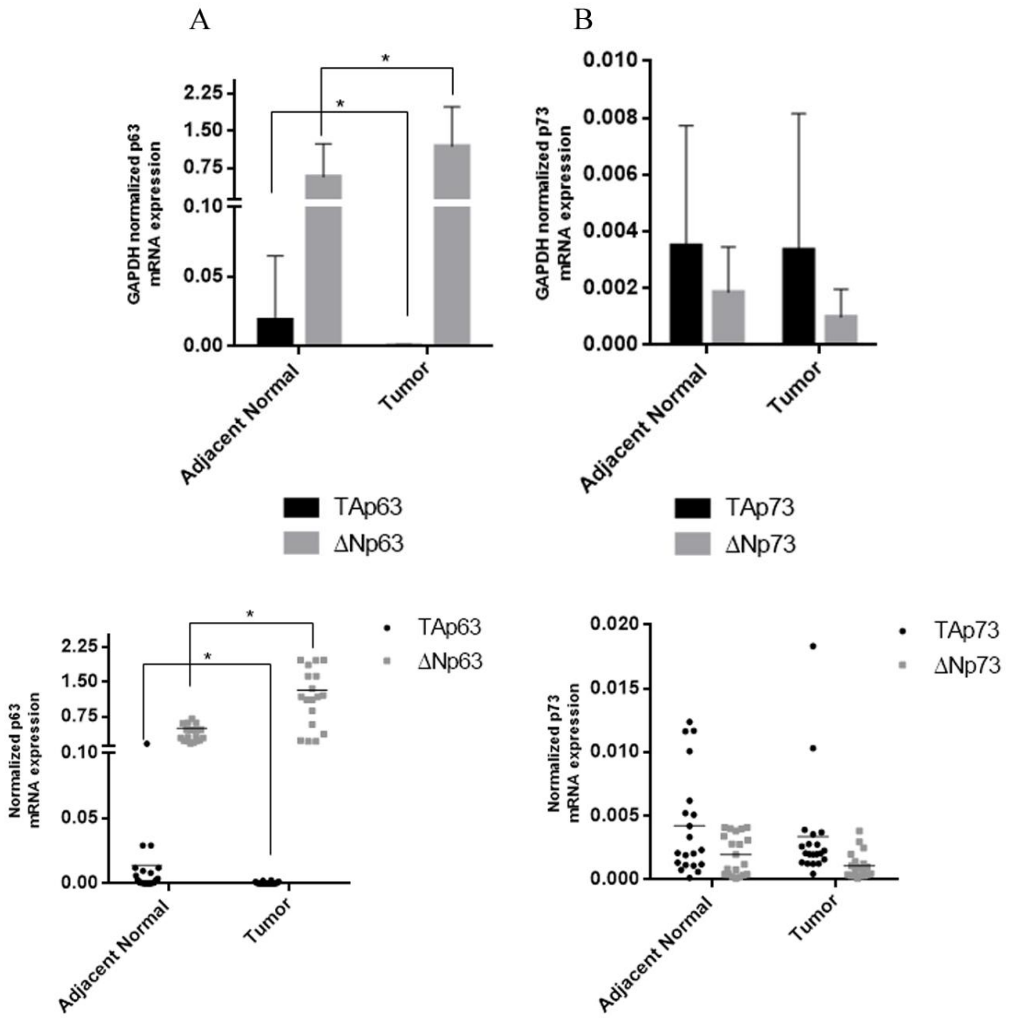


Figure 5-3: Expression of TA and ΔN isoforms of p63 and p73 in oral tissues. The expression of the isoforms of p63[A] and p73[B] was determined in 20 oral tumor and adjacent normal tissues. Error bars

represent standard error of mean. ΔNp63 was predominantly expressed over TAp63 in tissues as observed in cell lines and an upregulation of ΔNp63 was observed in oral tumor tissues as compared to adjacent normal tissues. No significant difference was observed in the expression of the isoforms of p73 in oral tumor tissues as compared to the adjacent normal tissues. * indicates $p < 0.05$

5.1.2 Expression of clusterin in oral cell lines and tissues

Clusterin transcript expression was determined by real time PCR using primers directed against the secretory form of clusterin. Primers against the nuclear form of clusterin were also tested, however nCLU expression was not detected in any of the cell lines tested and since the transcript was retracted from NCBI, only the expression of sCLU was tested in cell lines and tissues. Real time PCR analysis showed expression of sCLU in all the oral cell lines studied which was also reflected at the protein level as studied by western blotting. The expression of sCLU transcript was also determined in 20 oral tumor and adjacent normal tissues. Interestingly, although sCLU expression was observed in all the cell lines at transcript and protein level, in tumor tissues the clusterin transcript was found to be downregulated as compared to the expression in adjacent normal tissues.

Figure 5-4: Expression of clusterin transcript and protein in cell lines and tissues

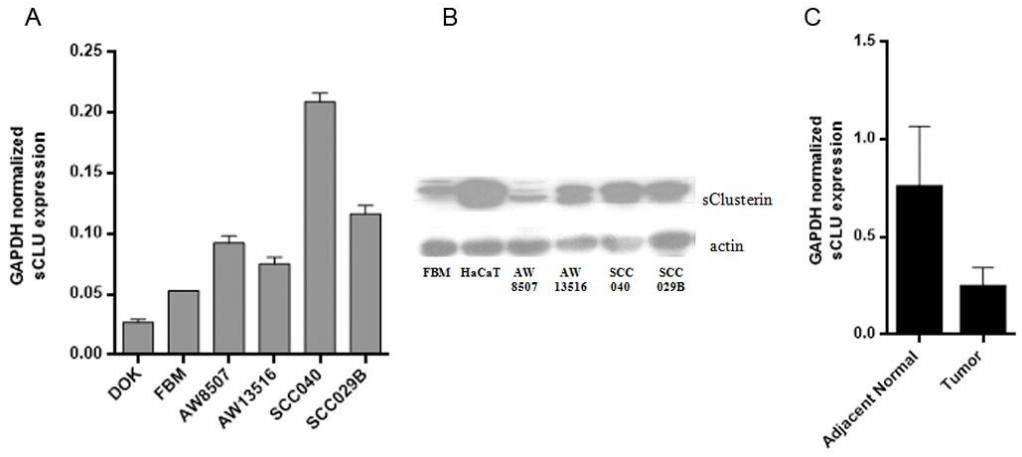


Figure 5-4: Expression of clusterin transcript and protein in cell lines and tissues. (A) Expression of sCLU in cell lines showed clusterin expression in all the cell lines with higher expression in two oral cancer cell lines, SCC040 and SCC029B. (B) Western blot analysis showed clusterin protein expression in all the cell lines studied. (C) Downregulation of sCLU transcript expression is observed in oral tumor tissues as compared to adjacent normal tissues (n=20).

5.1.3. Expression of survivin and its isoforms in oral cell lines and tissues.

The expression of the six survivin isoforms was determined by real time PCR in four oral cancer cell lines, one dysplastic cell line and two immortalized oral cell lines. As is shown in Figure 5-5, among the survivin splice variants, survivin wt was the predominantly expressed isoform in cell lines, followed by survivin ΔEx3 and survivin 2B. Survivin wt expression had a 13-fold higher expression in cell lines compared with normal tissues. Survivin 2 B and survivin DEx3 had close to 10-fold higher expression in oral cancer cell lines compared with normal tissues. However all the isoforms showed only 1.5 fold difference in expression in cancer cell lines as compared to tumor tissues. DOK expression showed 12 fold higher levels of survivin wt, and 1.5 fold higher expression as compared to normal tissues from healthy individuals. However the expression of

survivin wt in DOK was only 3 fold higher when compared to the expression in adjacent normal tissues. Currently isoform specific antibodies are not available for detecting endogenous levels of the survivin isoforms and hence only the total survivin protein is detected by western blotting. Our analysis showed survivin protein expression in all the cell lines studied albeit with differing levels.

Figure 5-5: Expression of survivin splice variants in oral cell lines.

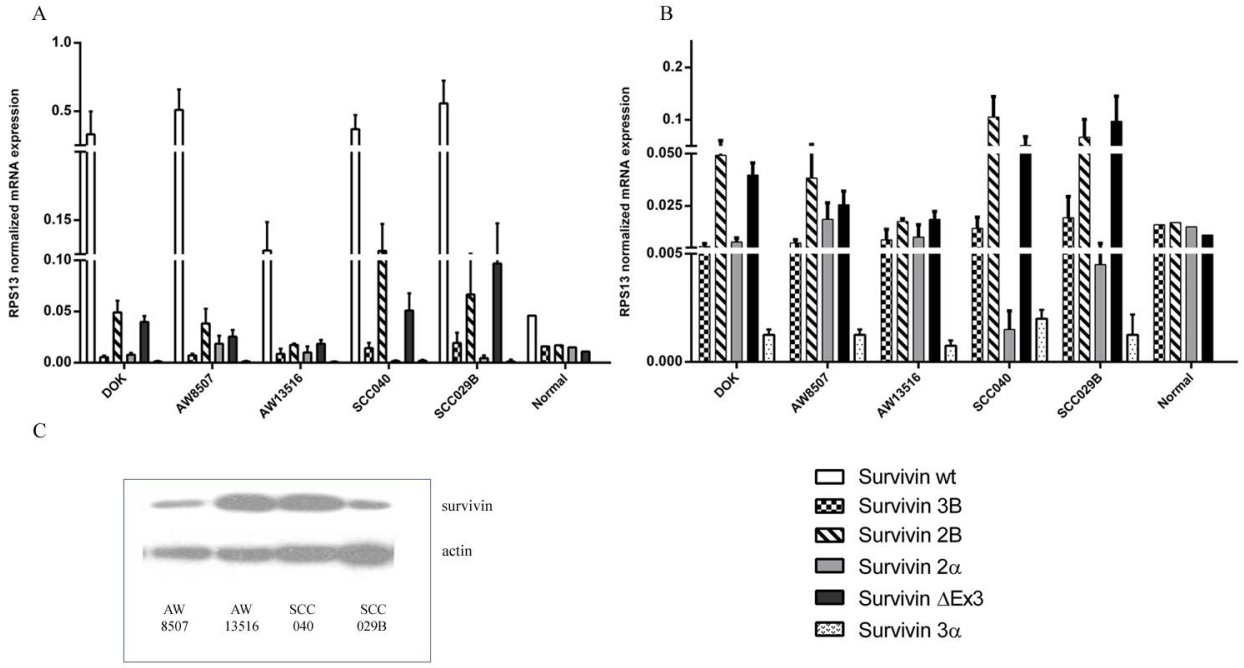


Figure 5-5: Expression of survivin splice variants in oral cell lines. (A) Normalized expression of the six survivin splice variants. Error bars represent the standard error of mean. Survivin wt was the predominantly expressed isoform in dysplastic as well as oral cancer cell lines. (B) Among the minor isoforms, survivin ΔEx3 and survivin 2B were dominantly expressed in all the cell lines. The expression of survivin 3α in the cell lines was low as compared to the other isoforms, it was however negligibly expressed in normal oral tissues. (C) Survivin protein expression in the four oral cancer cell lines used in the study. Actin was used as the loading control.

We also determined the expression of the six survivin isoforms in 75 oral tumor and adjacent normal tissues by real time PCR. Various studies have shown that in the case of oral cancers, since the entire oral cavity is exposed to the carcinogen, there may be certain molecular changes in the adjacent tissue as well [226-228]. Hence, to determine the expression of the survivin isoforms in normal tissues, we also analyzed the expression in 12 normal oral tissues from healthy individuals. The relative mRNA expression of the survivin isoforms in paired oral tumor and adjacent normal tissues and in 12 normal oral tissues from healthy individuals is shown in Figure 5-6. Survivin wt was found to be overexpressed in 80% cases when compared to adjacent normal tissues, whereas in comparison to the expression with normals from healthy individuals it was overexpressed in 88% cases. As observed in cell lines, in tissues survivin ΔEx3 and survivin 2B were found to be the next predominantly expressed isoforms with 64% and 76 % cases showing overexpression of survivin ΔEx3 and survivin 2B respectively. Survivin 3B showed overexpression in 33% cases while overexpression of survivin 2α and survivin 3α was observed in 41% cases 50% cases respectively. The fold change in expression of the survivin isoforms in tumors over normals showed upregulation of all isoforms in tumors, with survivin wt showing significant upregulation ($p < .01$) (Figure 5-7). As observed with normal tissues, the fold change of survivin wt, survivin 2 B, and survivin DEx3 also exhibited upregulation in tumors compared with adjacent normal samples. In comparison with adjacent normals survivin ΔEx3 was upregulated in 69% cases while Survivin 2 B and survivin 3α were overexpressed in 76% and 50% cases, respectively. Interestingly, however, the average expression of survivin 3 B and survivin 2α was lower in tumors compared with adjacent normal samples

Figure 5-6: The expression of survivin splice variants in paired oral tumor and adjacent normal tissues and in normal tissues from healthy individuals

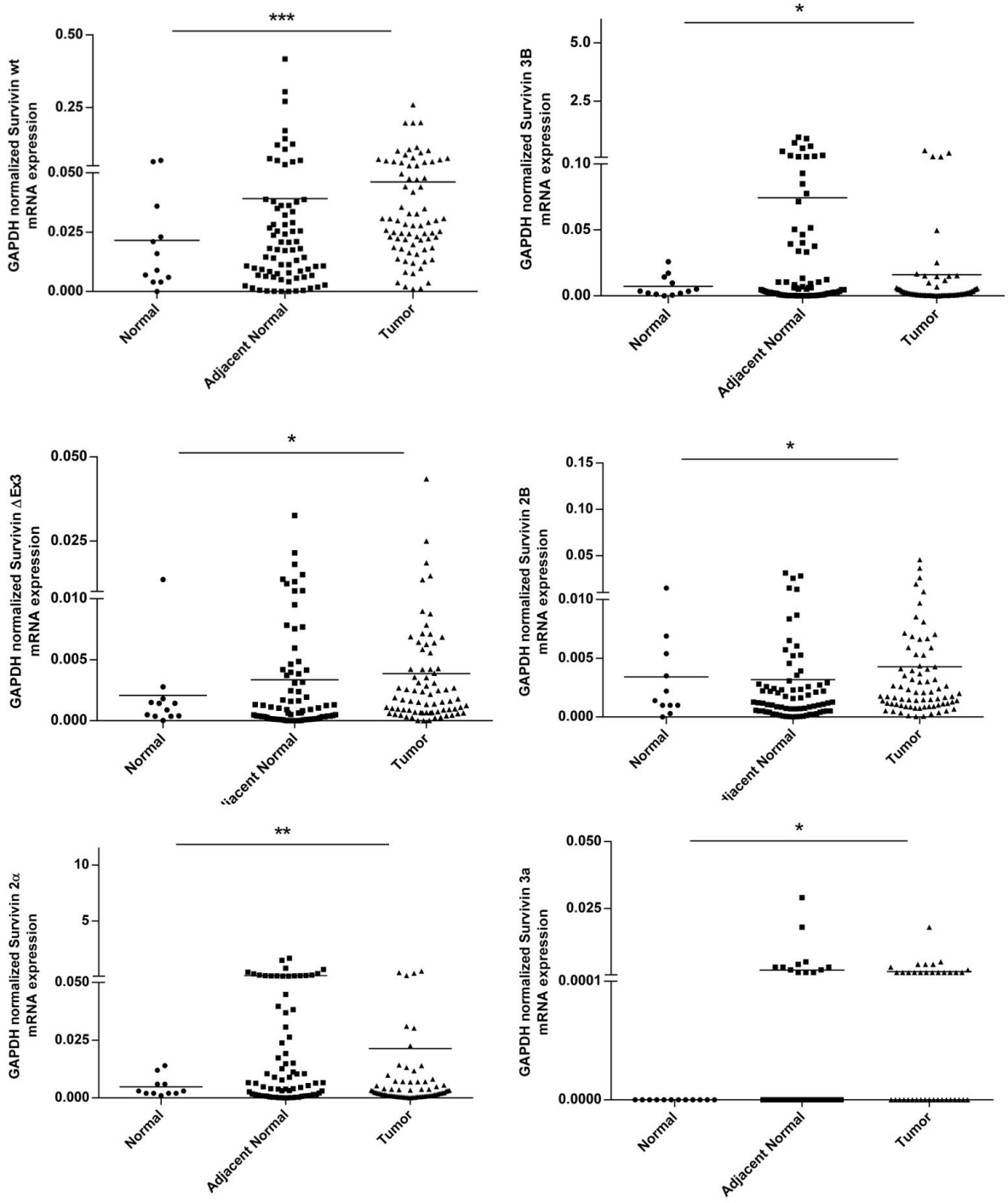


Figure 5-6: The expression of survivin splice variants in paired oral tumor and adjacent normal tissues and in normal tissues from healthy individuals. The expression of the six survivin splice variants showed

significant upregulation in tumor tissues as compared to the expression in normal tissues from healthy individuals. The expression of survivin 3B and survivin 2α was higher in the adjacent normal tissues as compared to the expression in normal as well as tumor tissues. *indicates $p < 0.05$, ** $p < 0.01$and *** $p < 0.001$

Figure 5-7: Fold change of survivin isoform expression in tumor tissues.

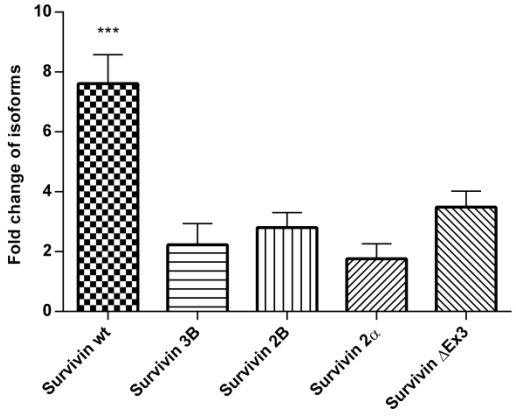


Figure 5-7: Fold change of survivin isoform expression in tumor tissues. The fold change of survivin isoform expression in tumor tissues compared with normal was calculated as $2^{-\Delta\Delta Ct}$. In tumor tissues, survivin wt showed an 8-fold upregulation over normal tissues. The minor isoforms of survivin showed <5-fold upregulation in tumor tissues compared with normal tissues. Error bars represent standard error of mean.

Thus the six survivin isoforms were found to be upregulated in oral tumors as compared to normals from healthy individuals. However the expression of survivin 2B and survivin 2α was higher in the adjacent normals as compared to tumors. In order to understand the clinical significance of these observations, the association of the survivin isoform expression with clinicopathological characteristics was determined by chi-square test. Survivin 3B expression

was found to be associated with poorly differentiated tumors which may imply a role for survivin 3B in an aggressive phenotype. Survivin wt is known to function as a dimer. The isoforms of survivin are thought to be capable of forming heterodimers with survivin wt and thus affecting it function. Three isoforms of survivin, viz. survivin wt, survivin 3B and survivin ΔEx3 are anti-apoptotic while survivin 2B and survivin 2α are proapoptotic. Thus the relative levels of the anti-apoptotic to the pro-apoptotic forms may regulate the function and role of survivin in the cell. Chi-square analysis of the overexpression of antiapoptotic forms of survivin and downregulation of the pro-apoptotic forms showed an association with TNM staging. Sixty-four percent of stage IV cancer showed an overexpression of at-least one anti-apoptotic isoform of survivin and downregulation of the pro-apoptotic forms. The ratio of the isoforms to survivin wt also showed a significant correlation among each other, although no association with clinicopathological parameters was observed.

5.1.4. Structural analysis of survivin isoforms and their heterodimerization with survivin wt.

The structure of the survivin wt homodimer has been reported by three groups [103, 229-231]. The homodimer of survivin wt is reported to have a bow-tie shaped arrangement and is anti-apoptotic. The structure of only one of the isoforms of survivin has been predicted by [114], while the structures of the other isoforms and their homodimerization with wt is not known. However one study has reported co-immunoprecipitation of survivin 2B and survivin ΔEx3 with survivin wt [22]. Survivin 2B is implied to be pro-apoptotic and heterodimerization of survivin 2B with survivin wt may attenuate the function of survivin wt. To determine the ability of the isoforms to heterodimerize with survivin wt, the structures of the survivin isoforms were first

modeled using I-Tasser Protein structure prediction, Zhang Labs, University of Michigan. Each of the predicted models was analyzed using SAVES analysis for Errat scores and Mol Probity analysis Ramachandran scores [232-234]. The scores of the models are shown in Table 5-1.

Table 5-1: Mol Probity Score for survivin isoform predictions and Errat score

Molecule	**Model**	**Mol Probity Score**	**Errat Score**	
Survivin 2B	M1	80.98	91.083	
	M2	76.69		
	M3	76.69		
	M4	*85.66*	*88.462*	*Loop Model*
	M5	82.82	85.713	
Survivin 3B	**M1**	**90.68**	**90.179**	
Survivin 2α	M1	88.89	90.769	
	M2	**93.06**	**95.455**	
	M3	81.94	95.385	
Survivin 3α	M1	86.84	86.567	
	M2	**90.69**	**97.101**	
Survivin ΔEx3	*M1*	*81.48*	*95.312*	*Loop Model*
	M2	81.48	85.496	
	M3	77.04		
	M4	81.48	95.349	
	M5	85.44	85.938	

The selected models for Survivin 3B, survivin 2α and Survivin 3α are indicated in bold. Survivin 2B Model 4 (M4) and Survivin ΔEx3 Model 1 (M1) were loop modeled using Mod loop.

As seen in Table 5-1, Survivin 3B Model 1, survivin 2α Model 2 and survivin 3α Model 2 were selected for docking and survivin 2B Model 4 and survivin ΔEx3 Model 1 were loop modeled

using Modloop [235]. Blind docking was performed using ClusPro for the selected models of survivin 3B, survivin 2α and survivin 3α with survivin wt [236]. Figure 5-5 shows the predicted structures of the selected models for survivin 3B, survivin 2α and survivin 3α and the structure of survivin wt monomer.

Figure 5-8: Predicted structures of survivin isoforms

Figure 5-8: Predicted structures of survivin isoforms. The predicted structures of survivin 3B, survivin 2α, survivin 3α and the survivin wt monomer are presented. The structures were viewed using PyMol. The alpha helical domain involved in cell cycle regulation is shown in red while the BIR domain which is essential for the anti-apoptotic function of survivin is shown in cyan.

The loop modeling for survivin 2B Model 4 and survivin ΔEx3 Model 1 is detailed in Table 5-2. The final structures used for further analysis are represented in bold.

Table 5-2: Loop modeling results for survivin 2B and survivin ΔEx3 using Mod Loop

Structure	Mol Probity Score (%)	Errat Score (%)
Survivin 2B Model 4	85.66%	88.462
Loop Model Cycle 1	88.34	95.972
Loop Model Cycle 2	**89.57**	**91.026**
Survivin ΔEx3 Model 1	81.48	95.312
Loop Model Cycle 1	82.96	96.094
Loop Model Cycle 2	85.19	97.638
Loop Model Cycle 3	87.41	96.850
Loop Model Cycle 4	88.15	95.480
Loop Model Cycle 5	**88.89**	**93.701**

The final selected models are depicted in bold.

Homodimerization of survivin wt monomers

The survivin dimer is reported to form a bow-tie shaped structure with the two helices resulting in a hydrophobhic pocket at the dimerization interface. The survivin dimer structure with the highest resolution of $2.17A^0$ 1F3H was used for analysis and comparison with the heterodimers. The dimer interface shows a symmetrical bonding with the Leu98 of one molecule interacting with the Leu 96 and Leu 98, and Phe 101. The surface visualization of the survivin dimer structure, 1F3H, is presented in Figure 5-9

Figure 5-9 : Survivin dimer 1F3H

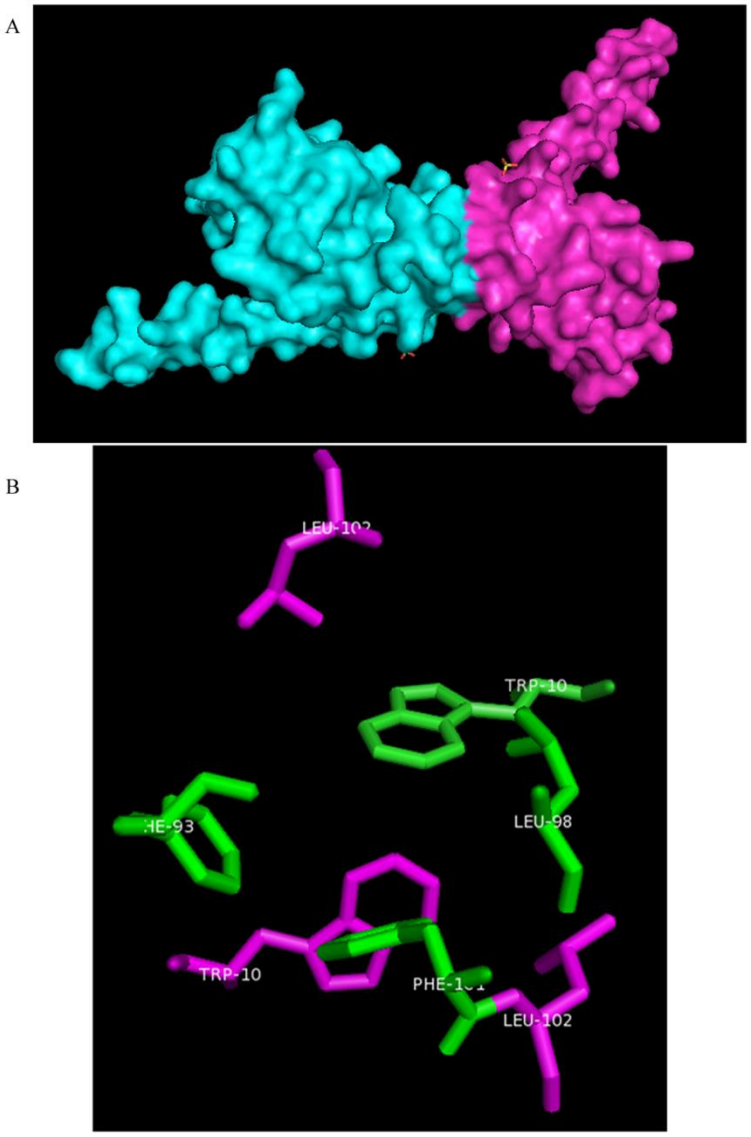


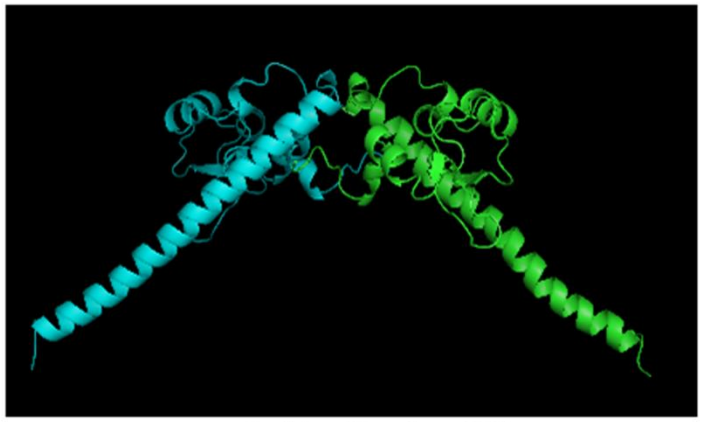
Figure 5-9: Survivin dimer 1F3H. (A) Surface view of the survivin wt dimer. The two survivin monomers are colored in Cyan and Magenta (B) The hydrophobic pocket of the survivin dimer depicting the hydrophobic residues.

Heterodimers of Survivin 2B : Survivin wt and Survivin 3B : Survivin wt

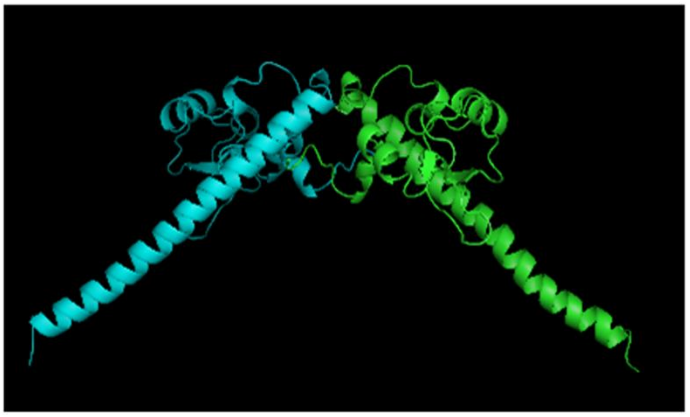
The loop modeled structures were then used for docking analysis with survivin wt in ClusPro. Survivin wt homodimer was also predicted using ClusPro and compared to the previously reported crystal structure for survivin; 1F3H [230]. The predicted survivin wt homodimer was comparable to the crystal structure of the homodimer reported in literature. The dimerization of the two survivin monomers was via the interaction of the linker peptide between the alpha helices and stabilized by the BIR domain.

Since the predicted homodimer was comparable to the reported crystal structure, the heterodimerization of the minor isoforms of survivin with survivin wt was also determined using ClusPro prediction. The docked models with the lowest energy were then visualized in PyMol. Analysis of the heterodimers identified that the survivin wt survivin 2B heterodimer and the survivin wt survivin 3B heterodimer dimerized using the same residue, Leu 98, as the homodimer of survivin wt. The linker peptide in the two isoforms was also involved in the heterodimer, indicating that the formation of these heterodimers may be competing with the homodimer formation. Figure 5-10 depicts the homodimer structure 1F3H and the structures predicted using ClusPro. Further invitro studies mutating the Leu98 residue may help understand the stability of the heterodimers of survivin 2B and survivin 3B with survivin wt.

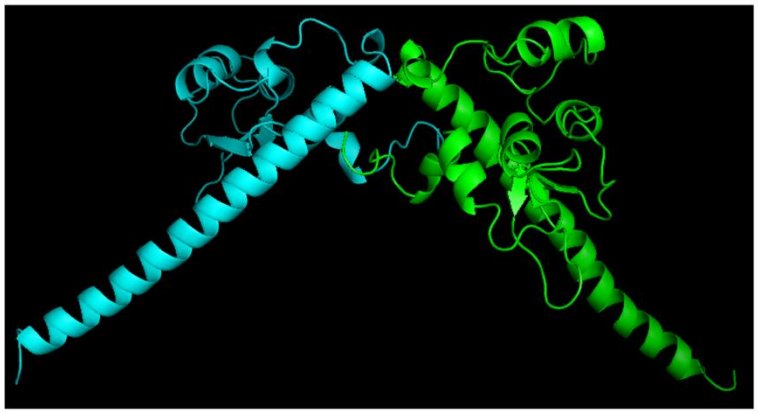
Figure 5-10: Docking analysis of survivin homodimer and heterodimer formation.



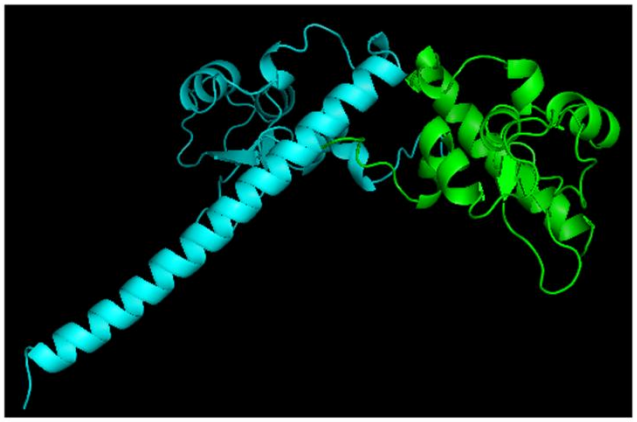
Survivin wt Homodimer 1F3H



Survivin wt Homodimer Predicted



Survivin wt Survivin 2B Heterodimer Predicted



Survivin wt Survivin 3B Heterodimer Predicted

Figure 5-10: Docking analysis of survivin homodimer and heterodimer formation. Survivin wt is depicted in cyan. In the heterodimers survivin 2B and survivin 3B is presented in green.

Docking analysis of the other minor isoforms also showed heterodimerization of survivin wt with the minor isoforms. However due to the absence of the C terminal alpha helix in survivin 2α and survivin 3α, the dimerization interface involves the BIR domain. In survivin ΔEx3 although a truncated alpha helix is observed, the lowest energy structure is formed by the packing of the survivin wt and survivin ΔEx3 alpha helical structures.

5.2 To examine whether p63 regulates survivin and/or clusterin expression in oral cancer cells in the background of a mutant or no p53 protein using siRNA strategies and luciferase assays.

The expression of p53 family proteins, survivin and clusterin was determined in four oral cancer cell lines. Among these AW8507 and SCC040 showed the expression of p63 protein. AW8507 is known to harbor an R273H mutation in p53 whereas p53 was not detectable at the protein level in SCC040. Immunofluorescence analysis of p53 and p63 in the two cell lines showed nuclear localization of both the proteins in AW8507, and although p63 was observed in SCC040, in keeping with the western blot results, p53 was not detected in SCC040.

Figure 5-11: Immunofluorescence staining of (A) p53 and (B) p63 in AW8507 and SCC040.

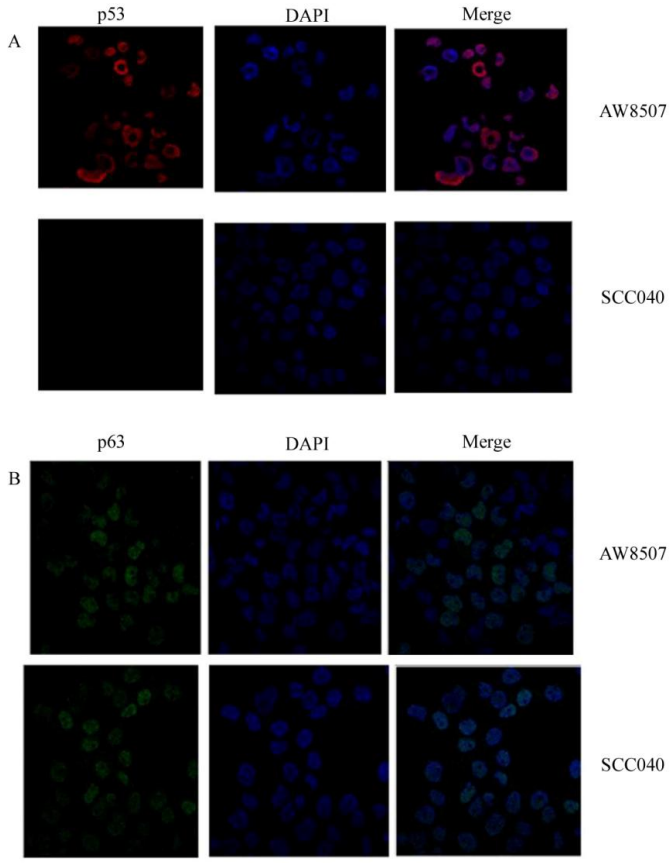


Figure 5-11: Immunofluorescence staining of (A) p53 and (B) p63 in AW8507 and SCC040. Representative images showing nuclear localization of p53 and p63 in AW8507 and SCC040 was

determined by immunofluorescence. DAPI was used for nuclear staining and images were obtained on a confocal microscope.

To determine if a functional p53 protein is present in SCC040, p21 induction post radiation was analyzed. As depicted in Figure 5-12 , p21 induction was observed in HCT116 cells which contain wild type p53 but not in SCC040. Taken together, the results indicate that p53 protein may be absent in SCC040.

Figure 5-12: p21 induction post radiation in HCT116 and SCC040

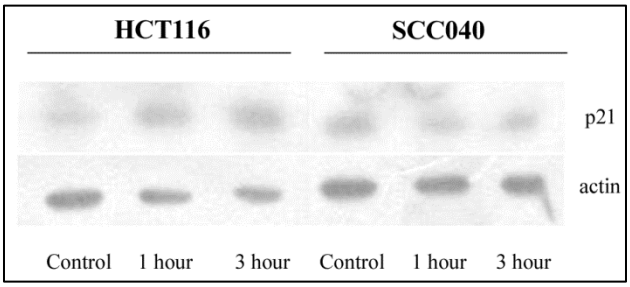


Figure 5-12: p21 induction post radiation in HCT116 and SCC040. p21 expression was determined in HCT116 and SCC040 at 1hour and 3 hours post radiation. Induction of p21 was observed in HCT116 but not in SCC040 upon radiation. Actin was used as loading control

Hence these two cell lines were chosen for further studies to delineate whether p63 plays a role in the regulation of survivin and/or clusterin expression.

Sequence analysis of p63 in AW8507 and SCC040

Since AW8507 shows the presence of a mutant p53 protein while SCC040 does not show the presence of p53 as detected by western blotting, the mutational status of p63 was analyzed by

sequencing the p63 genome in the two cell lines. Currently no reported hot spot mutations of p63 are reported in cancer. In the current study 10 exons of p63 were sequenced and analyzed for the presence of mutations. No mutations in the 10 exons of p63 were detected in the two cell lines.

5.2.1 Knockdown of p63 by siRNA in AW8507 and SCC040

Pan p63 knockdown was achieved using siRNA directed against all the isoforms of p63. As seen in Figure 5-13 knockdown of p63 was achieved with 50nM of siRNA in AW8507 and SCC040 cell lines, a scrambled siRNA was used as a control. The effect of the siRNA was persistent upto 60 hours post transfection. ΔNp63 transcripts were downregulated post p63 knockdown in both the cell lines, while TAp63 levels were extremely low in both the cell lines.

Figure 5-13: Transient knockdown of p63 in AW8507 and SCC040 using siRNA

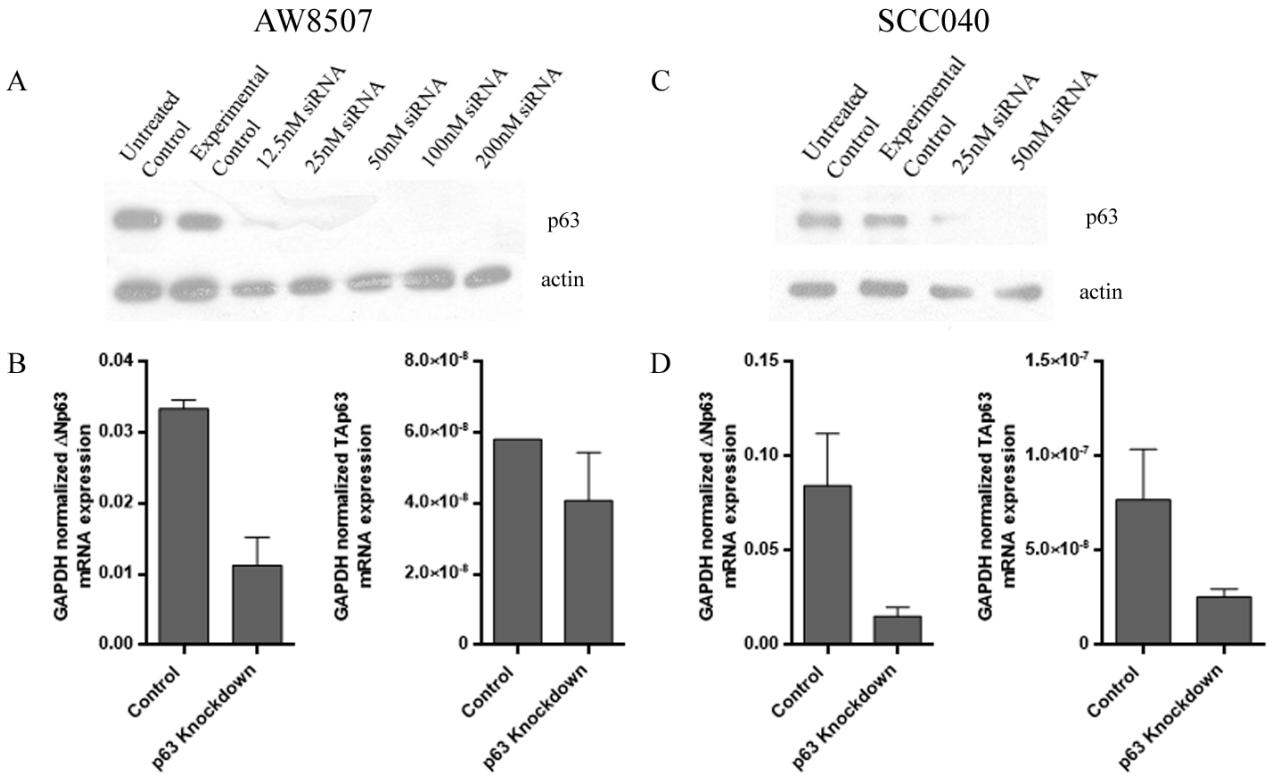


Figure 5-13: Transient knockdown of p63 in AW8507 and SCC040 using siRNA. Complete knockdown of p63 was achieved as demonstrated at the protein level by western blotting in (A) AW8507 and (C) SCC040 with 50nM of TP63 siGenome SmartPool siRNA (Dharmacon). ΔNp63 transcripts were also found to be downregulated post p63 knockdown in the (B) AW8507 and (D) SCC040.

5.2.2 Expression of survivin and clusterin transcripts post p63 knockdown in AW8507 and SCC040.

The expression of the survivin transcripts was analyzed post p63 knockdown in AW8507 and SCC040 by real time PCR. In AW8507, post p63 knockdown a significant downregulation of survivin wt and survivin 2B expression was observed while survivin 2α was found to be

upregulated post p63 knockdown. On the other hand in SCC040 survivin wt was found to be significantly upregulated post p63 knockdown.

Clusterin, was found to be upregulated post p63 knockdown in both AW8507 and SCC040 although the upregulation was not statistically significant.

Figure 5-14: Effect of p63 knockdown on survivin and clusterin transcript expression

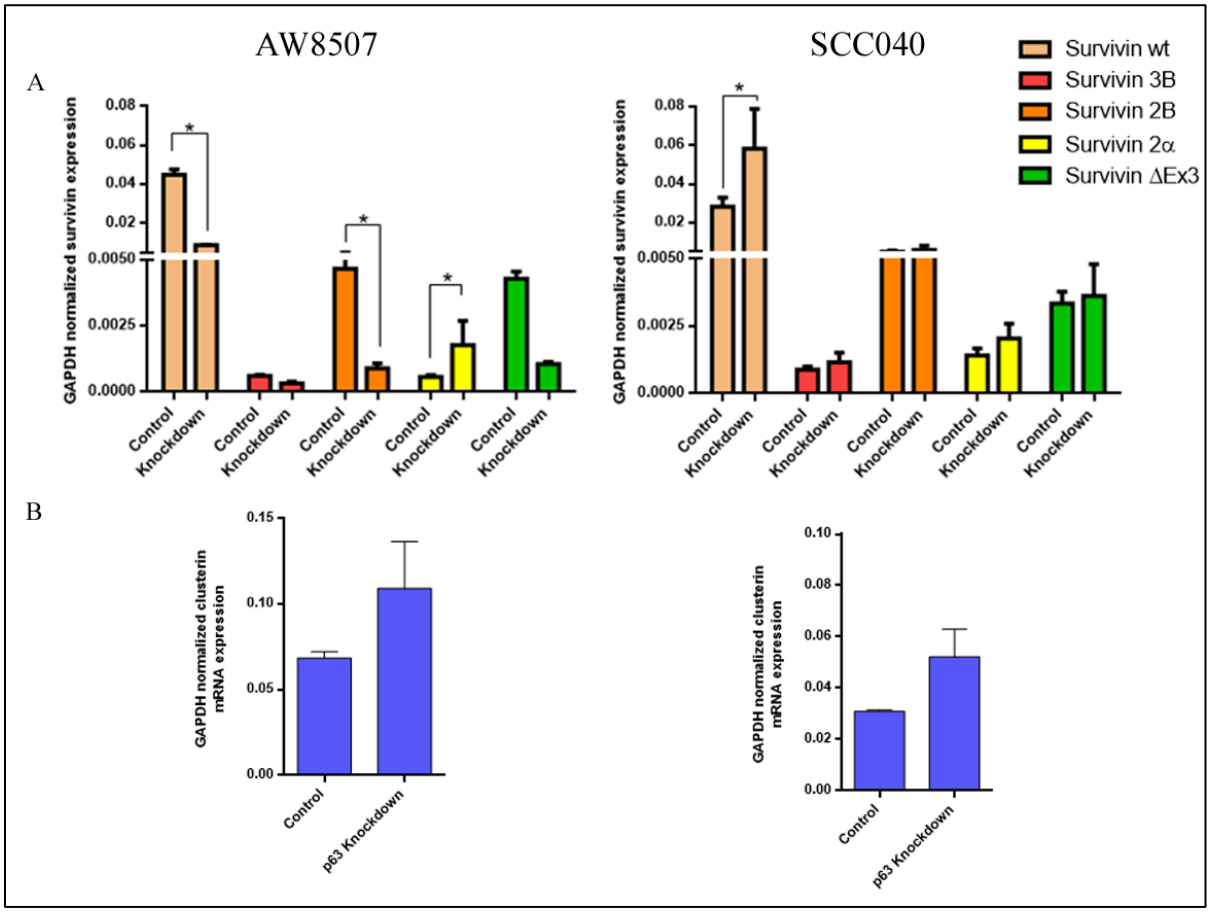


Figure 5-14: Effect of p63 knockdown on survivin and clusterin transcript expression (A) Survivin isoform expression in AW8507 and SCC040 post p63 knockdown. Survivin wt was significantly downregulated in AW8507 but upregulated in SCC040 post p63 knockdown. (B) Secretory clusterin expression post p63 knockdown. Clusterin was found to be upregulated post p63 knockdown in AW8507 as well as SCC040. *indicates $p < 0.05$.

5.2.3 Effect of p63 knockdown on survivin and clusterin promoter activity.

The activity of survivin and clusterin promoter post p63 knockdown was determined by luciferase assay. The minimal promoter of survivin showed increased luciferase activity post p63 knockdown in AW8507 but a marginal decrease in activity in SCC040 which did not reflect the transcript expression observed post knockdown. Interestingly, the extended promoter of survivin (1500 bases upstream of minimal promoter) showed a significant reduction in luciferase activity post p63 knockdown in SCC040, as seen in survivin wt transcript expression as well. However in AW8507 the extended promoter also showed a marginal decrease in activity as seen with the minimal promoter.

Clusterin promoter activity showed marginal increase post p63 knockdown in AW8507 as well as SCC040 thus reflecting the transcript expression (Fig 5.15).

Figure 5-15: Luciferase activity of the survivin and clusterin promoters in (A) AW8507 and (B) SCC040

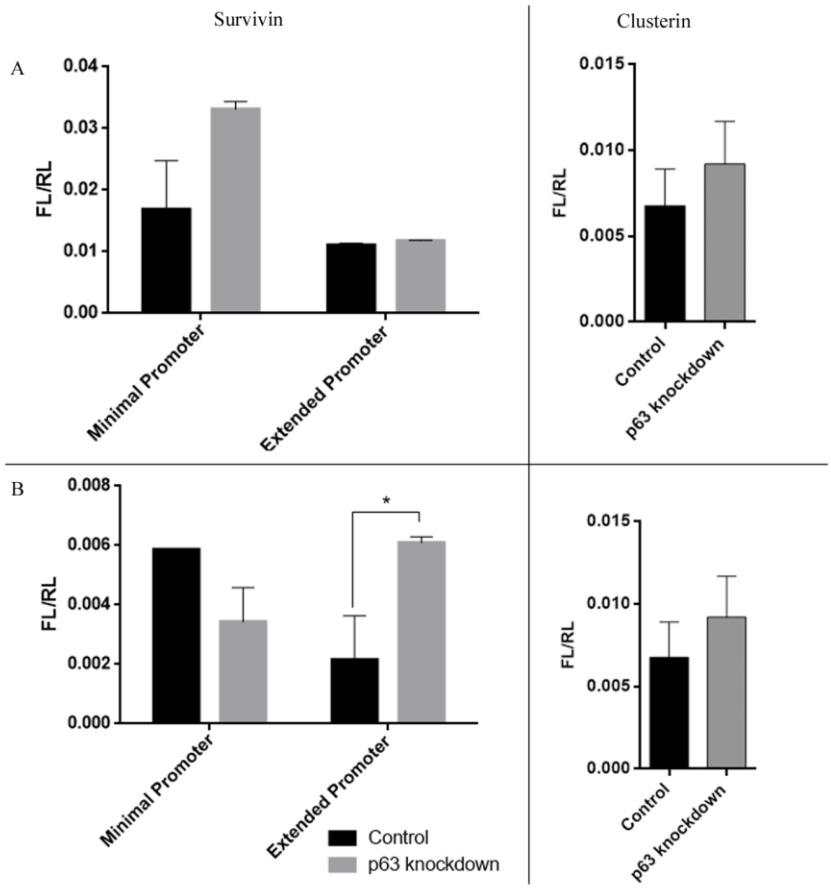


Figure 5-15: Luciferase activity of the survivin and clusterin promoters in (A) AW8507 and (B) SCC040. The luciferase activity was measured and calculated as the ratio of the activity of firefly luciferase to renilla luciferase and normalized with the protein concentrations.

5.2.4 Chromatin Immunoprecipitation Assays

The luciferase activity of the survivin promoters showed that the extended promoter of survivin reflected the transcript expression data in SCC040 while the upregulation observed in the minimal promoter in AW8507 was lost when the extended promoter activity was determined. The ability of the mutant p53 protein in AW8507 to bind to p63 was determined by co-

immunoprecipitation of the proteins in AW8507. Figure 5-16 shows the precipitation of p63 with p53 in AW8507.

Figure 5-16: Co-immunoprecipitation of p63 and mutant p53 in AW8507

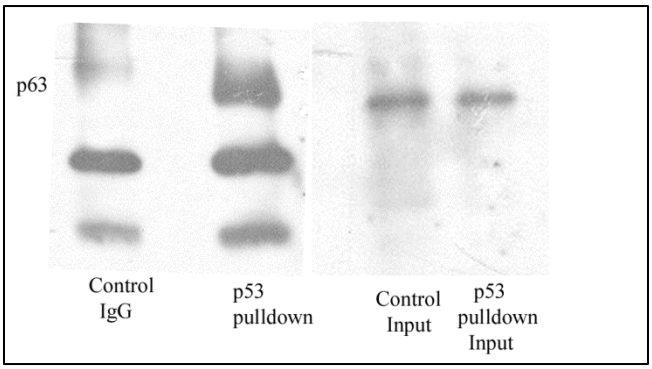


Figure 5-16: Co-immunoprecipitation of p63 and mutant p53 in AW8507. Antibody against p53 was used for pulldown and the protein lysates were then probed for the presence of p63. p63 was detected in the input and the p53 pulldown and was not detected in the IgG control .

PCR for p53 binding sites on the Survivin Promoter.

The survivin extended promoter was for the presence of p53 binding sites using Algen Promo Sequence as described in materials and methods. A total of seven primers were designed covering all the predicted binding sites of p53 on the survivin promoter. Interestingly, no binding of the p53 - p63 complex was observed on the promoter at the position in which p53 is reported to bind to and repress survivin expression [18]. However, binding of the complex was detected at position -2727 to -2443 from the transcription start site on the extended promoter, approximately 2100 bases upstream of the reported binding site. The specificity of the ChIP was tested using the p21 promoter containing the p53 binding site as control. Figure 5-17 shows the enrichment of the binding site between 323-607 in the p53 pulldown. A cartoon depicting the

shift in the binding site from the reported binding site covered between the region -575 to -9 from the transcription start site is also represented in the Figure 5-17.

Figure 5-17: Binding of mutant p53 and p63 complex on survivin promoter

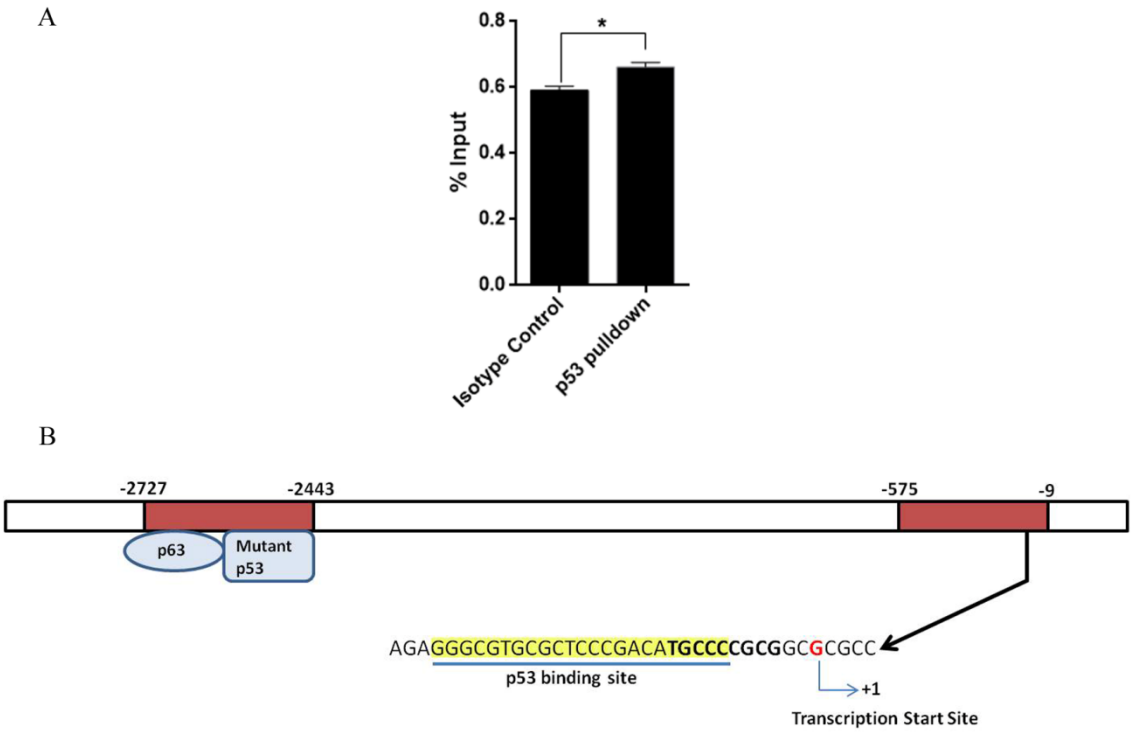


Figure 5-17: Binding of mutant p53 and p63 complex on survivin promoter (A) Survivin promoter PCR in p53 pull down. Data is presented as percentage enrichment of Input. * $p < 0.05$ (B) Cartoon depicting the shift in binding of mutant p53 and p63 complex on the survivin promoter in AW8507. The reported binding site for p53 in repression of survivin is indicated in yellow. The site overlaps with the consensus region of the E2F transcription factor indicated in bold.

5.3 To determine the role of p63 protein in oral cancer cells using different phenotypic assays, microarray analysis and radiation treatment post p63 knockdown in oral cancer cells.

5.3.1 Knockdown of p63 using shRNA in AW8507 and SCC040.

The expression analysis showed a predominant expression of ΔNp63 in the oral cell lines over TAp63. Knockdown of p63 by siRNA directed against both the isoforms of p63 showed an effect on the expression of survivin and clusterin in AW8507 and SCC040. To determine the phenotypic effects of p63 knockdown, ΔNp63 was knockdown using shRNA directed against ΔNp63. Figure 5-18 shows the knockdown of ΔNp63 by shRNA in AW8507 and SCC040.

Figure 5-18: Knockdown of ΔNp63 in AW8507 and SCC040

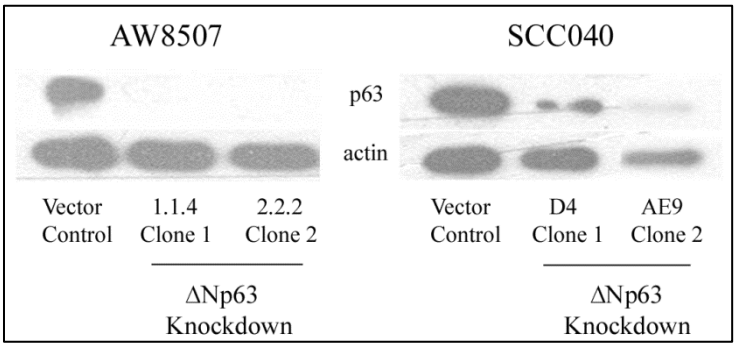


Figure 5-19: Knockdown of ΔNp63 in AW8507 and SCC040. Complete knockdown of p63 was observed in AW8507 using shRNA and greater than 70% knockdown was achieved in SCC040. β actin was used as a loading control.

5.3.2 Effect of ΔNp63 knockdown on colony formation in AW8507 and SCC040.

The effect of ΔNp63 knockdown on the colony forming ability in AW8597 and SCC040 was determined by soft agar assay. In AW8507 a significant increase in soft agar colony formation

was observed in the ΔNp63 knockdown clones as compared to vector control. On the other hand in SCC040 there was no significant difference in colony formation in the ΔNp63 knockdown and vector control cells.

Figure 5-19: Soft agar colony formation post ΔNp63 knockdown in AW8507 and SCC040

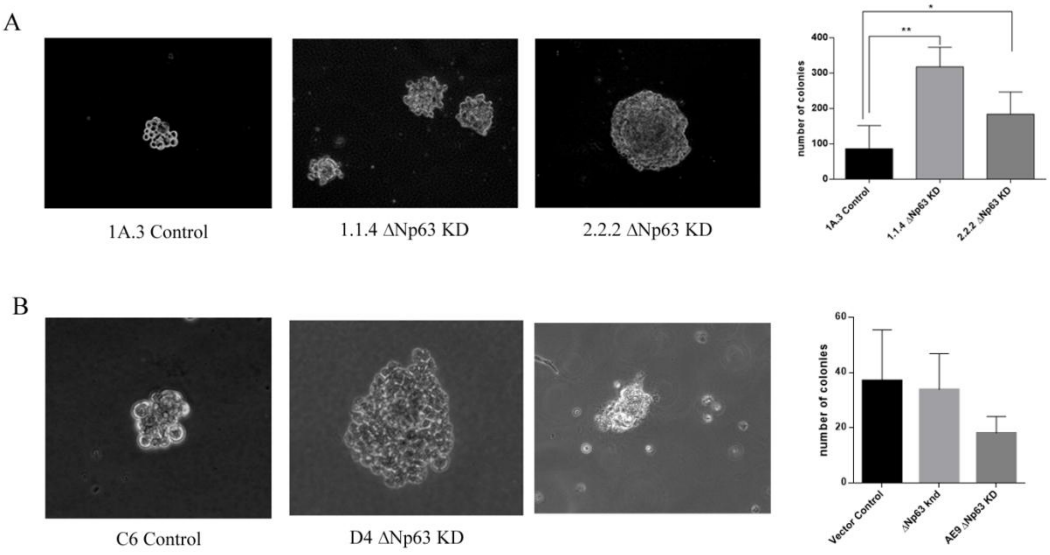


Figure 5-19: Soft agar colony formation post ΔNp63 knockdown in AW8507 and SCC040. The soft agar colony formation ability was tested in the vector control and ΔNp63 knockdown cells for both cell lines. Representative DIC images were taken on an inverted microscope. The number of colonies from three independent experiments were counted and plotted, error bars represent standard error of mean. (** $p < 0.01$, * $p < 0.05$)

5.3.3 Effect of ΔNp63 on invasive capability of AW8507 and SCC040.

The effect of ΔNp63 knockdown on invasive ability of AW8507 and SCC040 was determined by the matrigel assay. As described earlier, cells were seeded in the Boyden chamber in incomplete medium and the number of cells that invaded through the matrigel were determined by DAPI staining. As shown in Figure 5-20, in AW8507 there was no significant difference in the invasive capability post ΔNp63 knockdown as compared to the vector control whereas in SCC040 a significant increase in invasion was observed in ΔNp63 knockdown clones as compared to vector control.

Figure 5-20: Effect of ΔNp63 knockdown on Invasion

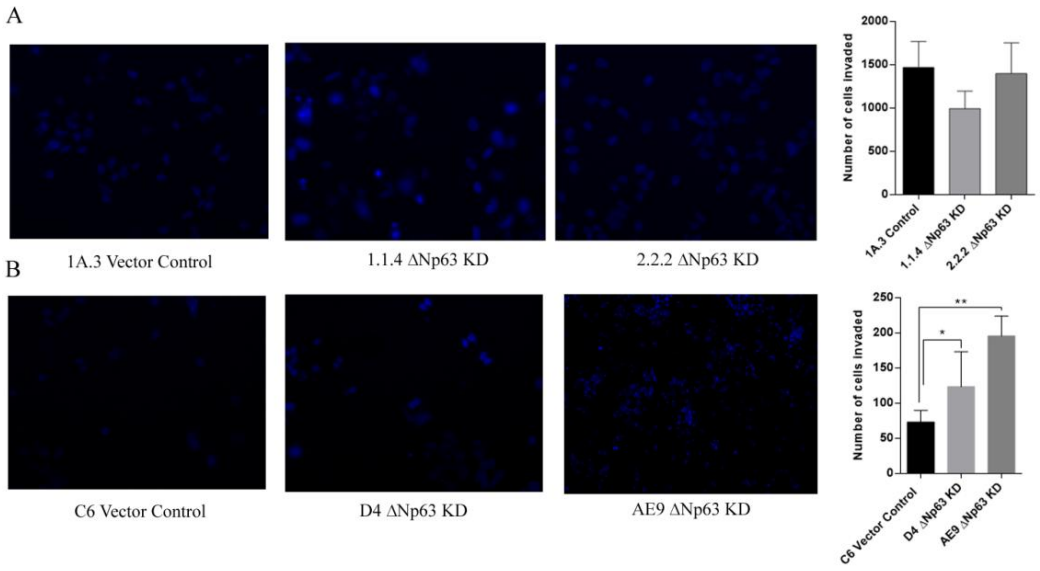


Figure 5-20: Effect of ΔNp63 knockdown on Invasion. Invasion ability post p63 knockdown was determined by invasion assay in Boyden Chamber using Matrigel. (A) In AW8507 post p63 knockdown no significant difference was observed in the ΔNp63 knockdown clones as compared to the vector control clone. (B) In SCC040 a significant increase post ΔNp63 knockdown was found as compared to the vector control. *indicates $p < 0.05$.

5.3.4 Effect of ΔNp63 knockdown on migration

Migration ability was determined by scratch wound assay in AW8507 and SCC040 post p63 knockdown. Briefly, cells were seeded such that complete confluence was achieved and a wound was made. The area of wound healed in control versus ΔNp63 knockdown clones was determined in three independent experiments. In AW8507, ΔNp63 knockdown resulted in an increase in migration as compared to the vector control cells. However in SCC040 no significant difference in migration was observed upon ΔNp63 knockdown.

Figure 5-21: Effect of ΔNp63 Knockdown on migration.

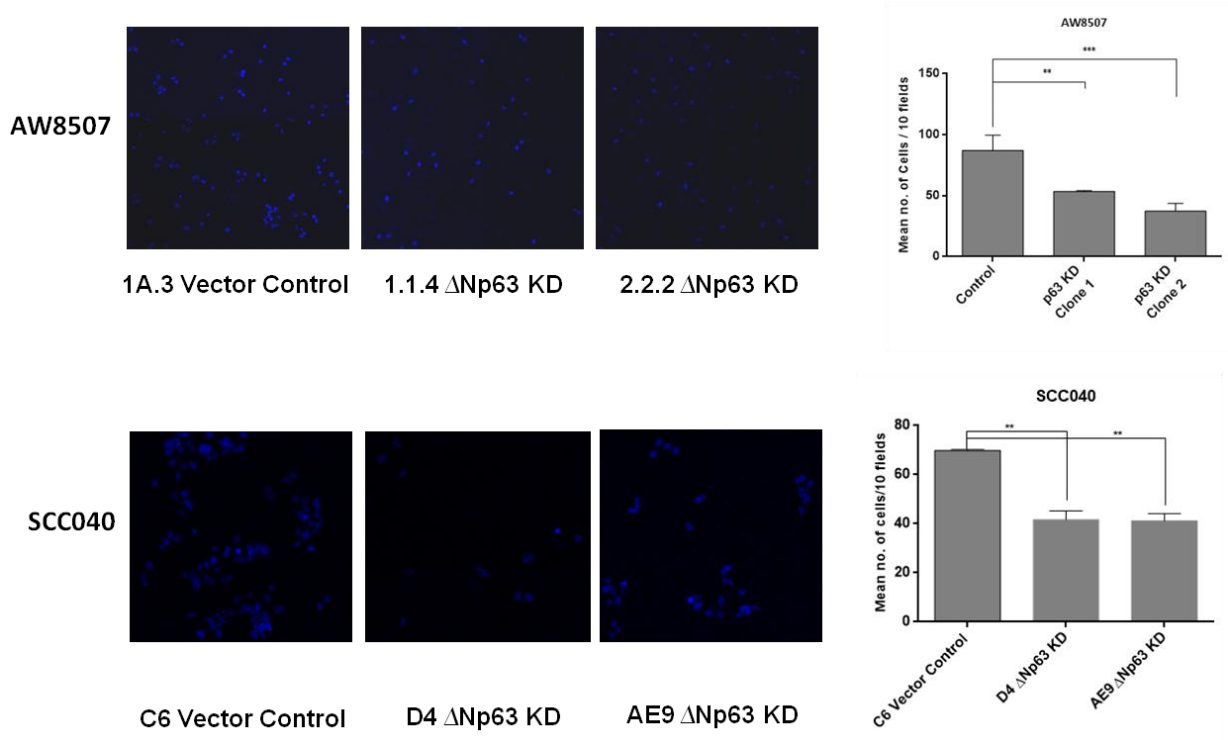


Figure 5-21: Effect of ΔNp63 Knockdown on migration. (A) In AW8507 a significant decrease in migration was observed post p63 knockdown. (B) In SCC040 migration was not affected significantly

post p63 knockdown. The images are representative images, results from three independent experiments were plotted with error bars showing standard error of mean. *** $p < 0.001$

5.3.5 Effect of ΔNp63 knockdown on proliferation.

MTT reduction assay was conducted to assess cell proliferation upon ΔNp63 knockdown in AW8507 and SCC040 cell lines. As described in section 4.2.16, cells were seeded in 96 well dishes and viability was assessed at periodic intervals. The ΔNp63 knockdown clones showed significantly reduced proliferation as compared to vector control cells of AW8507. However in SCC040 no significant change was observed in viability and proliferation of the knockdown clones and vector control cells.

Figure 5-22: MTT reduction assay for proliferation post ΔNp63 knockdown in AW8507 and SCC040 cell lines

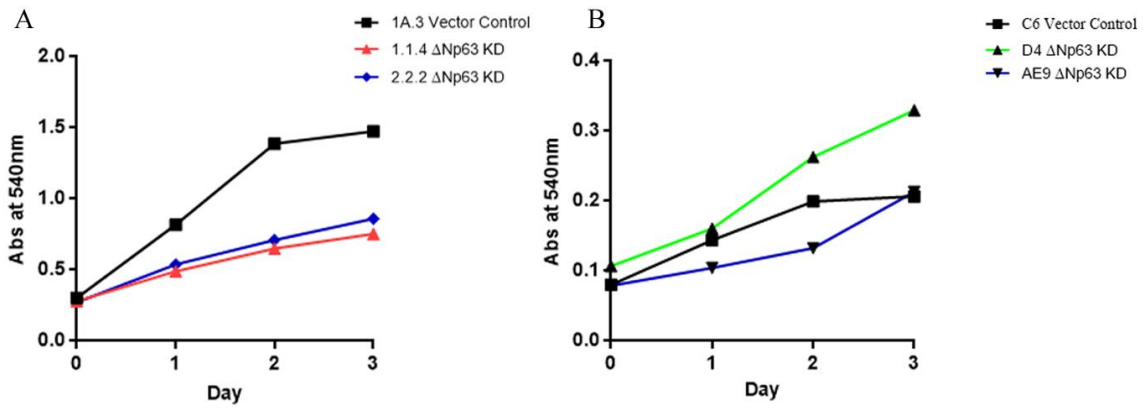


Figure 5-22: MTT reduction assay for proliferation post ΔNp63 knockdown in AW8507 and SCC040 cell lines (A) MTT cell survival assay showed a significant reduction in survival post knockdown in AW8507 as compared to the vector control ($p < 0.001$). (B) MTT cell survival showed no difference in survival post ΔNp63 knockdown in SCC040.

5.3.6 Combined effect of knockdown and radiation on expression of survivin and clusterin transcripts.

The expression of Survivin and clusterin is known to be induced upon exposure of cells to radiation. Several studies have shown an association of survivin overexpression with radio-resistance. siRNA mediated knockdown of p63 resulted in a differential effect on survivin isoform expression in AW8507 and SCC040 cell lines, however no significant difference was observed on the expression of clusterin transcripts upon p63 knockdown.

Hence we determined the combined effect of radiation and p63 knockdown on the expression of survivin splice variants in the two cell lines, AW8507 (with mutant p53) and SCC040 (absence of p53). As shown in Figure 5-23, treatment of AW8507 cells with 5Gy (D0 dose) radiation resulted in induction of survivin expression at 1 hour post treatment.. The knockdown of p63 resulted in downregulation of survivin expression , as was previously observed. Interestingly, in a combination of radiation and p63 there is a delay in the induction of survivin wt expression, with an increase observed only between 12-24 hours post irradiation

Figure 5-23: Combined effect of radiation and p63 knockdown in AW8507

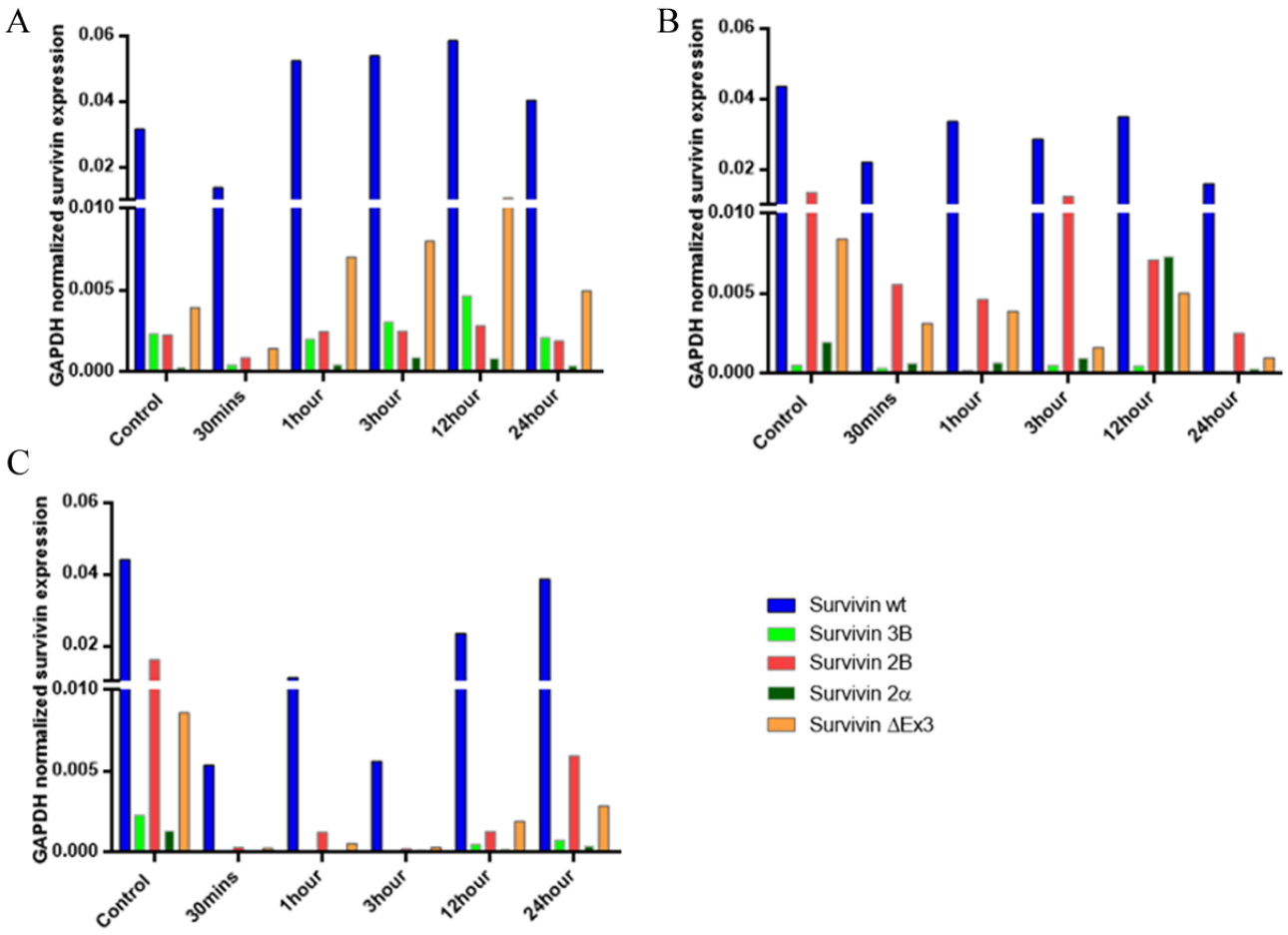


Figure 5-23: Combined effect of radiation and p63 knockdown in AW8507 (A) Expression of survivin splice variants post radiation treatment in AW8507 (B) Expression of survivin splice variants post p63 knockdown (C) Expression of survivin splice variants post p63 knockdown and radiation.

On the other hand, in SCC040 cells a marginal increase in survivin wt expression was seen post radiation treatment at 1hour. As seen previously, post p63 knockdown an increase is observed in survivin wt expression. Radiation treatment and p63 knockdown in combination showed an increase in survivin wt expression at 3 hours post radiation (Figure 5-24).

Our results suggest that p63 may play a role in survivin induction post radiation, specifically in the presence of mutant p53 as observed in AW8507 cells.

Figure 5-24: Combined effect of radiation and p63 knockdown in SCC040

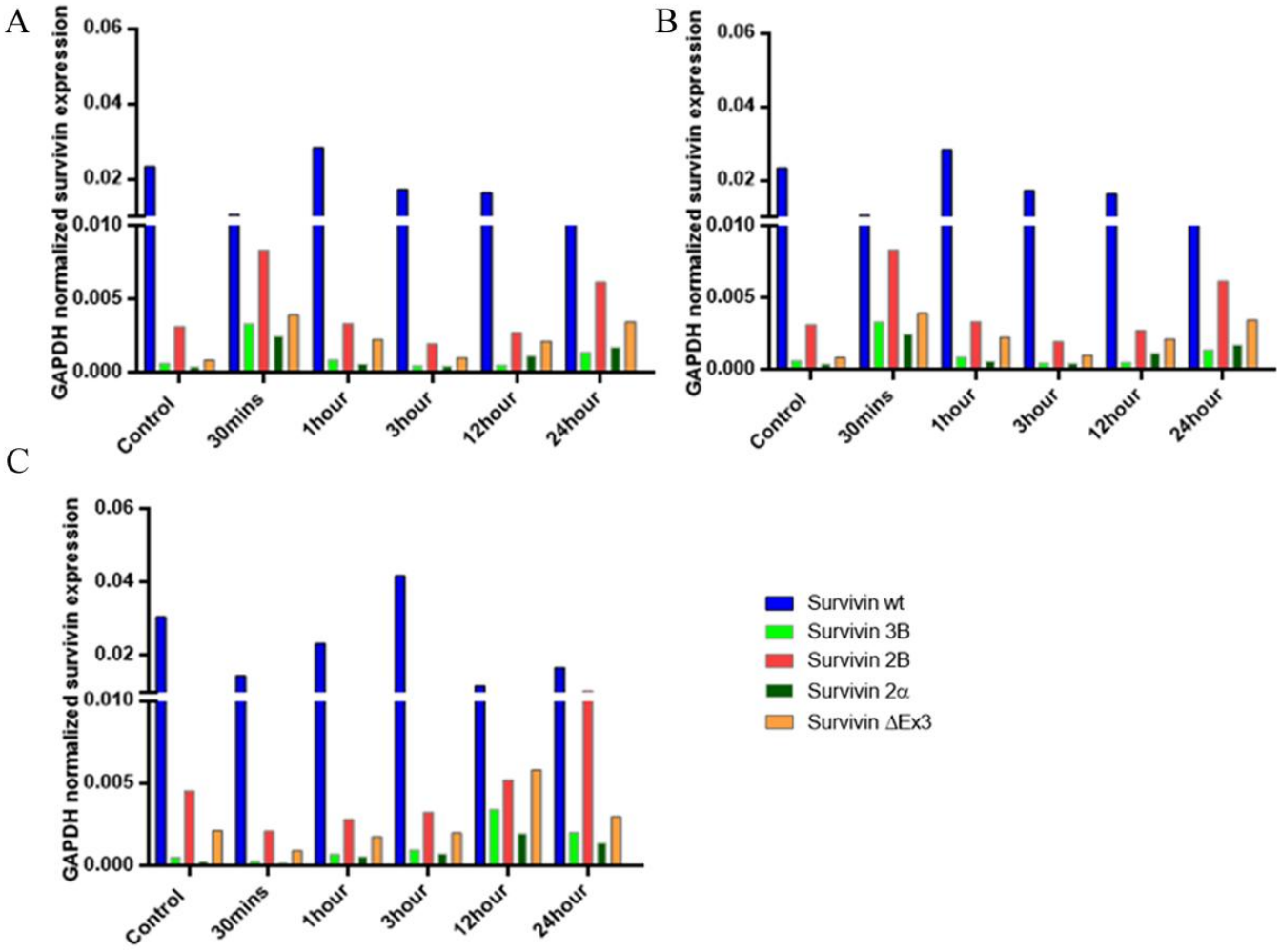


Figure 5-24: Combined effect of radiation and p63 knockdown in SCC040. (A) Survivin splice variant expression post radiation in SCC040 (B) Expression of survivin splice variants post p63 knockdown. (C) Expression of survivin splice variants post p63 knockdown and radiation.

The phenotypic assays conducted post p63 knockdown in AW8507 and SCC040 cells demonstrated a differential effect of ΔNp63 knockdown in the two cell lines, most likely due to

the differential status of p53 in the two cell lines. In AW8507 cells, which shows the presence of mutant p53, a reduction in migration was observed post knockdown however in SCC040 which shows no p53 protein, an increase in invasive capability was observed post p63 knockdown. Table 5-3 summarizes the results of the phenotypic assays in the two cell lines.

Table 5-3: Phenotypic effects of ΔNp63 knockdown in AW8507 and SCC040.

Post ΔNp63 knockdown	AW8507 (R273H mutant p53)	SCC040 (No p53 protein)
Soft agar Colony formation	Increase	Not significant
Migration (Wound Healing)	Decrease	Not Significant
Invasion	Not Significant	Increase
Cell viability/Proliferation	Decrease	Not Significant

5.3.7 Identification of differentially expressed genes in AW8507 and SCC040 cells upon knockdown of p63 by genome wide gene expression analysis.

The phenotypic properties of AW8507 and SCC040 were found to be altered upon knockdown of p63. Interestingly, although proliferation and migration was found to be reduced upon p63 knockdown in AW8507, there was an increase in invasion in SCC040. To identify the genes that may be regulated by p63 and to identify specifically the differentially expressed genes in AW8507 and SCC040 genome wide gene expression analysis was conducted in both the cell lines upon p63 knockdown. The cDNA microarray analysis was performed in two sets and the average values of the two were analyzed.

In AW8507, of the 44,296 genes 4304 showed differential expression on p63 knockdown among which 341 showed greater than 1.5 fold change. Among the 341 genes, 17 genes were upregulated while 324 genes were downregulated by more than 1.5 fold as compared to the

control. The list of the differentially expressed genes is presented in Annexure I. The differentially expressed genes were further analyzed in PANTHER, KEGG and the REACTOME databases to identify the pathways that are affected in AW8507 post p63 knockdown. The genes downregulated post p63 knockdown contributing to the annotated pathways by PANTHER, REACTOME or KEGG databases are presented in Table 5-4 and Table 5-5. A pie chart of the functional classification of the genes downregulated post p63 knockdown in AW8507 using the three databases is presented in Figure 5-25 and Figure 5-26.

Table 5-4: Functional Annotation of downregulated genes post p63 knockdown in AW8507 using PANTHER and REACTOME databases

PANTHER PATHWAYS				
Term	**Genes**	**Count**	**%**	**P Value**
P00049:Parkinson disease	HSPA5, HSPA8	2	12.5	0.103362
P00006:Apoptosis signaling pathway	HSPA5, HSPA8	2	12.5	0.129476
P00005:Angiogenesis	CTNNB1	1	6.25	1
P00012:Cadherin signaling pathway	CTNNB1	1	6.25	1
P00004:Alzheimer disease-presenilin pathway	CTNNB1	1	6.25	1
P04398:p53 pathway feedback loops 2	CTNNB1	1	6.25	1
P00057:Wnt signaling pathway	CTNNB1	1	6.25	1
P00017:DNA replication	TOP1	1	6.25	1
REACTOME PATHWAYS				
Term	**Genes**	Count	%	P Value
REACT_604:Hemostasis	ATP1B1, HSPA5	2	12.5	0.349702
REACT_15380:Diabetes pathways	ATP5A1, HSPA5	2	12.5	0.409039
REACT_1698:Metablism of nucleotides	ATP5A1	1	6.25	1
REACT_125:Processing of Capped Intron-Containing Pre-mRNA	HNRNPA1	1	6.25	1
REACT_152:Cell Cycle, Mitotic	CDK11A, CDK11B	1	6.25	1
REACT_1505:Integration of energy metabolism	ATP5A1	1	6.25	1
REACT_71:Gene Expression	HNRNPA1	1	6.25	1
REACT_6167:Influenza Infection	HNRNPA1	1	6.25	1

REACT_11123:Membrane Trafficking	HSPA8	1	6.25	1
REACT_578:Apoptosis	CTNNB1	1	6.25	1
REACT_6900:Signaling in Immune system	ATP1B1	1	6.25	1
REACT_11045:Signaling by Wnt	CTNNB1	1	6.25	1

Table 5-4: Functional Annotation of downregulated genes post p63 knockdown in AW8507 using PANTHER and REACTOME databases TERM indicates the pathways under which the genes are classified, and count indicates the number of genes classified in the pathway. The percentage contributed by the genes is presented as %.

Figure 5-25: Top downregulated PANTHER and REACTOME pathways in AW8507.

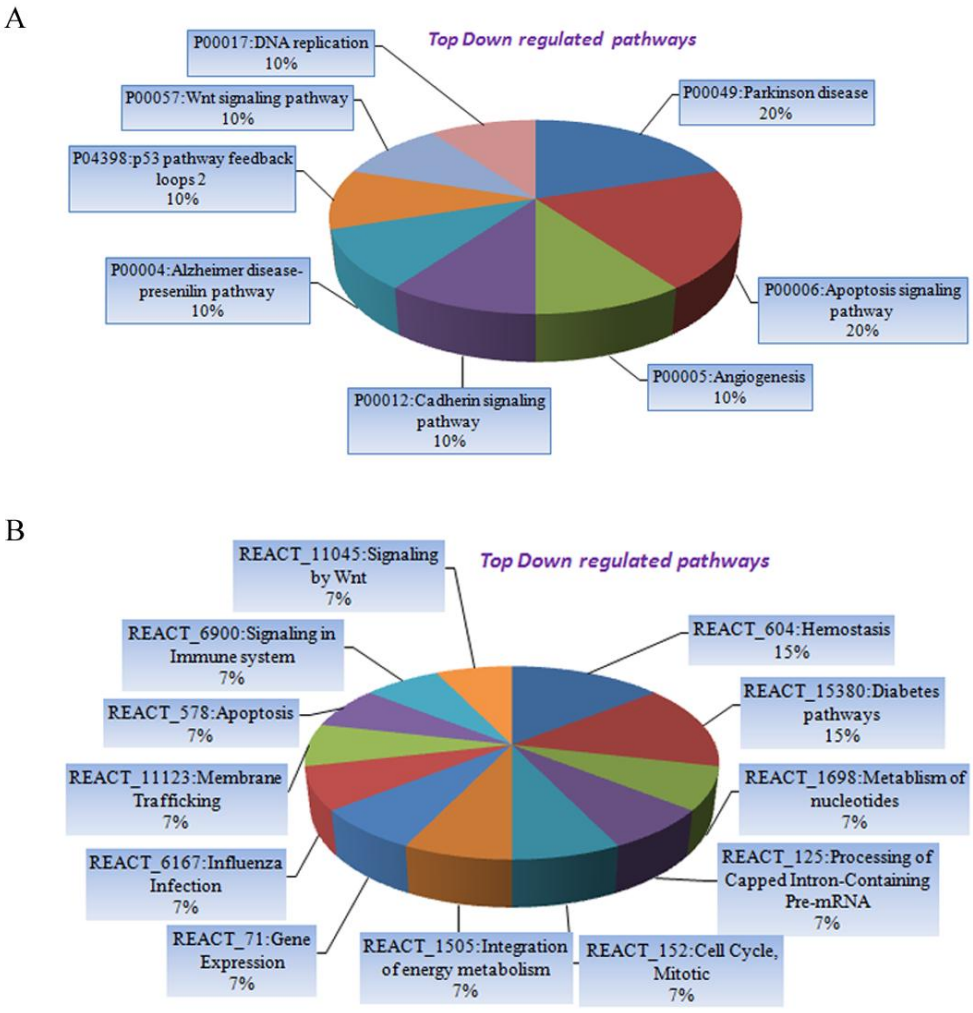


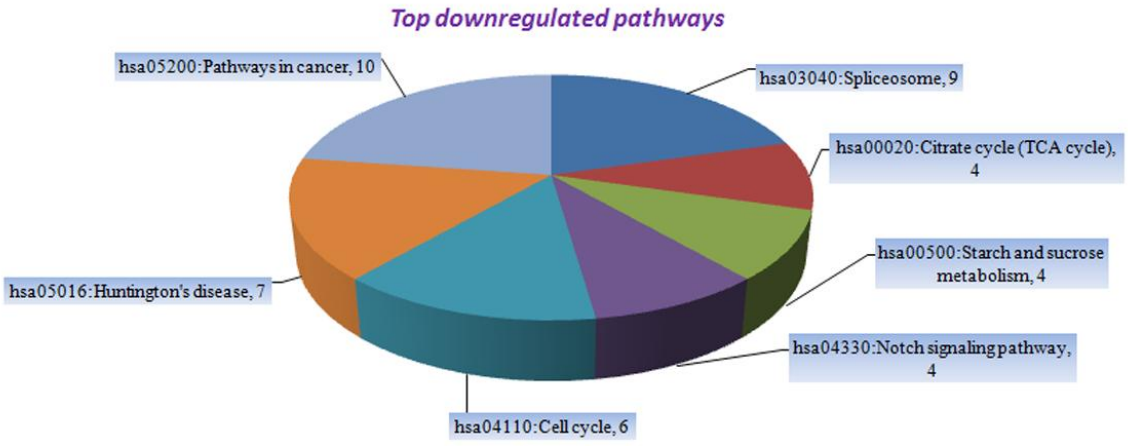
Figure 5-25: Top downregulated PANTHER and REACTOME pathways in AW8507. The significantly downregulated genes in AW8507 were functionally annotated in (A) PANTHER and (B) REACTOME pathways. The list of the genes involved in the pathways represented is presented in Table 5-

Table 5-5: KEGG annotation of downregulated genes in AW8507.

Term	Genes	Count	%	P Value
hsa03040:Spliceosome	NCBP2, RBM8A, TRA2A, LOC728643, PRPF3, HNRNPA1, RBMX, HNRNPU, HSPA8, DDX42	9	3.781513	0.001023
hsa00020:Citrate cycle (TCA cycle)	SDHA, SDHC, IDH1, ACLY	4	1.680672	0.014008
hsa00500:Starch and sucrose metabolism	AMY2B, AMY1C, AMY2A, AMY1B, UXS1, AMY1A	4	1.680672	0.031439
hsa04330:Notch signaling pathway	PSEN1, HDAC1, DTX3L, ADAM17	4	1.680672	0.041888
hsa04110:Cell cycle	RAD21, HDAC1, SMAD3, SMC3, ATM, TFDP1	6	2.521008	0.054849
hsa05016:Huntington's disease	SDHA, SIN3A, HDAC1, SDHC, ATP5A1, CLTC, NDUFA10	7	2.941176	0.075968
hsa05200:Pathways in cancer	MSH6, HIF1A, HDAC1, IL8, RHOA, SMAD3, STAT1, CRK, CTNNB1, AKT2	10	5.201681	0.090019

Table 5-5: KEGG annotation of downregulated genes in AW8507. Term indicates the pathways in which the genes were annotated. Count indicates the number of genes involved in the pathway and % indicates the contribution of the genes in the pathway.

Figure 5-26:The pathways enriched using the KEGG database for the downregulated genes in AW8507.



Similarly the genes that were upregulated more than 1.5 fold post p63 knockdown in AW8507 were also annotated for functional significance in the three databases. Table 5-6 and Table 5-7 enlist the upregulated genes annotated in pathways using PANTHER, REACTOME and KEGG databases. The pie chart depicting the contribution of each of the genes and the pathways as determined by the functional annotation is presented in Figure 5-27 and Figure 5-28.

Table 5-6: Functional Annotation of upregulated genes post p63 knockdown in AW8507 using PANTHER and REACTOME databases

PANTHER PATHWAYS				
Term	**Genes**	**Count**	**%**	**p Value**
P00056:VEGF signaling pathway	SH2D2A	1	6.25	1
P00018:EGF receptor signaling pathway	AREGB, AREG	1	6.25	1
P02769:Purine metabolism	NT5E	1	6.25	1
P00005:Angiogenesis	SH2D2A	1	6.25	1
P00034:Integrin signaling pathway	ITGA2	1	6.25	1
P00031:Inflammation mediated by chemokine and cytokine signaling pathway	ITGA2	1	6.25	1
P00050:Plasminogen activating cascade	MMP3	1	6.25	1
REACTOME PATHWAYS				
Term	**Genes**	**Count**	**%**	**P Value**
REACT_604:Hemostasis	ITGA2	1	6.25	1
REACT_1698:Metabolism of nucleotides	NT5E	1	6.25	1
REACT_17015:Metabolism of proteins	PFDN4	1	6.25	1
REACT_12472:Regulatory RNA pathways	DICER1	1	6.25	1
REACT_13552:Integrin cell surface interactions	ITGA2	1	6.25	1

Table 5-6: Functional Annotation of upregulated genes post p63 knockdown in AW8507 using PANTHER and REACTOME databases. TERM indicates the pathways under which the genes are

classified, and count indicates the number of genes classified in the pathway. The percentage contributed by the genes is presented as %.

Figure 5-27: Top upregulated genes annotated in PANTHER and REACTOME pathways in AW8507

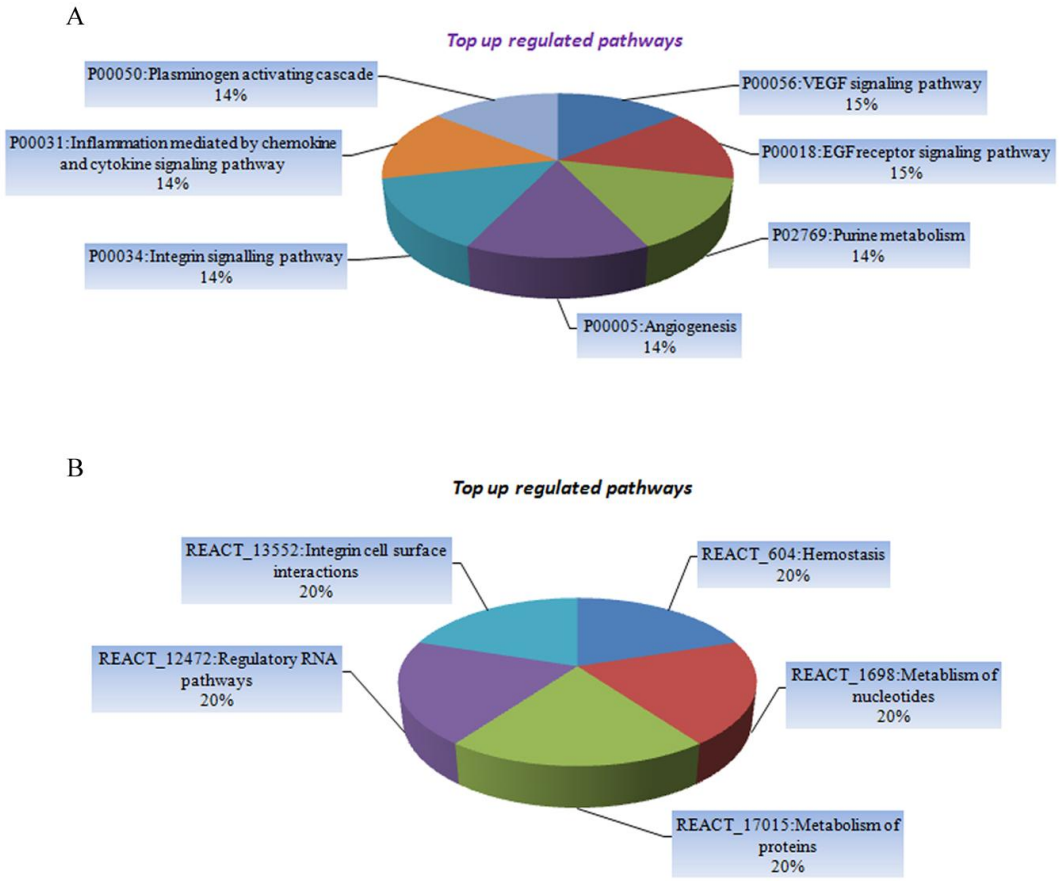


Figure 5-27: Top upregulated genes annotated in PANTHER and REACTOME pathways in AW8507. The significantly upregulated genes were functionally annotated in (A) PANTHER and (B) REACTOME pathways. The list of the genes involved in the pathways represented is presented in Table 5-6.

Table 5-7: Functional Annotation of upregulated genes post p63 knockdown in AW8507 using KEGG database.

Term	Genes	Count	%	P Value
hsa04640:Hematopoietic cell lineage	CSF2, ITGA2	2	12.5	0.127639
hsa04512:ECM-receptor interaction	ITGA2	1	6.25	1
hsa05414:Dilated cardiomyopathy	ITGA2	1	6.25	1
hsa04664:Fc epsilon RI signaling pathway	CSF2	1	6.25	1
hsa04650:Natural killer cell mediated cytotoxicity	CSF2	1	6.25	1
hsa04060:Cytokine-cytokine receptor interaction	CSF2	1	6.25	1
hsa05222:Small cell lung cancer	ITGA2	1	6.25	1
hsa00240:Pyrimidine metabolism	NT5E	1	6.25	1
hsa04370:VEGF signaling pathway	SH2D2A	1	6.25	1
hsa04630:Jak-STAT signaling pathway	CSF2	1	6.25	1
hsa04514:Cell adhesion molecules (CAMs)	PDCD1LG2	1	6.25	1
hsa05200:Pathways in cancer	ITGA2	1	6.25	1
hsa04810:Regulation of actin cytoskeleton	ITGA2	1	6.25	1
hsa04510:Focal adhesion	ITGA2	1	6.25	1
hsa05410:Hypertrophic cardiomyopathy (HCM)	ITGA2	1	6.25	1
hsa04012:ErbB signaling pathway	AREGB, AREG	1	6.25	1
hsa00760:Nicotinate and nicotinamide metabolism	NT5E	1	6.25	1
hsa00564:Glycerophospholipid metabolism	PGS1	1	6.25	1
hsa05412:Arrhythmogenic right ventricular cardiomyopathy (ARVC)	ITGA2	1	6.25	1
hsa00230:Purine metabolism	NT5E	1	6.25	1
hsa04660:T cell receptor signaling pathway	CSF2	1	6.25	1
hsa00600:Sphingolipid metabolism	ASAH2	1	6.25	1
hsa03320:PPAR signaling pathway	ANGPTL4	1	6.25	1

Table 5-7: Functional Annotation of upregulated genes post p63 knockdown in AW8507 using KEGG database. TERM indicates the pathways under which the genes are classified, and count indicates the number of genes classified in the pathway. The percentage contributed by the genes is presented as %.

Figure 5-28: The pathways enriched using the KEGG database for the significantly upregulated genes post p63 knockdown in AW8507.

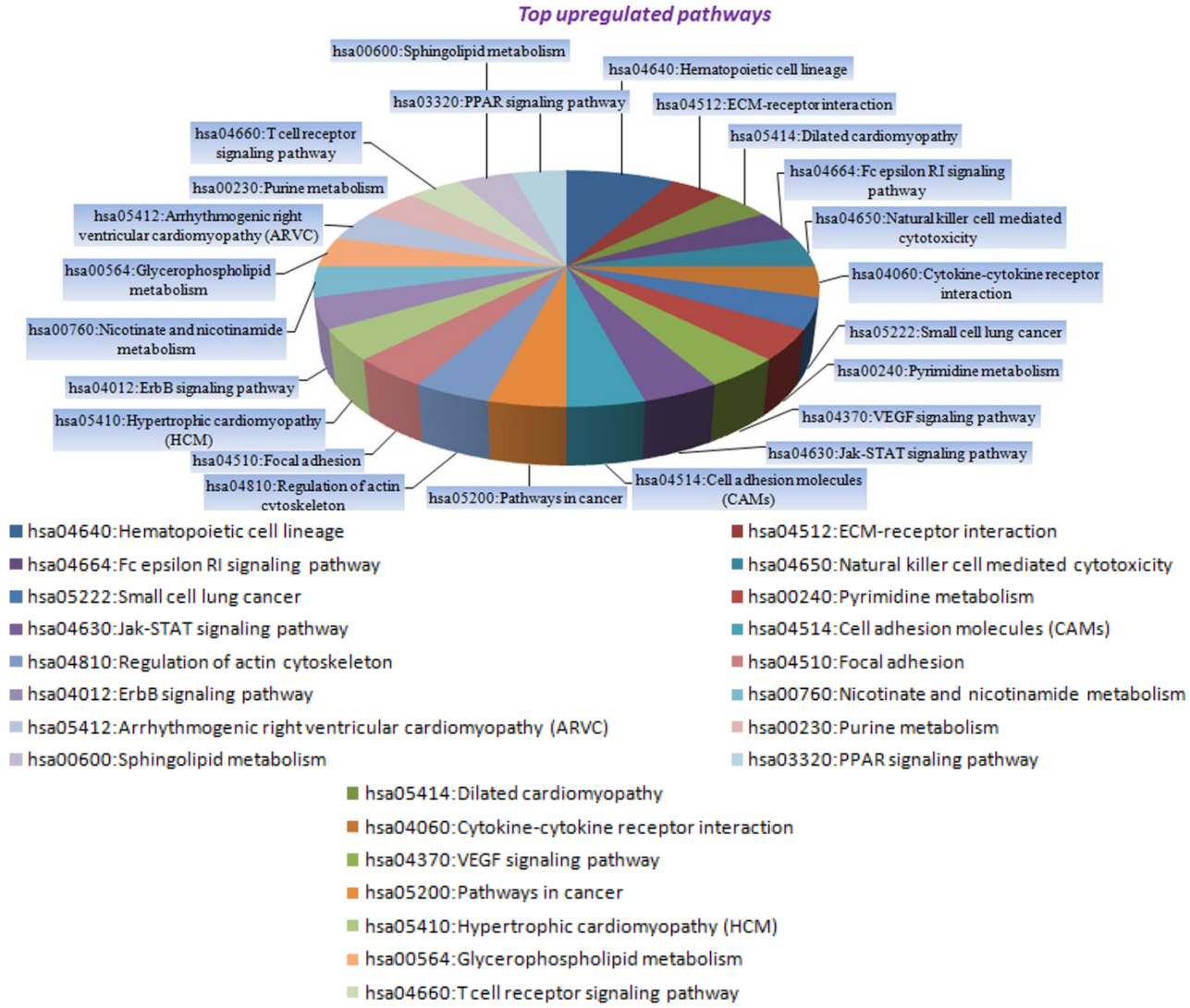


Figure 5-28: The pathways enriched using the KEGG database for the significantly upregulated genes post p63 knockdown in AW8507.

Functional annotation of the genes significantly upregulated or downregulated upon ΔNp63 knockdown demonstrated various genes involved in various signaling pathways such as VEGF, EGF and Inhibin signaling to be affected by knockdown of p63. Additionally, cell adhesion molecules and some genes involved in angiogenesis were also found to be altered.

Similarly functional annotation of the differentially expressed genes post ΔNp63 knockdown in SCC040 was also determined using PANTHER, REACTOME and the KEGG databases. The probe set consisting of 44,205 identified 12,939 genes that showed differential expression upon knockdown of p63 as compared to the vector control. Fold change cut off was set at 1.5 fold which identified 1556 genes, with 775 downregulated and 781 upregulated post knockdown. The list of the differentially expressed genes is presented in Annexure II.

Table 5-8 and 5.9 enlist the genes downregulated greater than 1.5 fold, annotated using the PANTHER and REACTOME pathways and the KEGG database respectively. Pie charts depicting the pathways identified in the databases is presented in Figure 5-29 and Figure 5-30.

Table 5-8: Functional Annotation of downregulated genes post p63 knockdown in SCC040 using PANTHER and REACTOME databases

PANTHER PATHWAYS				
Term	**Genes**	**Count**	**%**	**P Value**
P00034:Integrin signaling pathway	LAMA1, CAV1, COL13A1, ITGA2, COL12A1, LAMC2, COL4A6	7	5.132958	0.0022
P00031:Inflammation mediated by chemokine and cytokine signaling pathway	IFNAR2, PTGS1, ITGA2,	4	2.8169	0.32917
P00011:Blood coagulation	PROCR, SERPIN E1, SERPINB2	3	2.11268	0.05048
P00050:Plasminogen activating cascade	SERPINE1, SERPINB2	2	1.40845	0.137
P00059:p53 pathway	SERPINE1, THBS1	2	1.40845	0.55865
P00006:Apoptosis signaling pathway	IGF2R, HSPA8	2	1.40845	0.58551
P00026:Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway	ADRB2, GNAI1	2	1.40845	0.69599

P05913:Enkephalin release	GNAI1	1	0.70423	1
P00049:Parkinson disease	HSPA8	1	0.70423	1
P00033:Insulin/IGF pathway-protein kinase B signaling cascade	IGF2R	1	0.70423	1
P04378:Beta2 adrenergic receptor signaling pathway	ADRB2	1	0.70423	1
P05912:Dopamine receptor mediated signaling pathway	GNAI1	1	0.70423	1
P00018:EGF receptor signaling pathway	AREG	1	0.70423	1
P00039:Metabotropic glutamate receptor group III pathway	GNAI1	1	0.70423	1
P05917:Opioid proopiomelanocortin pathway	GNAI1	1	0.70423	1
P02762:Pentose phosphate pathway	RDH11	1	0.70423	1
P04373:5HT1 type receptor mediated signaling pathway	GNAI1	1	0.70423	1
P00043:Muscarinic acetylcholine receptor 2 and 4 signaling pathway	GNAI1	1	0.70423	1
P00040:Metabotropic glutamate receptor group II pathway	GNAI1	1	0.70423	1
P05916:Opioid prodynorphin pathway	GNAI1	1	0.70423	1
P05730:Endogenous_cannabinoid_signaling	GNAI1	1	0.70423	1
P00032:Insulin/IGF pathway-mitogen activated protein kinase kinase/MAP kinase cascade	IGF2R	1	0.70423	1
P05915:Opioid proenkephalin pathway	GNAI1	1	0.70423	1
P00048:PI3 kinase pathway	GNAI1	1	0.70423	1
P00052:TGF-beta signaling pathway	INHBA	1	0.70423	1
P05731:GABA-B_receptor_II_signaling	GNAI1	1	0.70423	1
REACTOME PATHWAYS				
Term	**Genes**	**Count**	**%**	**P Value**
REACT_604:Hemostasis	CD9, CAV1, SERPINE1, SERPINB2, ITGA2, THBS1,	9	6.33803	7.16E-04

	THBS2, SLC7A11, CALU			
REACT_15380:Diabetes pathways	GNAI1, PAPPA, SSR1, SLC30A7	4	2.8169	0.4666
REACT_14797:Signaling by GPCR	ADRB2, GNAI1, LTB4R, CXCR7	4	2.8169	0.93664
REACT_13552:Integrin cell surface interactions	LAMA1, ITGA2, THBS1	3	2.11268	0.15936
REACT_6900:Signaling in Immune system	CAV1, ULBP2, SLC7A11	3	2.11268	0.73259
REACT_9480:Gap junction trafficking and regulation	GJB6, GJB2	2	1.40845	0.23447
REACT_15518:Transmembrane transport of small molecules	SLC1A5, SLC7A11	2	1.40845	0.31252
REACT_16888:Signaling by PDGF	THBS1, THBS2	2	1.40845	0.43611
REACT_602:Metabolism of lipids and lipoproteins	FABP4, HADHA	2	1.40845	0.74344
REACT_13:Metabolism of amino acids	SLC1A5, SLC7A11	2	1.40845	0.77264
REACT_11061:Signalling by NGF	RTN4, RICTOR	2	1.40845	0.79862
REACT_17015:Metabolism of proteins	PIGF, EIF4A1	2	1.40845	0.8631
REACT_71:Gene Expression	EIF4A1, MED1	2	1.40845	0.96221
REACT_578:Apoptosis	DSG3	1	0.70423	1
REACT_15295:Opioid Signaling	GNAI1	1	0.70423	1
REACT_11123:Membrane Trafficking	HSPA8	1	0.70423	1
REACT_1762:3' -UTR-mediated translational regulation	EIF4A1	1	0.70423	1
REACT_12508:Metabolism of nitric oxide	CAV1	1	0.70423	1
REACT_9440:Synthesis of Cx26	GJB2	1	0.70423	1
REACT_13433:Biological oxidations	SLC35D1	1	0.70423	1

Table 5-8: Functional Annotation of downregulated genes post p63 knockdown in SCC040 using PANTHER and REACTOME databases. TERM indicates the pathways under which the genes are classified, and count indicates the number of genes classified in the pathway. The percentage contributed by the genes is presented as %.

Figure 5-29: Top downregulated PANTHER and REACTOME pathways in SCC040.

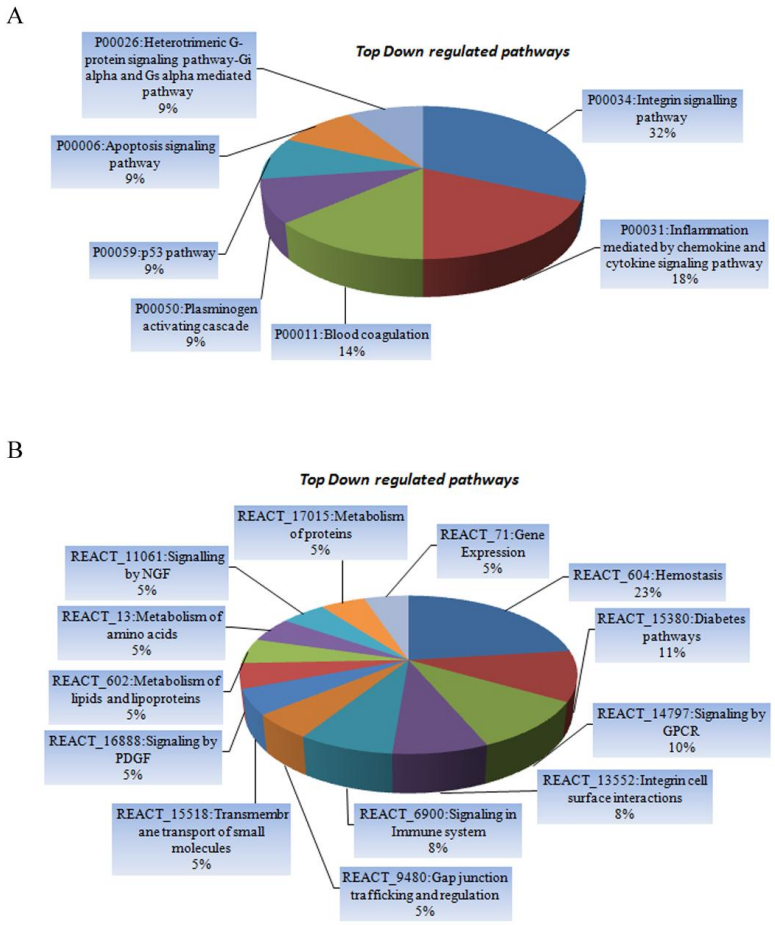


Figure 5-29: Top downregulated PANTHER and REACTOME pathways in SCC040. The significantly downregulated genes in SCC040 were functionally annotated in (A) PANTHER and (B) REACTOME pathways. The list of the genes involved in the pathways represented is presented in Table 5-8.

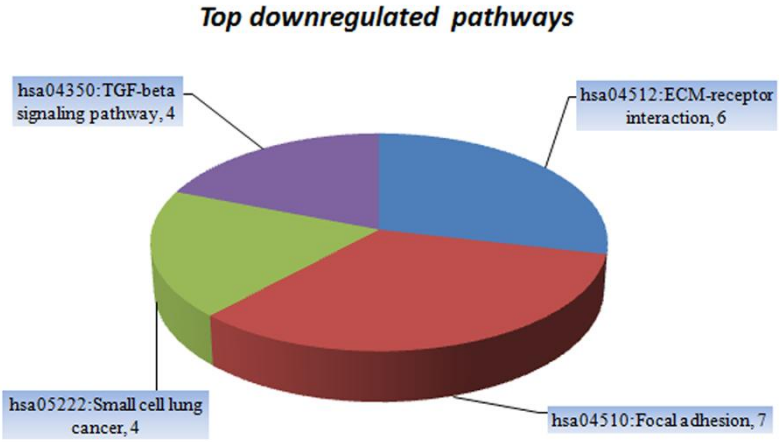
Table 5-9: Functional Annotation of downregulated genes post p63 knockdown in SCC040 using KEGG database.

Term	Genes	Count	%	p Value
hsa04512:ECM-receptor interaction	LAMA1, ITGA2, LAMC2, THBS1, THBS2, COL4A6	6	5.225352	8.79E-04
hsa04510:Focal adhesion	LAMA1, CAV1, ITGA2, LAMC2, THBS1, THBS2, COL4A6	7	5.1329577	0.008857

hsa05222:Small cell lung cancer	LAMA1, ITGA2, LAMC2, COL4A6	4	2.816901	0.039674
hsa04350:TGF-beta signaling pathway	INHBA, FST, THBS1, THBS2	4	2.816901	0.04332

Table 5-9: Functional Annotation of downregulated genes post p63 knockdown in SCC040 using KEGG database. TERM indicates the pathways under which the genes are classified, and count indicates the number of genes classified in the pathway. The percentage contributed by the genes is presented as %.

Figure 5-30: The pathways enriched using the KEGG database for the significantly downregulated genes post p63 knockdown in SCC040.



The genes significantly upregulated more than 1.5 fold in SCC040 were also functionally annotated. Table 5-10 and Table 5-11 present the list of upregulated genes classified into biological pathways using the databases and the contribution of each pathway is represented as pie charts in Figure 5-31 and Figure 5-32.

Table 5-10: Functional Annotation of genes upregulated post p63 knockdown in SCC040 using PANTHER and REACTOME databases.

PANTHER PATHWAY				
Term	**Genes**	**Count**	**%**	**P Value**
P00052:TGF-beta signaling pathway	FOXQ1, SMAD6, FOXA1, GDF15	4	5.210526	0.098814
P00033:Insulin/IGF pathway-protein kinase B signaling cascade	FOXQ1, FOXA1	2	2.105263	0.482813
P00005:Angiogenesis	PTPRB, HIF1A, CTNNB1	3	3.157895	0.49411
P00048:PI3 kinase pathway	FOXQ1, FOXA1	2	2.105263	0.579316
P00004:Alzheimer disease-presenilin pathway	TRIM2, CTNNB1	2	2.105263	0.630999
P00029:Huntington disease	ARL14, ARL4A	2	2.105263	0.707793
P00047:PDGF signaling pathway	VAV3, ELF3	2	2.105263	0.748277
P00036:Interleukin signaling pathway	FOXQ1, FOXA1	2	2.105263	0.803097
P00034:Integrin signaling pathway	ARL14, NTN4	2	2.105263	0.813795
P00012:Cadherin signaling pathway	CTNNB1	1	1.052632	1
P04372:5-Hydroxytryptamine degradation	ALDH3B1	1	1.052632	1
P02775:Salvage pyrimidine ribonucleotides	CDA	1	1.052632	1
P05918:p38 MAPK pathway	DUSP10	1	1.052632	1
P00059:p53 pathway	IGFBP3	1	1.052632	1
P04398:p53 pathway feedback loops 2	CTNNB1	1	1.052632	1
P02723:Adenine and hypoxanthine salvage pathway	PRTFDC1	1	1.052632	1
P00003:Alzheimer disease-amyloid secretase pathway	ADAM10	1	1.052632	1
P00046:Oxidative stress response	DUSP10	1	1.052632	1
P00053:T cell activation	VAV3	1	1.052632	1
P00056:VEGF signaling pathway	HIF1A	1	1.052632	1
P02774:Salvage pyrimidine deoxyribonucleotides	CDA	1	1.052632	1

P00022:General transcription by RNA polymerase I	SDPR	1	1.052632	1
P00010:B cell activation	VAV3	1	1.052632	1
P00031:Inflammation mediated by chemokine and cytokine signaling pathway	VAV3	1	1.052632	1
P02788:Xanthine and guanine salvage pathway	PRTFDC1	1	1.052632	1
P00006:Apoptosis signaling pathway	BIRC3	1	1.052632	1
P00057:Wnt signaling pathway	CTNNB1	1	1.052632	1
P04391:Oxytocin receptor mediated signaling pathway	OXTR	1	1.052632	1
P00045:Notch signaling pathway	ADAM10	1	1.052632	1
P00009:Axon guidance mediated by netrin	NTN4	1	1.052632	1
P00008:Axon guidance mediated by Slit/Robo	NTN4	1	1.052632	1
P02771:Pyrimidine Metabolism	CDA	1	1.052632	1
P00030:Hypoxia response via HIF activation	HIF1A	1	1.052632	1
REACTOME PATHWAY				
Term	**Genes**	**Count**	**%**	**p Value**
REACT_9417:Signaling by EGFR	ADAM10, ADAM12	2	2.105263	0.188895
REACT_1788:Transcription	PAPOLA, TCEA1	2	2.105263	0.446208
REACT_71:Gene Expression	PAPOLA, TCEA1	2	2.105263	0.804868
REACT_14797:Signaling by GPCR	GPER, OXTR	2	2.105263	0.954426
REACT_6259:HIV-1 elongation arrest and recovery	TCEA1	1	1.052632	1
REACT_604:Hemostasis	SLC16A1	1	1.052632	1
REACT_6900:Signaling in Immune system	SLC16A1	1	1.052632	1
REACT_6185:HIV Infection	TCEA1	1	1.052632	1
REACT_299:Signaling by Notch	ADAM10	1	1.052632	1
REACT_11044:Signaling by Rho GTPases	VAV3	1	1.052632	1
REACT_1892:Elongation arrest and recovery	TCEA1	1	1.052632	1
REACT_578:Apoptosis	CTNNB1	1	1.052632	1

REACT_1698:Metablism of nucleotides	CDA	1	1.052632	1
REACT_769:Pausing and recovery of elongation	TCEA1	1	1.052632	1
REACT_1046:Pyruvate metabolism and TCA cycle	SLC16A1	1	1.052632	1
REACT_6143:Pausing and recovery of Tat-mediated HIV-1 elongation	TCEA1	1	1.052632	1
REACT_15380:Diabetes 3pathways	IGFBP3	1	1.052632	1
REACT_11123:Membrane Trafficking	SEC23A	1	1.052632	1
REACT_11045:Signaling by Wnt	CTNNB1	1	1.052632	1
REACT_216:DNA Repair	TCEA1	1	1.052632	1
REACT_13433:Biological oxidations	CYP26A1	1	1.052632	1
REACT_125:Processing of Capped Intron-Containing Pre-mRNA	PAPOLA	1	1.052632	1
REACT_6844:Signaling by TGF beta	SMAD6	1	1.052632	1
REACT_13:Metabolism of amino acids	GATM	1	1.052632	1
REACT_6344:Tat-mediated HIV-1 elongation arrest and recovery	TCEA1	1	1.052632	1
REACT_152:Cell Cycle, Mitotic	CEP70	1	1.052632	1
REACT_12034:Signaling by BMP	SMAD6	1	1.052632	1
REACT_474:Metabolism of carbohydrates	SLC16A1	1	1.052632	1
REACT_6244:Pausing and recovery of HIV-1 elongation	TCEA1	1	1.052632	1

Table 5-10: Functional Annotation of genes upregulated post p63 knockdown in SCC040 using PANTHER and REACTOME databases. TERM indicates the pathways under which the genes are classified, and count indicates the number of genes classified in the pathway. The percentage contributed by the genes is presented as %.

Figure 5-31: Top upregulated PANTHER and REACTOME pathways in SCC040.

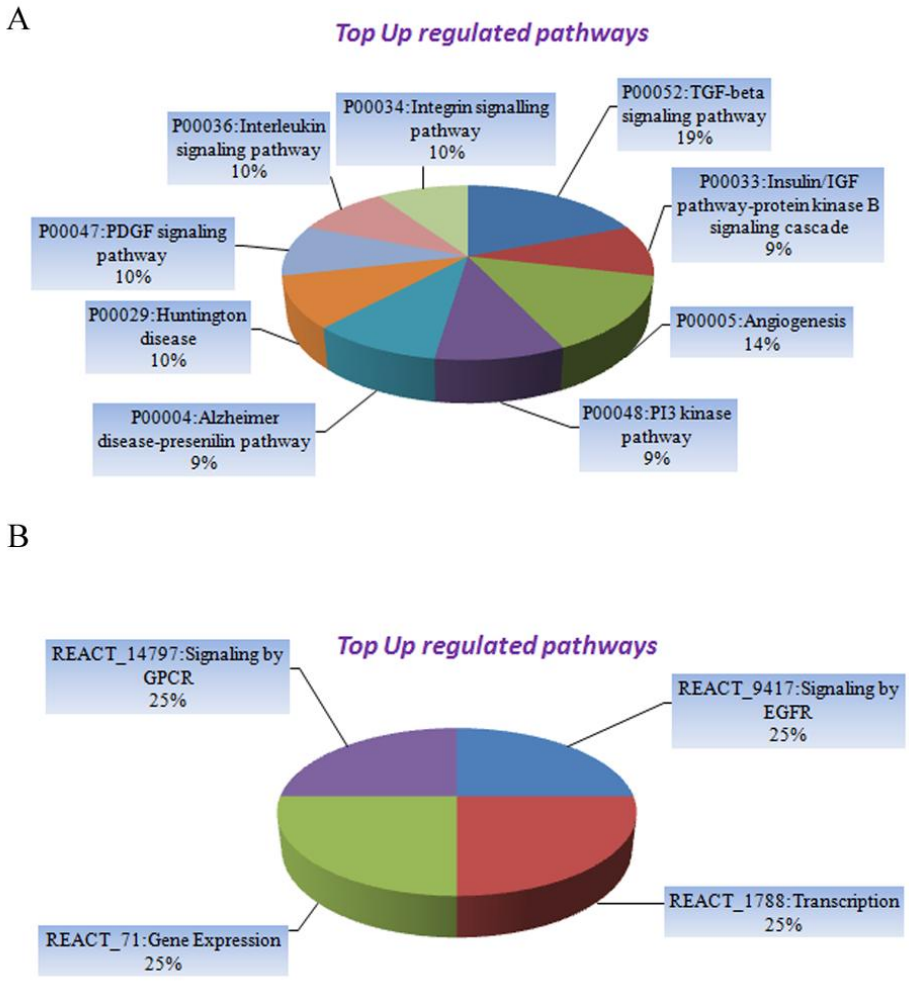


Figure 5-31: Top upregulated PANTHER and REACTOME pathways in SCC040. The significantly upregulated genes in SCC040 were functionally annotated in (A) PANTHER and (B) REACTOME pathways. The complete list of the genes upregulated more than 1.5 fold and their functional pathways is presented in Table 5-10.

Table 5-11: Functional Annotation of upregulated genes post p63 knockdown in SCC040 using KEGG database.

Term	Genes	Count	%	p Value
hsa05216:Thyroid cancer	PPARG, CTNNB1	2	2.105263	0.133527
hsa05200:Pathways in cancer	HIF1A, PPARG, BIRC3, CTNNB1	4	5.210526	0.21608
hsa00830:Retinol metabolism	BCMO1, CYP26A1	2	2.105263	0.234742
hsa04510:Focal adhesion	VAV3, BIRC3, CTNNB1	3	3.157895	0.259713
hsa04520:Adherens junction	PTPRB, CTNNB1	2	2.105263	0.317757
hsa04670:Leukocyte transendothelial migration	VAV3, CTNNB1	2	2.105263	0.444775
hsa04310:Wnt signaling pathway	PRICKLE1, CTNNB1	2	2.105263	0.530195
hsa04010:MAPK signaling pathway	DUSP10, PTPRR	2	2.105263	0.741196
hsa05222:Small cell lung cancer	BIRC3	1	1.052632	1
hsa05211:Renal cell carcinoma	HIF1A	1	1.052632	1
hsa04150:mTOR signaling pathway	HIF1A	1	1.052632	1
hsa04080:Neuroactive ligand-receptor interaction	OXTR	1	1.052632	1
hsa00350:Tyrosine metabolism	ALDH3B1	1	1.052632	1
hsa05010:Alzheimer's disease	ADAM10	1	1.052632	1
hsa04662:B cell receptor signaling pathway	VAV3	1	1.052632	1
hsa04666:Fc gamma R-mediated phagocytosis	VAV3	1	1.052632	1
hsa04020:Calcium signaling pathway	OXTR	1	1.052632	1
hsa04210:Apoptosis	BIRC3	1	1.052632	1
hsa04070:Phosphatidylinositol signaling system	INPP4B	1	1.052632	1
hsa04810:Regulation of actin cytoskeleton	VAV3	1	1.052632	1
hsa00983:Drug metabolism	CDA	1	1.052632	1
hsa00330:Arginine and proline metabolism	GATM	1	1.052632	1
hsa00982:Drug metabolism	ALDH3B1	1	1.052632	1
hsa05130:Pathogenic Escherichia coli infection	CTNNB1	1	1.052632	1

hsa00140:Steroid hormone biosynthesis	STS	1	1.052632	1
hsa05217:Basal cell carcinoma	CTNNB1	1	1.052632	1
hsa05215:Prostate cancer	CTNNB1	1	1.052632	1
hsa04650:Natural killer cell mediated cytotoxicity	VAV3	1	1.052632	1
hsa04530:Tight junction	CTNNB1	1	1.052632	1
hsa04664:Fc epsilon RI signaling pathway	VAV3	1	1.052632	1
hsa05120:Epithelial cell signaling in Helicobacter pylori infection	ADAM10	1	1.052632	1
hsa03410:Base excision repair	HMGB1	1	1.052632	1
hsa03320:PPAR signaling pathway	PPARG	1	1.052632	1
hsa05210:Colorectal cancer	CTNNB1	1	1.052632	1
hsa00562:Inositol phosphate metabolism	INPP4B	1	1.052632	1
hsa00150:Androgen and estrogen metabolism	STS	1	1.052632	1
hsa00360:Phenylalanine metabolism	ALDH3B1	1	1.052632	1
hsa03018:RNA degradation	PAPOLA	1	1.052632	1
hsa00260:Glycine, serine and threonine metabolism	GATM	1	1.052632	1
hsa04916:Melanogenesis	CTNNB1	1	1.052632	1
hsa00860:Porphyrin and chlorophyll metabolism	FTH1	1	1.052632	1
hsa00980:Metabolism of xenobiotics by cytochrome P450	ALDH3B1	1	1.052632	1
hsa05016:Huntington's disease	PPARG	1	1.052632	1
hsa05412:Arrhythmogenic right ventricular cardiomyopathy (ARVC)	CTNNB1	1	1.052632	1
hsa00340:Histidine metabolism	ALDH3B1	1	1.052632	1
hsa04120:Ubiquitin mediated proteolysis	BIRC3	1	1.052632	1
hsa04350:TGF-beta signaling pathway	SMAD6	1	1.052632	1
hsa00240:Pyrimidine metabolism	CDA	1	1.052632	1
hsa04115:p53 signaling pathway	IGFBP3	1	1.052632	1
hsa05213:Endometrial cancer	CTNNB1	1	1.052632	1
hsa00590:Arachidonic acid metabolism	PTGES	1	1.052632	1

hsa04360:Axon guidance	NTN4	1	1.052632	1
hsa04621:NOD-like receptor signaling pathway	BIRC3	1	1.052632	1
hsa04062:Chemokine signaling pathway	VAV3	1	1.052632	1
hsa00532:Chondroitin sulfate biosynthesis	CSGALNACT1	1	1.052632	1
hsa04660:T cell receptor signaling pathway	VAV3	1	1.052632	1
hsa00010:Glycolysis / Gluconeogenesis	ALDH3B1	1	1.052632	1

TERM indicates the pathways under which the genes are classified, and count indicates the number of genes classified in the pathway. The percentage contributed by the genes is presented as %.

Figure 5-32: The pathways enriched using the KEGG database for the genes significantly upregulated post p63 knockdown in SCC040

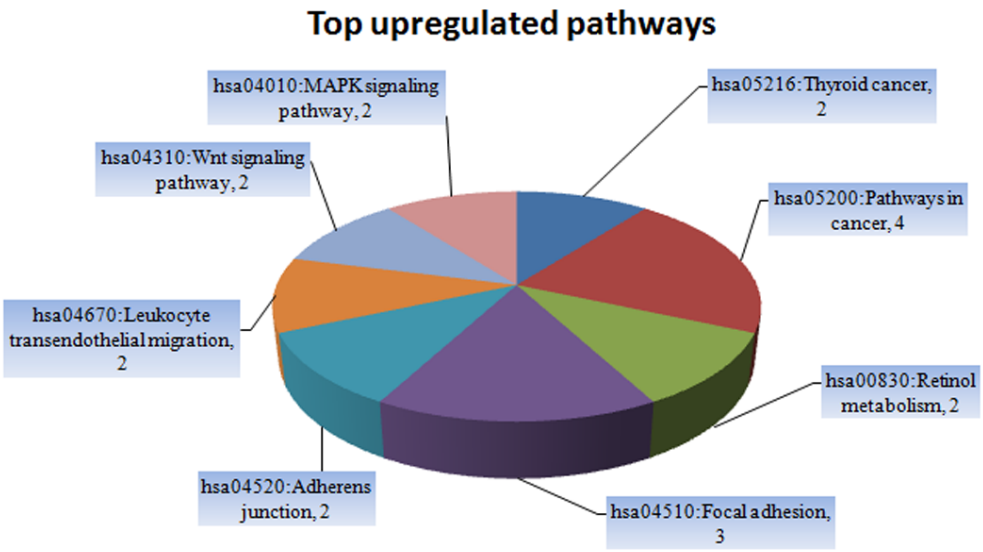


Figure 5-32: The pathways enriched using the KEGG database for the genes significantly upregulated post p63 knockdown in SCC040. The top five pathways annotated using the KEGG database for the DEGs upregulated more than 1.5 fold is presented in the Figure. The complete list of pathways and the genes involved is presented in Table 5-11.

As observed in AW8507, functional analysis of the DEGs in SCC040 demonstrated signaling pathways such as EGF, TGF and Integrin Pathways, as well as the p53 signaling pathways were affected post p63 knockdown. Interestingly the phenotypic assays showed differential effects of ΔNp63 knockdown in AW8507 and SCC040. Hence among the differentially expressed genes in the two cell lines, the genes that were common identified. Table 5-12 enlists the genes that are upregulated in AW8507 but downregulated in SCC040 and the genes that are upregulated in SCC040 but downregulated in AW8507.

Table 5-12: : Genes downregulated in AW8507 but upregulated in SCC040 and genes upregulated in AW8507 but downregulated in SCC040 post p63 knockdown.

Upregulated in AW8507 but Downregulated in SCC040	**Upregulated in SCC040 but downregulated in AW8507**
AREG	IGFBP3
ITGA2	HIF1A
NT5E	SLC16A1
ZNF750	XAF1
ANGPTL4	TRIM22
	STARD7
	CAPZA1
	TRA2A
	SAMD9L
	NAP1L4
	TUBGCP3
	SRSF5
	CLEC7A
	RAD21
	IDH1
	CTNNB1
	NCOR1
	CFI
	TFDP1
	ACTG1P4

	IL8
	SLITRK6
	SAFB
	LMNB1
	FAM111B
	DNAJB6
	NUCKS1
	RAD23B
	TOP1
	CALD1
	ZNF292
	HNRNPH3
	ALDH1A3

A representative heat map of the differentially expressed genes in the two cell lines is presented in Figure 5-33

Figure 5-33: Representative heat maps of the differentially expressed genes in (A) SCC040 and (B) AW8507

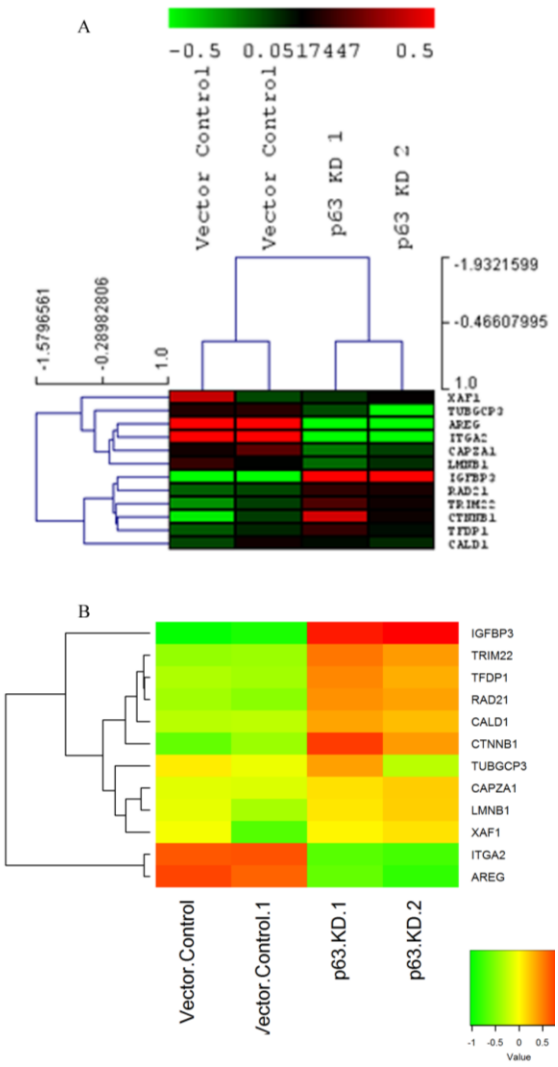


Figure 5-33: Representative heat maps of the differentially expressed genes in (A) SCC040 and (B) AW8507. Red color indicates downregulation, green color indicates upregulation.

5.3.8 Validation of microarray results by qRT-PCR.

The genes identified in the microarray analysis were validated by selecting the top up and downregulated genes contributing to a few major pathways known to be associated with carcinogenesis. A few genes known to be under the regulation of p63 were also validated as

positive controls. Figure 5-33 and Figure 5-34 show the validation of the microarray results for the positive control genes and genes involved in NOTCH signaling in both AW8507 and SCC040.

Figure 5-34: Validation of microarray genes by real time PCR.

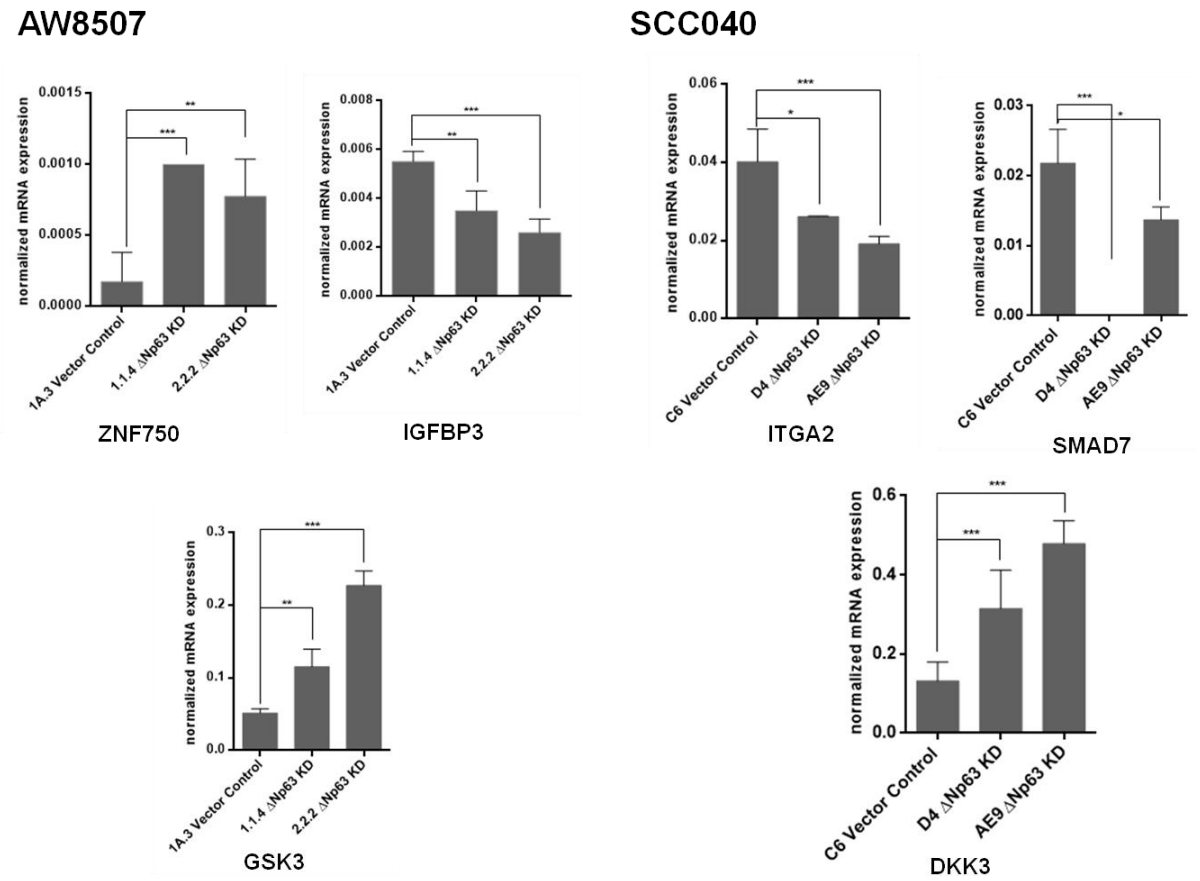


Figure 5.33: Validation of microarray genes by real time PCR. In AW8507, the p63 target IGFBP3 was downregulated while ZNF750 and GSK3 (a WNT pathway inhibitor) were upregulated. In SCC040 ITGA2, a p63 target was downregulated while SMAD7 was downregulated and DKK3 (a WNT pathway inhibitor was upregulated)

Figure 5-35: Validation of NOTCH pathway genes in AW8507 and SCC040.

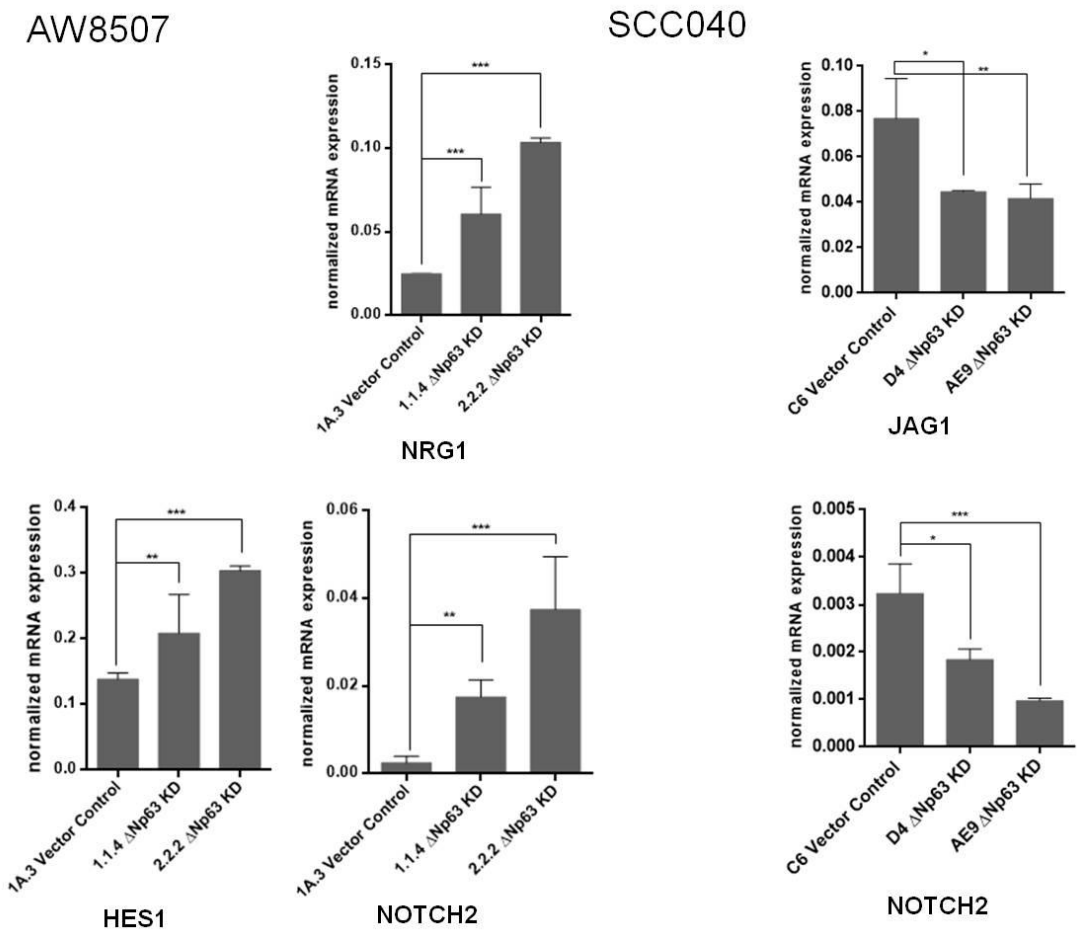


Figure 5-34: Validation of NOTCH pathway genes in AW8507 and SCC040. In AW8507 the NOTCH receptors NRG1 and NOTCH2 and the NOTCH pathway target HES1 was upregulated while in SCC040 the NOTCH receptors JAG1 and NOTCH2 were downregulated.

6 . Discussion

The role of the tumor suppressor gene p53 in the pathogenesis of oral cancers has been extensively studied with approximately half of the oral cancer cases showing inactivation of p53. Earlier studies from the lab showed altered expression of the p53 repressed proteins clusterin and survivin in oral cancer cell lines and tissues. The current study identified the predominantly expressed isoforms of p63, p73, survivin and clusterin in oral cancers. The present study for the first time demonstrates the ability of p63 to repress survivin in the absence of a functional p53. Our studies also show a gain of function role for the R273H p53 mutant which binds to p63 and results in activation of survivin expression. We further show that the knockdown of the predominantly expressed ΔNp63 isoform of p63 affects the migratory, invasive and proliferative ability of the oral cancer cell lines, thus demonstrating an oncogenic role of ΔNp63 in these cells.

6.1 ΔNp63, TAp73 are the predominantly expressed isoforms of p63 and p73.

Real time PCR analysis of the TA and ΔN isoforms of p63 and p73 in oral tumor tissues demonstrated overexpression of the ΔNp63 isoforms in tumor tissues as compared to normal. In oral cancer cell lines as well ΔNp63 expression was predominant over TAp63 expression. Previous studies have also reported predominant expression of ΔNp63α in head and neck cancer cell lines and the salivary gland [237, 238]. In squamous cell carcinoma cell lines ΔNp63 levels have been reported to be 100 times more than the TAp63 levels [239, 240].

In the current study, the levels of p73 did not differ significantly in tissues although in cell lines TAp73 expression was higher than ΔNp73 levels. The expression level of p73 was also significantly lower as compared to p63 in the tissues studied. This has been reported in neuroblastoma and prostate cancers as well where the levels of p73 are reported to be relatively

lower as compared to p63 and p53 [241-243] and the TAp73 isoforms are reported to be predominant over the ΔNp73 isoforms [243, 244].

6.2 sClu expression in oral cancer cell lines and tissues.

Secretory clusterin was expressed in the oral cancer cell lines although the transcript was found to be downregulated in oral tumor tissues as compared to adjacent normal tissues. Clusterin is reported to be upregulated in response to stress in prostate, kidney, breast and colon cancers [245-248] although various studies have also reported downregulation of clusterin in prostate, oral and salivary gland cancers [249-251].

6.3 Survivin wt , survivin 2B and survivin ΔEx3 are the predominant isoforms of survivin in oral cancers.

Our studies show that survivin wt was the predominantly expressed isoform in oral cancer cell lines and oral tumor tissues . Survivin protein expression in oral cell lines has been previously reported [252] and was also detected by western blotting in the four oral cancer cell lines used in the study. In keeping with previous literature survivin wt was the predominant isoform with 80-88% upregulation in oral tumor tissues as compared to normal tissues from healthy individuals [23, 138, 139, 141, 146]. Survivin ΔEx3 was the next most overexpressed isoform in oral tumor tissues which was also reported by De Maria et. al.[20]. Survivin ΔEx3 has been found to be upregulated in glioblastomas, breast and thyroid cancers [137, 146, 149]. It was found to be significantly upregulated in thyroid tumors as compared to adjacent non-neoplastic and non tumoral tissues. Survivin 3B interestingly was found to be associated with differentiation status of the tumor, with higher expression correlating with poorly differentiated tumors. Studies in breast cancers have shown an inverse correlation of survivin 3B with 10 proapoptotic genes and 5 antiapoptotic genes [143] while overexpression in non-tumor cells has been reported to result

in tumor formation in nude mice [111]. Although survivin 2B was found to be upregulated in the current study, association of survivin 2B with tumorigenic properties is ambiguous. Survivin 2B expression was found to increase with higher tumor grade in oral cancers [20], and association has been shown with progressive T stage and poor clinical outcome[140, 142]; in colorectal cancers, reduced expression in advanced stages has been reported [147] with overexpression of survivin 2B inhibiting ovarian tumor growth [148]. The second proapoptotic form survivin 2α was first described by Caldas et. al. in medulloblastoma cell lines [114]. The expression of survivin 2α and survivin 3B was found to be higher in the adjacent normals as compared to normal as well as tumor tissues. Higher expression of survivin 2α has been reported in adjacent non-neoplastic tissues as compared to non-tumoral and thyroid and breast tumors indicating a possible role for survivin 2α in early stages of tumorigenesis. [150] [143]. Survivin 3α was detected first in 2006 and since then two reports have determined its expression in cancer [115] though no functional role has as yet been ascribed to it although in the current study, survivin 3α was undetectable in normal tissues, but was overexpressed in oral tumor tissues.

Interestingly our studies show an association of the combined expression of the three antiapoptotic isoforms of survivin with TNM staging of the tumors, with sixty four percent Stage IV tumors showing overexpression of at least one of the antiapoptotic survivin isoforms. Survivin is known to function as a homodimer, though the minor isoforms are also hypothesized to be capable of forming heterodimers with survivin wt. We found a significant correlation between the expression of the survivin minor isoforms with survivin wt indicating that the relative levels of the isoforms may be important in determining cell fate. Pavlidou et. al. have shown a correlation in the expression of survivin wt with survivin 2B and survivin 3B and have

also found that the ratio of survivin ΔEx3 to survivin wt significantly correlated to poor prognosis.

6.4 Dimerization of the survivin monomer is stabilized by a hydrophobic pocket formed by a linker peptide and the alpha helices.

The minor isoforms of survivin are hypothesized to form heterodimers with survivin wt that may attenuate the function of survivin wt. However only three of the survivin isoforms have been shown to co-immunoprecipitate with survivin wt (Caldas 2005). The structure of the survivin wt homodimer was solved independently by three groups and is reported to have a bow-tie shaped structure [103, 229, 230]. The structures of the survivin 2α and survivin ΔEx3 have been predicted but the ability of the minor isoforms to heterodimerize with survivin wt is not yet reported [114, 253]. The current study is the first to predict the structures of the minor isoforms of survivin and determine the ability of the minor isoforms to heterodimerize with survivin wt. Comparison of the survivin wt homodimer predicted using ClusPro and the crystal structure 1F3H in the RCSB PDB database both revealed the dimerization interface to involve the linker peptide between the alpha-helix and the BIR domain of the survivin monomers.

Docking analysis of the minor isoforms with survivin wt predicted that the pro-apoptotic survivin 2B and the anti-apoptotic isoform survivin 3B can dimerize with survivin wt using the same linker peptide as observed in the homodimer. Analysis of the interacting residues also showed that the Leu 98 involved in the dimeric pocket in the survivin homodimer is also the residue involved in the survivin wt-survivin 2B and survivin wt-survivin 3B heterodimers. Leu 98 is conserved in all the IAPs except in XIAP and may thus be important for the apoptotic function as well. The dimerization of survivin 2B which is pro-apoptotic to the antiapoptotic survivin wt may attenuate the anti-apoptotic function of survivin wt. The involvement of the

same residue further suggests a competitive binding of survivin homodimers versus the heterodimers with survivin 2B and survivin 3B. Thus the balance of the pro and the anti-apoptotic isoforms of survivin may indeed be the determining factor in cell fate. Further in vitro studies to identify the importance of the Leu 98 in dimerization by site directed mutagenesis, may help understand the kinetics of the survivin dimer formation.

6.5 p63 knockdown results in downregulation of survivin wt in AW8507 but upregulation in SCC040.

Our expression analysis showed overexpression of survivin and clusterin transcripts in oral cancer cell lines, both of which are repressed by p53 and predominant expression of the p63 transcripts over p73 transcripts. p53 dependent apoptosis is also reported to require the association of p63 and p73 [254]. Previous reports suggest ability of the p63 and p73 to transactivate p53 targets [242, 255-259]. Our studies demonstrate downregulation of survivin wt expression upon p63 knockdown in AW8507 which shows the presence of a mutant p53 implying that p63 may be activating survivin expression in the presence of a mutant p53. On the other hand in SCC040 survivin wt was upregulated upon p63 knockdown indicating that in the absence of a functional p53, p63 represses survivin expression. Although previously mutant p53 proteins were known to accumulate in the cell due to increased stability, these were considered to be inactive. However, recent in vivo studies have shown higher tumor formation in mice harboring mutant p53 as compared to p53 null mice, indicating a role for mutant p53 in tumorigenesis [260]. Recent evidence suggests that some of the mutant p53 proteins may be capable of interacting with p63 and affecting its function [213, 261-263]. The results of the present study also suggest that p63 may not be regulating the expression of clusterin in oral cancers. Immunoblotting analysis in the cell line harboring mutant p53 (AW8507) demonstrated

the ability of p53 mutant R273H to bind to p63. Our studies also demonstrate that the complex of mutant p53 and p63 in AW8507 does not utilize the site utilized by p53 for repression of survivin [18] but binds 2000bases upstream of this canonical site. Earlier studies in keratinocytes have identified that the binding of mutant p53 to p63 may be capable of shifting the position of p63 binding on the promoter and may also affect its regulatory function. This was demonstrated in HaCaT cells wherein the presence of mutant p53 resulted in the activation of KLF4 by p63 instead of repression as observed in normal keratinocytes [213]. Our studies suggest that in AW8507 as well, p63 may be activating survivin in the presence of the p53 mutant R273H and that this may be due to a shift in the binding position of p63 on the survivin promoter. Our studies thus demonstrate a gain of function role for the p53 mutant R273H in oral cancers.

6.6 Knockdown of ΔNp63 affects the migration and invasive properties of AW8507 and SCC040.

Our studies show a decrease in migration in AW8507 and SCC040 upon ΔNp63 knockdown but increase in invasion in SCC040. We also demonstrate a reduced proliferative capacity in AW8507 upon ΔNp63 knockdown. Previous studies in keratinocytes have demonstrated a decrease in cell proliferation on p63 knockdown which was rescued on simultaneous knockdown of p53 implying that inhibition of cell proliferation may be p53 dependent [259, 264, 265]. This has also been demonstrated in murine models wherein overexpression of ΔNp63 in squamous cell carcinomas promoted cell survival [266]. In osteosarcoma cell lines overexpression of ΔNp63α showed enhanced wound healing and anchorage independent growth [238, 267] while knockdown showed reduced wound healing ability in osteosarcoma and neuroblastoma cell lines and reduced tumor growth [267, 268]. Although these results suggest an oncogenic role for ΔNp63 isoforms, ΔNp63 expression has also been associated with tumor suppressor roles. p63

expression is reported to be reduced in metastatic lesions in breast, prostate and lung cancers and was found to progressively decrease with increasing stages of the disease [269]. Studies in breast and lung adenocarcinoma have also shown overexpression of ΔNp63 to inhibit cell invasion but not affect cell proliferation ability and apoptotic index [269]. Knockdown of ΔNp63 is also reported to increase invasion in lung and bladder cancer cell lines [269, 270] while Tran et. al. 2013 have reported increased invasive ability of bladder cancer cells upon knockdown of ΔNp63α [271].

Thus although the overexpression of ΔNp63 isoforms has been associated with metastatic ability certain studies also demonstrate it to be reduced in metastatic lesions. In the current study as well in AW8507, ΔNp63 exhibited an oncogenic role evidenced by reduced migration and proliferation upon its knockdown but in SCC040 ΔNp63 knockdown resulted in an increased invasive ability. Tran et. al. 2013 suggest a partial role for ΔNp63 in EMT implying EMT- like characteristics to be required for escaping from the primary tumors but a further transition from mesenchymal to epithelial transition to facilitate proliferation of the secondary tumors [271]. Ratovitski et. al. 2001 have further reported the ability of wild type p53 to bind to and induce degradation of ΔNp63, with half life of p63 reducing from greater than 2hours to less than 45mins in the presence of wt p53 [272]. AW8507 harbors a mutation in the DNA binding domain of p53 (R273H) which is reported to be capable of binding but does not induce degradation as efficiently as wt p53. In the current study as well, p63 is demonstrated to co-immunoprecipitate with mutant p53 in AW8507 and may contribute to some of the oncogenic properties of ΔNp63 in these oral cancer cells.

6.7 Identification of genes regulated by p63 differentially in AW8507 (p53 R273H mutant) and SCC040 (no p53 protein)

The phenotypic studies in AW8507 and SCC040 and the differential survivin expression post p63 knockdown in the two cell lines implied different functional roles of p63 in the two cell lines possibly due to the differing p53 status. To identify the genes that may be contributing to these phenotypic effects cDNA microarray analysis was performed post p63 knockdown in AW8507 and SCC040. The differentially expressed genes in both the cell lines were classified using the PANTHER, REACTOME and KEGG databases.

Pathway analysis of the differentially expressed genes in AW8507 showed Notch signaling, Pathways in Cancer, Cell cycle and TCA cycle related genes to be downregulated. In SCC040 as well Notch Pathway was downregulated, so were genes involved in the Pathways in cancer, focal adhesion and ECM-receptor interaction while Wnt signaling, Tight junction related molecules and cell cycle related genes were upregulated. Previous studies have also demonstrated adherens junctions, focal adhesion, cell adhesion pathways to be regulated by p63 in prostate cancer cells as well as keratinocytes [273 {Wu, 2012 #288]}. Gene expression analysis post p63 knockdown in head and neck cancer identified pathways associated with migration, adhesion and invasion to be differentially regulated by p63 [274]. The phenotypic assays in the current study also showed decrease in migration in AW8507 and increase in invasion in SCC040 post ΔNp63 knockdown.

Analysis of the microarray demonstrated downregulation of Inhibin βA upon p63 knockdown in the two cell lines. Validation of the βA subunit transcripts by real time PCR also showed significant downregulation of the βA subunit in AW8507 and SCC040 upon p63 knockdown.

Inhibin and Activins are heterodimeric glycoproteins involved in Activin signaling, a part of the TGFβ superfamily [275, 276]. The proteins are formed either by the dimerization of the β subunits which forms Activins or heterodimerization with the α subunits which forms the Inhibin proteins. However since cDNA microarray would identify the transcript levels of the subunits, the identification of the Inhibin βA would indicate the downregulation of the βA transcript as validated by real time PCR which may be capable of forming either Inhibins or Activins. Activin A is known to be involved in various cancers including OSCC wherein it is associated with increased tumorigenic properties such as increased migration and invasion and with poor prognosis [277].

Among the upregulated genes in AW8507 ITGA2 was identified by PATNHER, REACTOME and KEGG Pathway analysis and validated by real time PCR (P00034 Integrin Signaling Pathway, P00031 Inflammation mediated by Chemokine and Cytokine Signaling Pathway, REACT_604 Hemostasis, REACT_13552 Integrin cell surface interactions, hsc04640 Hematopoietic cell lineage). On the other hand in SCC040 ITGA2 was identified to be downregulated by PANTHER, REACTOME and KEGG analysis (P00034 Integrin Pathway, P00031 Inflammation mediated by chemokine and cytokine signaling pathway, REACT_604 Hemostasis, REACT_13552 Integrin Cell surface interactions, hsa04512 ECM receptor interaction, hsa4510 focal adhesion, hsa05222 small cell lung cancer. ITGA2 loss is reported to play an important role in cancer metastasis [278-281] though the mechanism of action is as yet unclear. ITGA2 loss is associated with increased migration in breast cancers but does not affect invasive properties while in colon cancers ITGA2 loss is associated with reduced migration [281, 282].

We further validated CTNNB1 involved in the Wnt pathway to be downregulated in AW8507. CTNNB1 encodes the beta-catenin (cadherin associated protein) which is responsible for transcriptional activation of the WNT gene expression in complex with TCF/LEF. The WNT/ β-catenin signaling association with cancer is well documented and is also being targeted for anti-cancer therapy [283-286]. β-catenin is also shown to be associated with colorectal cancers, breast cancers and as observed in this study also in oral cancers [283, 287-290].

Pathway analysis identified downregulation of a few genes involved in NOTCH signaling in SCC040 post p63 knockdown which were validated by real time PCR as well. NOTCH signaling has been associated with increased migration in breast cancer cells [291, 292]. In the current study we found migration to be reduced in SCC040 upon p63 knockdown which may be due to the downregulation of genes involved in the NOTCH pathway upon knockdown.

We also demonstrated increased anchorage independence in AW8507 upon p63 knockdown but reduced migration. It is possible that different pathways may be contributing to the contrasting phenotypic results observed in the study. Upregulation of Hes1 as validated by real time PCR was observed in AW8507. Increased HES1 expression has been associated with increased anchorage independent growth and metastatic capacities in colon cancers.

The gene expression analysis thus identified various genes involved to be differentially upregulated or downregulated in the two cell lines that may contribute to the differential phenotypic effects of p63 knockdown in the two cell lines. Our studies identify NOTCH signaling pathway, Wnt signaling to be regulated by p63. Further studies determining the role of the genes contributing to these pathways may help in identifying the exact signaling cascade that are regulated by p63 in oral cancer cells and which of these would be affected by the presence of a mutant p53 protein.

7 . Summary and Conclusions

The results of the current study demonstrate that in oral cancer cell lines and tissues, ΔNp63 isoforms are predominantly expressed over the TAp63 isoforms. We have also observed a downregulation of sCLU transcripts in tumor tissues as compared to normal tissues. Overexpression of all survivin splice variants was seen in oral tumor tissues as compared to normal tissues. This study is the first to report the upregulation of survivin 2α and survivin 3α in oral cancer cell lines and tissues. Among the isoforms of survivin, in keeping with previous studies, survivin wt was found to be the predominantly expressed isoform in oral cancer cell lines and tissues. Additionally survivin 2B and survivin ΔEx3 were the predominant minor isoforms expressed in oral cancer cell lines. This is the first study to demonstrate an underestimation of survivin upregulation in tumor tissues when compared to adjacent normal tissues. The current study also demonstrates that the overexpression of at least one anti-apoptotic form and downregulation of the pro-apoptotic forms significantly correlated with TNM staging with 64% stage IV tumors showing overexpression of the anti-apoptotic forms,

Survivin is reported to function as a homodimer to inhibit apoptosis in response to DNA damage. Our docking analysis has indicated possible heterodimerization of survivin wt- survivin 2B and survivin wt-survivin 3B. We have identified Leu 98 to be one of the key residues involved in the dimerization interface. Among the minor isoforms, it was observed that the heterodimerization of survivin 3B (anti-apoptotic) and survivin 2B (pro-apoptotic) also involved the Leu 98 residues. Thus the same Leu98 residue seems to be utilized and survivin 2B may compete with survivin wt for the formation of a dimer. Further since survivin 2B is proapoptotic, the heterodimer of survivin 2B and survivin wt may result in reduced anti-apoptosis abilities. These results suggest

that the ratio of the pro-apoptotic (survivin 2B) isoforms to antiapoptotic (survivin wt, survivin 3B) may be an important determinant of the formation of homodimer or heterodimer. Further *in vitro* studies need to be conducted to identify the importance of Leu 98 on the dimerization ability of the survivin isoforms

Our studies also demonstrate that in the presence of mutant p53, p63 activates survivin expression whereas in the absence of a functional p53, p63 represses survivin expression. Clusterin expression however was not altered significantly upon p63 knockdown. The current study thus for the first time shows that the regulation of survivin by p63 is influenced by the presence of an R273H mutant p53 protein.

Our studies demonstrated the ability of the R273H p53 mutant to bind to p63 in AW8507 cells. Our results show that in AW8507 the mutant p53 p63 protein complex binds approximately 2100 bases upstream of the reported binding site for wild type p53. We thus demonstrate that in the presence of mutant p53 p63 activates survivin expression in oral cancer cells which may be possibly mediated by a shift in the binding of the mutant p53-p63 complex on the survivin promoter.

Further we demonstrated that in the presence of mutant p53, migration and proliferation of the oral cancer cells is reduced upon p63 knockdown. However in the absence of p53 invasive ability of the cells increases upon p63 knockdown (SCC040). Our results thus suggest that although p63 may result in reduced invasive ability and increased migration, the presence of mutant p53 might result in increased proliferation via the mutant p53 p63 complex.

Microarray analysis identified upregulation of EGFR receptor signaling, Integrin Signaling Pathway and Notch signaling in AW8507 while Cell cycle, signaling by WNT and Cadherin signaling pathway were downregulated. On the other hand in SCC040 TGFβ Signaling, EGFR signaling, focal adhesion and adherens junction to be upregulated while Integrin signaling and apoptosis signaling was downregulated. Analysis of the genes involved in the pathways identified CTNNB1, Wnt Signaling pathway to be upregulated in SCC040 but downregulated in AW8507 while ITGA2 was upregulated in AW8507 but downregulated in SCC040. Downregulation of ITGA2 is associated with reduced migration, while CTNNB1 overexpression is associated with increased metastatic ability. Downregulation of ITGA2 in SCC040 but upregulation in AW8507 post knockdown may indicate that in the presence of mutant p53, p63 represses ITGA2 expression and thus facilitates migration. In keeping with this phenotypic assays also showed reduced migration in AW8507 post knockdown. However CTNNB1 was found to be upregulated in SCC040 but downregulated in AW8507 indicating again that in the presence of mutant p53, p63 may be activating CTNNB1 expression and thus facilitating metastatic potential. Our gene expression analysis thus shows NOTCH, TGFβ and Wnt signaling pathways to be altered in oral cancer cells upon p63 knockdown. The three pathways are known to be associated with tumorigenic properties however, the association of NOTCH and Wnt with p63 has been demonstrated for the first time in this study. Further studies exploring the role of the genes involved in these pathways may give a better understanding of the signaling mechanisms of p63 and their role in oral tumorigenesis.

Conclusions

The current study identified that the ΔNp63, sCLU and survivin wt isoforms of p63, clusterin and survivin respectively were predominantly expressed in oral cancer cell lines and may be associated with oral tumorigenesis. The results of the current study also demonstrate that p63 is capable of repressing survivin in the absence of a functional p53, however in the presence of mutant p53, p63 activates survivin expression in oral cancer cells. We also show that the metastatic potential of oral cancer cells is enhanced by the presence of mutant p53 by affecting the regulation of genes such as ITGA2, CTNNB1 and NOTCH by p63. Currently targeting pathways that are regulated by mutant p53 is one of the approaches utilized in cancer drug discovery [214]. The present study has determined the possible pathways regulated by p63 and by the mutant p53-p63 complex that may be targeted in oral cancers. Further studies determining the role of the genes involved in these pathways would provide a better approach to cancer drug discovery.

8 . References

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9 . Annexure

Annexure I: List of Genes downregulated and upregulated in AW8507 upon p63 knockdown
Fold change > 1.5, p <0.05

Downregulated		
Gene Symbol	**Log FC**	**p Value**
CAPZA1	-1.547587148	4.05E-04
HSPA5	-1.44114883	9.11E-03
TRA2A	-1.398513272	8.68E-04
HSPA5	-1.377527925	1.41E-02
CTNNB1	-1.369367705	1.92E-02
HSPA5	-1.341878489	1.35E-02
SPTBN1	-1.321391994	1.08E-02
HSPA8 /// SNORD14C /// SNORD14D	-1.282361305	1.23E-02
CTNNB1	-1.282307559	9.34E-03
CTNNB1	-1.16415306	5.85E-03
CTNNB1	-1.152879883	3.66E-03
ARL6IP1	-1.151910685	4.15E-04
SET /// SETP4	-1.137098987	3.91E-02
ATP1B1	-1.112075021	3.88E-04
SPTBN1	-1.110547789	1.48E-02
FUCA1	-1.109073094	1.64E-03
TOP1	-1.082340639	3.10E-02
HNRNPA1	-1.06033033	8.46E-03
ATP5A1	-1.035991804	1.08E-02
ATP5A1	-1.03296103	2.01E-02
CTNNB1	-1.02369832	2.35E-03
CCNL1	-1.021672932	2.46E-04
NREP	-1.020589326	3.99E-03
CDK11A /// CDK11B	-1.015909232	6.71E-05
HDAC1	-0.987950374	5.21E-03
CFB	-0.986899112	1.86E-03
ERP29	-0.983003425	7.26E-03
SNX3	-0.97795986	2.69E-05
ACAD11 /// NPHP3 /// NPHP3-ACAD11	-0.972508739	2.39E-04
SRSF1	-0.965069702	6.30E-04
EEF1A1	-0.962788332	1.06E-02
NAP1L4	-0.947422604	2.20E-02
NAP1L4	-0.944471294	1.91E-02

Upregulated		
Gene Symbol	**Log FC**	**p Value**
AREG /// AREGB	0.797509	1.30E-04
ANGPTL4	0.682924	2.58E-04
NT5E	0.680514	1.22E-02
ESM1	0.679761	5.53E-03
MMP3	0.672745	9.42E-04
DICER1	0.641478	4.02E-03
PGS1	0.632795	1.82E-02
IER5L	0.627367	1.52E-02
ASAH2 /// ASAH2B	0.620364	1.43E-03
PDCD1LG2	0.61349	4.17E-04
SH2D2A	0.613221	2.72E-04
CSF2	0.613133	4.26E-03
ZNF750	0.602579	2.63E-02
NR1I3	0.592185	2.45E-02
ITGA2	0.589693	2.72E-03
RAB6A	0.574784	1.57E-03
VWA2	0.571379	1.57E-03

KIF14	-0.942352645	4.96E-04			
MSH6	-0.942305239	6.42E-03			
RPL15	-0.93877227	2.65E-03			
STARD7	-0.927407021	8.78E-03			
DDX42	-0.926794356	4.83E-03			
ALDH1A3	-0.925283352	2.73E-03			
HSPD1	-0.916021958	1.20E-02			
EIF2S3	-0.912862246	1.40E-02			
EIF3L	-0.910147434	1.83E-02			
EXOSC10	-0.906063751	2.78E-03			
SIN3A	-0.899200982	5.13E-03			
ALDH1A3	-0.890638492	1.11E-02			
CTNNB1	-0.887016516	1.42E-02			
NBPF10 /// NBPF11 /// NBPF12	-0.88234956	3.50E-03			
DOCK9	-0.876955405	3.78E-02			
XAF1	-0.876939402	1.02E-03			
CSE1L	-0.866960169	1.91E-03			
APOL1	-0.86674442	1.98E-02			
SLC12A2	-0.865795595	5.44E-04			
PRRC2C	-0.858605486	3.06E-02			
WDR45B	-0.858443666	4.15E-03			
PDS5A	-0.857159679	4.63E-03			
AHCYL1	-0.857105575	1.23E-02			
CAST	-0.85657748	3.01E-03			
NCBP2	-0.85639907	1.13E-03			
CFI	-0.847932706	1.16E-02			
AKAP10	-0.847802968	2.11E-04			
UBE2I	-0.847153796	2.55E-04			
LMNB1	-0.846206823	4.55E-03			
PPP2R5C	-0.844324708	5.67E-04			
ANP32A	-0.844231788	2.51E-02			
XAF1	-0.838769725	2.91E-04			
HIF1A	-0.837454207	1.13E-04			
PDIA6	-0.836845527	9.31E-03			
PTGES3	-0.836822957	1.87E-03			
C7orf55-LUC7L2 /// LUC7L2	-0.835529335	4.09E-03			
SLC16A1	-0.832991461	9.09E-03			
DDX42	-0.824835946	7.50E-03			
ZDHHC7	-0.824212251	3.57E-03			

MALAT1	-0.82255075	2.40E-02			
NPTN	-0.822536992	1.01E-02			
CLPX	-0.817219899	2.74E-02			
HNRNPA1 /// HNRNPA1P10 /// HNRNPA1P33	-0.815298527	9.16E-03			
HDAC8	-0.806507866	2.25E-02			
SMPX	-0.80052615	2.29E-03			
NUCKS1	-0.795949997	7.63E-03			
ZNFX1	-0.792313333	5.24E-04			
XAF1	-0.790560979	1.06E-04			
GOLGA8A /// GOLGA8B	-0.790246942	2.97E-04			
UPF3A	-0.78620719	4.82E-03			
IGFBP3	-0.786181847	2.46E-02			
SRRM1	-0.784420838	2.45E-03			
DNAJB6	-0.781496622	2.12E-02			
NCOR1	-0.780733154	6.48E-03			
SRSF5	-0.779901211	1.49E-02			
CRK	-0.777526141	3.55E-02			
HNRNPF	-0.775815604	2.82E-02			
UXS1	-0.775705329	1.30E-02			
STAG3 /// STAG3L1 /// STAG3L3	-0.773529156	3.78E-03			
HSPA9	-0.772333315	3.40E-02			
ACADM	-0.769189814	7.25E-04			
PRPF3	-0.765428039	1.45E-02			
IL8	-0.764429677	9.37E-03			
ADPGK	-0.761850563	6.64E-03			
CD44	-0.75943524	1.60E-02			
MPHOSPH8	-0.754930996	4.97E-03			
SAMD9L	-0.754911042	4.01E-02			
RASA1	-0.753435778	8.96E-04			
HSPA8 /// SNORD14C /// SNORD14D	-0.75191521	3.77E-03			
KLRC4-KLRK1 /// KLRK1	-0.751808493	3.59E-04			
HIF1A	-0.750677042	9.20E-04			
NCBP2	-0.748965796	7.70E-04			
PRC1	-0.747620728	3.47E-02			
RBM5	-0.747248983	8.62E-04			
TRIM22	-0.743892305	9.58E-04			

CAST	-0.742149999	1.59E-04			
HSPD1	-0.739155942	1.68E-02			
ADAM17	-0.73661988	7.57E-03			
CTNNB1	-0.735577205	1.91E-02			
DDX3X	-0.735506049	9.60E-03			
TFDP1	-0.734204989	1.76E-02			
EZH2	-0.733383157	9.53E-04			
RAD23B	-0.733070466	4.29E-04			
TOP2A	-0.730311975	3.99E-03			
TMEM183A /// TMEM183B	-0.726612837	1.20E-03			
HNRNPDL	-0.726392252	2.60E-03			
DTX3L	-0.725763454	3.40E-04			
TTC19	-0.724296163	6.02E-03			
MAT2A	-0.723627546	1.69E-02			
CCNL1	-0.721936126	2.02E-04			
TRIM22	-0.721135285	2.84E-04			
CAPRIN2	-0.720932951	1.34E-02			
MAPK6	-0.720093	2.74E-04			
SRSF5	-0.719348572	3.34E-03			
DKC1 /// MIR644B /// SNORA56	-0.718198071	1.04E-02			
ATP8B5P /// ZFAND6	-0.718083286	1.80E-04			
TRA2A	-0.714397497	1.26E-03			
SSFA2	-0.711523608	5.73E-04			
SRSF5	-0.710172135	3.81E-03			
CLTC	-0.710162791	2.42E-03			
SVIL /// SVILP1	-0.710080567	4.62E-03			
HADHA	-0.709439009	2.66E-02			
APOL1	-0.708329109	2.12E-02			
NUP85	-0.708099536	4.40E-02			
RAD21	-0.708073938	8.20E-04			
CLTC	-0.707710204	2.48E-02			
RHOA	-0.706342727	4.39E-04			
PAF1	-0.702131759	4.55E-02			
NREP	-0.701657961	1.94E-04			
SMC3	-0.700943183	2.19E-03			
F3	-0.700868067	1.87E-02			
RIPK4	-0.700835034	3.75E-03			
SAFB	-0.700751928	3.67E-02			
PSEN1	-0.700295603	3.61E-03			

AKR1C1 /// AKR1C2	-0.698937033	9.00E-03			
SPINK5	-0.698672028	4.51E-04			
AKT2	-0.697870102	2.32E-02			
HDAC8	-0.696227638	1.54E-02			
DMXL2	-0.693341012	3.41E-03			
FHL1	-0.693145953	3.18E-02			
LINC00969 /// SDHA /// SDHAP1 /// SDHAP2	-0.691590144	2.29E-02			
HNRNPDL	-0.690691021	6.01E-03			
TRA2A	-0.688066685	1.41E-03			
NFATC2IP	-0.687134708	2.96E-02			
HSPA8 /// SNORD14C /// SNORD14D	-0.68408871	2.29E-02			
KLF5	-0.683668	8.34E-03			
AKAP9	-0.682755924	1.81E-02			
R3HDM1	-0.682357898	4.83E-03			
LMNB1	-0.681841565	4.31E-03			
XAF1	-0.680143725	3.12E-04			
PRKAG1	-0.679622776	8.16E-03			
LOC101060541 /// RBM8A	-0.67928429	4.60E-04			
CTPS1	-0.677674325	3.24E-02			
RHOA	-0.677467694	9.53E-04			
HSPD1	-0.675551373	1.59E-02			
SH3KBP1	-0.674568951	5.72E-03			
UBE2I	-0.674037475	1.83E-03			
PGK1	-0.67346054	7.95E-04			
ZFAND6	-0.672050526	1.07E-03			
IFI16	-0.67185883	7.38E-03			
USP7	-0.671525907	8.88E-03			
DROSHA	-0.670434051	1.89E-02			
IDH1	-0.670378113	6.59E-04			
TUBGCP3	-0.67005278	2.91E-03			
ARHGAP11A /// ARHGAP11B	-0.669643563	3.68E-02			
EIF4A2 /// SNORA81 /// SNORD2	-0.667424321	2.19E-02			
NDUFA10	-0.667180695	1.12E-02			
NUP153	-0.664295985	1.29E-02			
CDK13	-0.66355156	4.80E-04			

RTCB	-0.66317693	1.78E-02			
GOLGA8A /// GOLGA8B	-0.662773619	4.37E-04			
SMAD3	-0.662751559	2.16E-02			
MCCC1	-0.660645523	1.11E-02			
NAP1L4	-0.660555072	9.42E-03			
RAD23B	-0.660300833	4.48E-03			
UBE2I	-0.659317636	2.65E-03			
ANXA2	-0.6586895	1.52E-02			
HNRNPF	-0.657304308	3.03E-02			
CALD1	-0.65624911	1.05E-02			
METTL3	-0.655021473	1.17E-02			
SRSF5	-0.654170258	3.83E-03			
AAGAB	-0.653940206	1.86E-02			
PNN	-0.653254032	1.06E-02			
DMTF1	-0.651993532	4.69E-02			
ZFAND6	-0.650272502	3.32E-04			
C22orf43	-0.650191335	1.06E-02			
SLC25A14	-0.649325191	6.96E-04			
MPHOSPH8	-0.648902841	6.42E-04			
BCLAF1	-0.64872007	1.26E-03			
QRICH1	-0.646448208	1.70E-02			
HNRNPDL	-0.642787755	1.17E-02			
GOLGA8A /// GOLGA8B	-0.642499512	9.77E-03			
HNRNPD	-0.642436137	7.34E-03			
HSPA8 /// SNORD14C /// SNORD14D	-0.640045185	1.29E-02			
SRSF5	-0.63739131	2.13E-03			
TTC3 /// TTC3P1	-0.63728596	4.08E-04			
KLRC3	-0.63706701	1.64E-02			
KLRC3	-0.636948708	1.30E-02			
NPTN	-0.636652238	2.24E-02			
RHEB	-0.636447751	1.01E-03			
MSL1	-0.636414059	4.12E-03			
SSRP1	-0.634955252	2.82E-02			
LOXL2	-0.63435602	2.42E-03			
GOLGA8A /// GOLGA8B	-0.634151942	1.12E-02			
MTMR4	-0.633682856	6.51E-04			
NAP1L4	-0.633278751	9.46E-03			
PPIA	-0.632287564	1.07E-02			

SUMF2	-0.632178613	3.10E-03			
CASP2	-0.630655131	1.40E-02			
BCCIP	-0.630197004	1.61E-02			
GALNT10	-0.629665116	4.05E-02			
SPDYE2B	-0.629498913	2.46E-03			
SECISBP2	-0.628352366	2.48E-03			
LETMD1	-0.627696373	1.56E-02			
KDM4C	-0.626766098	2.87E-02			
RACGAP1	-0.626670605	5.59E-03			
FER	-0.626160775	1.11E-03			
JARID2	-0.625939131	2.73E-02			
TMEM2	-0.625594801	1.37E-03			
ZFP62	-0.625146565	8.53E-04			
MAEA	-0.624794722	9.10E-03			
SDHC	-0.623972419	5.65E-03			
CP	-0.621607762	1.32E-03			
ZNF292	-0.621440501	1.15E-03			
GTF2H2 /// GTF2H2B /// GTF2H2C /// GTF2H2D	-0.621346937	1.07E-03			
CLEC7A	-0.621334723	1.85E-02			
CFB	-0.62010158	1.28E-02			
RBMX /// SNORD61	-0.61918346	3.75E-02			
TRIM22	-0.618575368	1.77E-03			
LAMP1	-0.618499293	1.68E-02			
ANXA5	-0.616875052	2.15E-02			
SRRM2	-0.61649854	1.59E-03			
ZKSCAN5	-0.616496097	3.76E-04			
RBFOX2	-0.615858257	7.62E-03			
UTP6	-0.615751604	4.23E-02			
STMN1	-0.614787707	9.83E-04			
TRA2A	-0.614461678	8.86E-04			
ACTG1P4 /// AMY2A /// AMY2B	-0.614314397	1.63E-03			
SPTAN1	-0.613276717	6.69E-03			
POM121 /// POM121C	-0.61200414	3.42E-02			
EIF2B1	-0.61173543	2.07E-02			
STAT1	-0.611363722	1.23E-02			
KLRC3	-0.61119905	1.15E-03			
NREP	-0.610715386	3.44E-04			

WHSC1	-0.610598712	3.98E-02			
APMAP	-0.610571902	4.36E-02			
EBNA1BP2	-0.609304575	7.43E-03			
SMARCA2	-0.60828595	2.17E-03			
SLITRK6	-0.607528652	1.48E-03			
CTGF	-0.607282928	1.81E-02			
PGK1	-0.607112208	1.04E-03			
MCM8	-0.606228935	2.00E-02			
MMP13	-0.605453979	1.46E-03			
GDI2	-0.604175219	1.98E-03			
TFDP1	-0.60405671	2.71E-02			
ACLY	-0.604048727	1.22E-02			
OGT	-0.602469442	1.05E-02			
SRSF5	-0.60245055	1.30E-02			
NPTN	-0.600897913	4.62E-03			
SMARCA2	-0.600257751	1.58E-03			
ACTG1P4 ///AMY2A /// AMY2B	-0.60007916	5.48E-03			
HNRNPU	-0.599531597	1.77E-02			
RAD21	-0.599501587	2.85E-03			
TOP2A	-0.599496694	1.99E-02			
LOC100505519 /// SCAF8 /// TIAM2	-0.599041084	2.82E-02			
USP14	-0.597786519	5.74E-03			
SMC2	-0.596892954	3.86E-02			
CAPZA1	-0.596016143	2.87E-04			
GFPT2	-0.595991914	9.07E-03			
COPG2	-0.595271532	2.98E-02			
PLK2	-0.594529927	4.48E-02			
TNNI2	-0.593681814	1.62E-03			
MAEA	-0.592690397	1.50E-02			
STAU1	-0.592034589	3.73E-03			
KRT15	-0.589765922	8.84E-04			
IL8	-0.589412605	1.88E-02			
ATM	-0.587982116	1.65E-02			
PARG	-0.586565426	4.65E-03			
MAEA	-0.586428641	1.24E-02			
FAM111B	-0.586244376	2.08E-03			
CCDC150	-0.586180781	1.68E-02			
DDX42	-0.585293612	3.93E-03			
UBE2I	-0.585274903	3.04E-03			

SLC4A1AP	-0.585220572	3.50E-02			
RNF145	-0.584822683	2.03E-03			
LETMD1	-0.583037981	3.99E-02			
MRPS27	-0.58240351	4.43E-02			
STAG3L1 /// STAG3L2 /// STAG3L3	-0.581631485	2.62E-03			
SART3	-0.581102975	2.36E-02			
RNF145	-0.579868073	9.69E-03			
HMGN1	-0.578909892	2.47E-03			
MED9	-0.578128431	1.67E-02			
HEATR5B	-0.577527631	4.35E-03			
SGK494 /// SPAG5	-0.57728589	3.67E-02			
KIAA1598	-0.576493154	1.40E-02			
ZNF232	-0.575503699	2.65E-03			
RBM5	-0.575411884	4.88E-03			
HNRNPH3	-0.575210669	2.54E-02			
UBE2I	-0.574181999	2.39E-03			
MEST	-0.573534736	2.95E-02			
LUC7L3	-0.572891436	8.15E-04			
MRRF	-0.572426111	1.40E-02			
NRD1	-0.572353833	1.77E-02			
CHRNA9	-0.571650915	1.13E-03			
TPM3	-0.571592756	6.35E-03			
ENSA	-0.571390078	1.11E-02			
SLC25A27	-0.570998365	2.26E-02			

Annexure II: List of downregulated and upregulated genes in SCC040 upon p63 knockdown

Fold Change > 1.5 fold, $p < 0.05$

Downregulated		
Gene Symbol	**Log FC**	**p Value**
COL12A1	-2.469357382	2.28E-07
CA12	-2.345104594	5.76E-07
SAA1 /// SAA2 /// SAA2-SAA4	-2.038119494	3.03E-07
GJB2	-1.893475699	1.24E-06
ACER3	-1.883370635	1.64E-06
FST	-1.858934888	1.02E-06
DKK3	-1.853392382	2.16E-06
KRT6B	-1.820844868	7.10E-07
RTN4	-1.817521167	7.97E-07
GJB6	-1.805015819	6.78E-07
IL36G	-1.791354905	1.11E-06
MAN2A1	-1.683992182	1.09E-06
SPRR2E	-1.63129607	1.93E-05
HIPK2	-1.595789934	1.52E-06
USP31	-1.587299512	6.00E-06
THBS2	-1.586923599	2.35E-06
S100A9	-1.567431261	4.54E-06
CALU	-1.544166204	3.15E-06
LAMC2	-1.510664383	2.49E-06
KIAA1430	-1.506683603	6.43E-06
SPRR1A /// SPRR1B	-1.503338312	2.87E-06
SSR1	-1.473289917	1.42E-05
PAPPA	-1.466369778	3.40E-06
CERS3	-1.46375569	4.06E-06
TENM2	-1.45242314	2.30E-06
SPRR2D	-1.431097908	7.45E-06
SDAD1	-1.429617137	4.23E-06
AREG /// AREGB	-1.41693962	1.20E-05
DHX33	-1.402545789	3.55E-06
BPTF	-1.402075897	7.42E-05
ITGA2	-1.396818916	3.10E-06
KRT16 /// KRT16P1 /// KRT16P2	-1.390054527	4.75E-06
AHCYL1	-1.384856898	4.14E-06
CAV1	-1.377549617	3.71E-06
FEZ1	-1.359609001	4.51E-05

Upregulated		
Gene Symbol	**Log FC**	**p Value**
GDF15	2.022466	1.21E-06
KRT8	1.994256	4.18E-07
IGFBP3	1.922963	6.81E-07
ALDH3B1	1.919863	6.97E-07
RHOBTB3	1.896448	7.75E-07
TCEA1	1.727931	1.11E-06
IKZF2	1.67057	5.20E-06
HIF1A	1.621001	1.47E-06
FYB	1.594569	1.61E-06
BIRC3	1.590701	8.82E-06
ARL14	1.546802	2.37E-06
PPARG	1.54635	2.61E-05
BCMO1	1.49865	1.04E-04
VAV3	1.485147	5.42E-06
DUSP10	1.459306	2.95E-06
C1orf63	1.454876	6.39E-06
ERP27	1.44445	2.03E-04
SMAD6	1.44392	2.30E-04
RAB6A	1.416003	3.25E-05
PTPRB	1.414087	4.92E-05
EPS8	1.361459	2.91E-06
GATM	1.358218	3.11E-06
ANKRD10	1.356736	7.28E-06
SNX10	1.341331	1.32E-05
NTN4	1.339931	1.42E-05
FTH1	1.335544	3.18E-06
SLC16A1	1.324373	1.89E-05
XAF1	1.314903	4.37E-06
TMEM27	1.314029	3.81E-06
INPP4B	1.305798	1.79E-05
DNAJC15	1.304279	9.34E-06
CSGALNACT1	1.300675	4.77E-06
PTPRR	1.300626	8.58E-06
FOXA1	1.298319	7.94E-06
FER1L4	1.29507	3.99E-06

RICTOR	-1.354975105	7.86E-06	PTGES	1.274071	5.61E-06
CST3	-1.338286879	1.87E-05	TNFAIP2	1.272211	4.97E-06
RUFY3	-1.328629756	4.61E-06	SEC23A	1.264109	5.07E-06
SLC7A11	-1.326919439	5.70E-05	TRIM22	1.238957	1.31E-05
FABP4	-1.322440617	8.24E-06	TRIM2	1.187386	7.73E-06
PI3	-1.318750314	9.62E-06	MBNL1	1.1849	7.40E-05
COL4A6	-1.315488597	3.51E-05	ADAM12-OT1	1.182055	3.30E-04
LAMA1	-1.313685291	8.65E-06	SCEL	1.179126	6.34E-05
ANTXR2	-1.295768421	1.08E-05	PKP3	1.176457	1.50E-04
MAFB	-1.292777145	6.16E-06	EVI2A	1.174007	1.87E-04
FABP5 /// FABP5P3 /// LOC101060453	-1.284891111	1.45E-05	ELF3	1.163897	2.50E-05
SORL1	-1.282658245	5.36E-06	PAPOLA	1.162199	4.49E-05
SERPINB2	-1.271338818	8.47E-06	FNDC3B	1.161068	8.63E-05
AMTN	-1.264612363	7.00E-06	NEBL	1.158902	1.68E-04
FKBP14	-1.264598615	7.41E-06	NUSAP1	1.148305	2.98E-05
PLTP	-1.260540119	5.57E-06	CDA	1.138338	5.11E-05
MAGT1	-1.248323164	5.73E-06	STARD7	1.132941	5.41E-05
CXCL14	-1.247197478	5.58E-06	NDRG4	1.129637	2.78E-05
KRT6C	-1.237814739	2.31E-05	ANXA4	1.117654	3.83E-05
YIPF6	-1.229074687	5.20E-06	CMBL	1.114494	3.32E-05
S100A8	-1.223934723	1.65E-05	PRICKLE1	1.106914	1.00E-05
RDH11	-1.221024571	8.29E-06	GABPB1	1.103048	3.41E-05
ATP6V1A	-1.216580698	5.30E-06	BBS10	1.098935	1.33E-03
GLIPR1	-1.208473999	7.61E-06	TWF1	1.095867	7.23E-05
INHBA	-1.207775428	9.41E-06	HDAC9	1.091918	1.84E-05
PDPN	-1.19871964	8.72E-06	ADAM10	1.085244	7.11E-05
EIF4A1	-1.196788199	2.00E-05	ARHGAP21	1.081147	1.58E-05
BTG2	-1.192741408	1.68E-05	SCOC	1.073663	1.49E-04
QKI	-1.192554614	6.29E-06	CCDC80	1.071482	6.84E-05
HADHA	-1.189458204	7.59E-05	ECM2	1.068812	6.26E-05
RRAGC	-1.175327166	7.47E-06	SDPR	1.060559	1.37E-04
SIGMAR1	-1.175312695	7.59E-05	DDX60L	1.056429	1.33E-05
HSP90B1 /// MIR3652	-1.172188263	4.09E-05	CXXC5	1.051526	2.00E-05
PDE7A	-1.168356977	2.39E-05	FNDC3A	1.051024	2.10E-04
ECM1	-1.163812957	1.72E-05	LIMCH1	1.045207	3.33E-05
SLC26A2	-1.159704655	9.00E-05	ARRDC3	1.04401	1.80E-05
IGF2R	-1.158468947	1.06E-05	ASPH	1.041857	1.29E-04
ULBP2	-1.157484755	1.17E-04	PARP14	1.038483	8.92E-05
KTN1	-1.157440647	2.58E-05	NTRK2	1.034789	7.92E-03
ASXL2	-1.152199775	1.00E-05	OXTR	1.031725	1.66E-04
SPRR2A	-1.146565171	6.56E-05	CAPZA1	1.031399	1.54E-05

KIAA1715	-1.143228892	5.26E-04	CALB2	1.031141	4.41E-05
MED1	-1.136472232	1.96E-05	CLIP4	1.028552	1.13E-04
PTGS1	-1.135621656	8.13E-06	STS	1.025809	2.30E-04
LTB4R	-1.133007047	8.38E-05	HMGB1	1.023888	2.12E-05
AKR1C1	-1.132362236	1.84E-05	GOLPH3	1.021778	6.96E-05
SPRR1A	-1.124980438	1.47E-05	GPER	1.019546	2.57E-05
NAV1	-1.124828079	2.90E-05	RMI1	1.017911	4.43E-05
SLPI	-1.121057102	2.00E-05	ADAM12	1.016816	2.05E-05
PRRC2C	-1.118953064	2.28E-05	OLFML3	1.013669	3.67E-05
GM2A	-1.116274867	1.47E-05	PRR15	1.012093	2.44E-05
MOSPD2	-1.11425411	1.38E-05	ARL4A	1.012027	2.20E-05
RPL7L1	-1.111926457	9.27E-06	CYP26A1	1.01185	1.74E-04
KRT5	-1.109895138	2.48E-05	ANKRD13C	1.0097	1.74E-05
TP53AIP1	-1.101256072	2.37E-05	FA2H	1.009559	2.38E-05
DSC2	-1.098474988	1.26E-05	LSM12	1.007589	3.41E-05
SDK2	-1.096960334	3.09E-05	UTRN	1.006349	2.97E-05
SERPINE1	-1.094259775	2.64E-04	GPR39	1.004195	1.42E-03
COL13A1	-1.091247424	2.62E-05	FOXQ1	1.001238	2.02E-05
BPTF /// LOC146880	-1.08962914	3.30E-05	SRSF3	0.99554	2.58E-05
AKR1C1 /// AKR1C2 /// LOC101060798	-1.088595497	3.83E-05	PRTFDC1	0.99016	1.10E-04
SLC30A7	-1.088521273	1.61E-05	SCNN1A	0.988666	6.37E-05
RAET1L /// ULBP2	-1.080679833	2.90E-05	AASDHPPT	0.98722	3.62E-05
MPZL2	-1.079645903	2.28E-05	BHLHE41	0.987129	5.72E-05
MLEC	-1.075685309	2.44E-05	TRA2A	0.986114	6.97E-05
SLC39A6	-1.072915181	1.96E-05	MYO5C	0.985818	2.65E-05
STK4	-1.07194683	8.20E-05	PKN2	0.980955	1.19E-04
PIGF	-1.06836068	4.48E-05	PPP1CB	0.979032	4.85E-05
PROCR	-1.063690829	3.01E-05	CLDN4 /// LOC100996451	0.977225	2.05E-05
THBS1	-1.061710006	1.36E-05	IL1R1	0.973831	1.58E-04
GDPD2	-1.058944027	2.95E-05	METTL18	0.971567	5.02E-05
SAA1	-1.056717758	2.83E-04	CEP70	0.967215	2.63E-05
GNAI1	-1.051739908	3.16E-05	TRIM55	0.967207	5.19E-04
BRI3BP	-1.051181502	1.46E-05	HRCT1	0.961983	8.48E-05
DEFB103A /// DEFB103B	-1.051139666	3.52E-05	SP3	0.961037	3.07E-05
XPR1	-1.05037908	1.94E-05	IBTK	0.958342	3.49E-05
DSG3	-1.047494429	2.06E-05	STYX	0.94925	1.21E-04
GYLTL1B	-1.045033951	3.32E-05	RTKN2	0.948408	2.15E-04
ZFP36L2	-1.041897499	2.55E-05	ARHGAP18	0.946005	1.21E-04
GDPD1	-1.038061298	6.03E-05	ALCAM	0.945933	2.91E-05
SLC1A5	-1.034209886	1.89E-05	DLEU2 ///	0.942482	5.76E-05

			DLEU2L		
HSPA8 /// SNORD14C /// SNORD14D	-1.030140321	1.54E-05	SAMD9L	0.940459	5.41E-05
CXCR7	-1.027952755	1.44E-05	REEP5	0.940327	5.13E-05
TXNDC12	-1.019835253	1.56E-05	GCA	0.939212	2.59E-05
TMEM41A	-1.017844382	2.03E-05	EPSTI1	0.934882	2.79E-05
CD9	-1.016027264	2.14E-05	CCDC50	0.933898	4.81E-05
FAM134B	-1.015428	5.01E-05	DOPEY2	0.931879	2.69E-04
NIPAL4	-1.014213253	2.92E-05	PCDH1	0.929656	3.98E-05
CNTN1	-1.011280859	9.32E-05	MAML2	0.922512	3.94E-05
POGLUT1	-1.011067776	9.80E-05	TPD52	0.922247	8.28E-05
OS9	-1.009335983	1.56E-05	GRAMD1C	0.922081	5.40E-05
CD99	-1.005445184	6.72E-05	PTP4A1	0.920124	2.94E-05
SNORD12B	-1.005247688	2.19E-05	UCA1	0.91904	3.28E-05
CTB-89H12.4 /// OTTHUMG00000163462	-1.005057245	2.42E-05	AK4 /// LOC100507855	0.918479	2.82E-05
YIPF5	-1.002844226	2.62E-04	ZNF488	0.916396	1.58E-04
ADRB2	-1.002311382	8.20E-05	PSIP1	0.914443	2.33E-04
RPSA /// SNORA6 /// SNORA62	-1.002161649	1.27E-04	ITGBL1	0.912795	4.24E-05
SEMA5A	-1.000247328	3.81E-04	DAPP1	0.910953	9.41E-05
EMP3	-0.999522625	2.57E-05	MMP28	0.910679	4.64E-05
SLC7A8	-0.999490821	2.29E-05	CDKN2B	0.909963	3.97E-05
SLC35B4	-0.996461069	9.87E-04	ATAD2B	0.905123	3.99E-05
EIF4A1 /// SNORA48 /// SNORA67 /// SNORD10	-0.996135322	2.78E-05	USP25	0.903516	7.27E-05
UTP14C	-0.991778783	4.75E-04	ARHGAP29	0.903264	4.55E-05
TNFRSF10A	-0.990279395	3.27E-05	EIF2AK2	0.902511	1.03E-04
CYP27B1	-0.990217504	4.02E-04	TMOD3	0.901506	4.28E-04
CKLF /// CMTM1	-0.988665826	3.20E-05	GALNT12	0.901475	7.83E-05
IL1R2	-0.987871895	4.63E-04	ANKRD12	0.901404	6.66E-05
CALML3	-0.987213718	2.09E-05	RBBP4	0.900407	6.04E-05
MMRN2	-0.987189856	6.88E-04	CASP8	0.89874	1.30E-04
CLPTM1	-0.986669965	8.74E-05	CALB1	0.898082	4.76E-05
RAET1G /// RAET1L /// ULBP2	-0.986603882	3.25E-05	KRT80	0.898078	6.07E-05
MTDH	-0.985773581	3.32E-05	CARD8	0.895969	1.14E-04
IL1B	-0.984894714	3.33E-05	ASF1A	0.890142	3.87E-05
TMEM87A	-0.983276352	2.21E-05	KCTD3	0.889384	7.97E-05
CEP350	-0.981008523	9.52E-05	TBL1XR1	0.886634	4.48E-05
APLN	-0.974436342	3.33E-05	ANKRD32	0.883779	4.31E-05
ITGB1	-0.972686541	4.26E-05	NAP1L4	0.88258	1.83E-04
SNORD17	-0.967614974	7.05E-04	BCL10	0.881876	1.72E-04

SH2D5	-0.966723779	2.71E-05	ARHGEF39	0.881761	3.82E-05
S100A3	-0.966476034	2.24E-05	FBXO32	0.881494	1.37E-04
KRT14	-0.962834667	2.60E-05	PDIK1L	0.881041	4.17E-05
FUCA1	-0.962401235	2.30E-05	GPR160	0.880707	4.53E-04
ATP6V0B	-0.960129706	2.34E-05	TWIST1	0.878307	7.15E-05
TNC	-0.959090022	4.23E-05	IFIH1	0.878109	9.43E-05
FAM217B	-0.956193712	6.24E-05	SMARCE1	0.875701	2.76E-04
SLC35D1	-0.954586504	2.91E-05	ST6GALNAC5	0.875314	7.33E-05
BCL2L11	-0.953846131	5.52E-05	PSMB10	0.873278	2.16E-04
TNRC6B	-0.953796249	7.92E-05	TUBGCP3	0.871037	1.08E-04
CMTM6	-0.949601407	6.52E-05	KIF18A	0.870238	8.37E-05
MIR3651 /// SNORA84	-0.945409829	4.86E-04	CCDC117	0.869736	4.88E-05
RAD50	-0.94486696	8.50E-05	CXCL6	0.869169	4.44E-04
FAM213A	-0.944377774	3.13E-05	CDKN2C	0.868895	1.46E-04
NSD1	-0.943297272	4.33E-04	ADAM9	0.866336	1.37E-04
ACVR1C	-0.942295594	1.35E-04	RBM25	0.864081	1.05E-04
FLRT2 /// LOC100506718	-0.941653497	8.43E-05	DNER	0.863486	6.12E-05
IL1RAP	-0.941641646	4.69E-05	HNRNPL	0.863314	3.88E-05
MBOAT2	-0.940244511	1.39E-04	SRSF5	0.863154	1.54E-04
SPRR1B	-0.936482617	2.36E-05	C15orf48	0.861119	9.56E-05
TMEM208	-0.935579897	4.38E-05	MARCKS	0.859075	4.45E-05
WDR66	-0.933962921	5.87E-05	SPATS2L	0.853794	5.26E-05
HSP90B1	-0.933248798	4.40E-05	RTP4	0.85306	4.61E-05
CNFN	-0.931002422	9.58E-05	SEC22B	0.852912	1.19E-04
GPATCH4	-0.930095318	3.27E-05	CAMK2N1	0.85229	6.74E-05
DAD1	-0.928683578	4.00E-05	PRPF40A	0.851014	4.26E-05
CADM1	-0.92845692	4.55E-05	RARRES3	0.850906	1.58E-04
NRG1	-0.925038902	5.03E-05	ARHGAP5	0.846843	1.42E-04
ZNF573	-0.924336867	4.16E-05	NUDT4 /// NUDT4P1	0.845196	4.24E-05
KRT13	-0.921809392	2.78E-05	TOB1	0.84508	2.16E-04
GOLT1B	-0.914305121	3.56E-05	ANK1	0.845015	1.09E-04
TSPAN5	-0.914179879	6.10E-05	SLC2A12	0.844272	1.03E-04
PRNP	-0.914133488	5.97E-05	LNX1	0.843367	5.36E-04
SCD5	-0.913360198	3.22E-05	TMED2	0.841501	8.46E-05
CD44	-0.913067378	3.72E-05	ASS1	0.841488	1.34E-04
CALR	-0.912614064	3.56E-04	G3BP1	0.837263	2.91E-04
SLC12A2	-0.910732401	3.93E-05	KAT6B	0.836374	2.43E-04
STEAP1 /// STEAP1B	-0.908637306	3.72E-05	CLEC7A	0.836285	3.35E-04
PRRG4	-0.908348764	1.36E-04	DCLRE1C	0.836144	2.20E-04
SLC2A5	-0.908301223	7.25E-05	DPP4	0.834024	5.53E-05
CTSC	-0.908053569	2.44E-04	PSMB9	0.830747	6.43E-05

RAB12	-0.905711436	1.88E-04	NAPEPLD	0.830556	5.38E-05
PHB2	-0.905375929	2.91E-05	SH3BGRL2	0.82823	1.77E-04
PPARA	-0.902050916	5.84E-05	UHRF1	0.826813	1.29E-04
SRPRB	-0.899693189	4.80E-05	RAD21	0.825997	8.49E-05
SLC7A1	-0.899224658	3.25E-05	LOC100996626 /// PNRC2	0.823738	6.20E-05
ITGB4	-0.896118832	8.01E-05	PAIP1	0.822867	4.40E-04
TPBG	-0.894364642	5.06E-05	XRN1	0.822197	5.40E-05
ATP10D	-0.894289707	5.63E-05	MECOM	0.821176	5.09E-05
LRP8	-0.891800298	3.32E-05	ZNF680	0.820387	4.96E-04
WLS	-0.889060247	1.48E-04	PBX1	0.8185	3.12E-04
SEC62	-0.88816976	3.62E-05	NADK2	0.817275	5.92E-05
TNFRSF19	-0.887637018	2.35E-04	ZFP91	0.817077	2.88E-04
CRK	-0.885812449	1.31E-04	TIMP4	0.816919	1.45E-04
TLN1	-0.885777762	1.85E-04	TSNAX	0.816595	2.10E-03
KRTDAP	-0.884677778	4.91E-05	SKAP2	0.815149	1.06E-04
TPST2	-0.882438526	3.36E-05	KPNA1	0.814221	2.76E-04
MFHAS1	-0.881751699	5.38E-05	COPS2	0.813174	3.59E-04
ERP29	-0.880871854	3.88E-05	IDH1	0.812447	6.68E-05
SMTN	-0.880217598	6.77E-05	PNISR	0.810323	6.37E-05
FABP5 /// LOC101060453	-0.877367315	1.98E-04	BTAF1	0.806626	2.74E-03
SNHG15 /// SNORA9	-0.875835128	1.91E-03	EFHD2	0.805949	1.25E-04
PTPRZ1	-0.87517569	5.09E-05	TMEM56	0.805893	1.18E-04
IL1A	-0.874927833	3.65E-05	PSTPIP2	0.805752	1.31E-04
OAF	-0.864918494	4.83E-05	CAPSL	0.805498	2.11E-04
MDM4	-0.862939866	4.03E-05	NXT2	0.805405	2.40E-03
KRT6A /// KRT6B /// KRT6C	-0.862393802	4.90E-05	DLEU2	0.805113	1.19E-04
VPS35	-0.85979653	1.43E-03	CTH	0.804064	1.13E-04
MRPS28	-0.858007445	8.52E-05	LCA5	0.803827	5.05E-04
GLI2	-0.856653226	4.06E-05	ACBD7	0.802575	2.11E-04
DRAP1	-0.856147668	8.78E-05	LYPD1	0.802194	9.10E-05
DKC1 /// MIR644B /// SNORA56	-0.85433184	2.44E-04	CHKA	0.801857	6.81E-05
PPIG	-0.85270867	6.28E-05	FMR1	0.801792	1.84E-04
SPRR3	-0.851487226	1.34E-03	PCNP	0.800846	1.11E-04
CSNK1G2	-0.851178744	1.64E-04	KMT2C	0.8002	9.44E-05
AKAP12	-0.848312307	2.03E-04	ID4	0.799548	1.13E-04
TIMM17A	-0.845172273	7.56E-05	KIAA1033	0.795741	3.47E-04
CDKL3	-0.844921096	5.15E-04	CMPK2	0.795386	9.83E-05
EMC4	-0.843727267	5.00E-05	CDKN1C	0.794477	5.16E-03
PRDX4	-0.842684932	5.54E-05	CTNNB1	0.793883	1.21E-04

SLC38A7	-0.841440258	5.68E-05	PDP1	0.79169	8.35E-05
IL7R	-0.839501586	1.19E-04	ANXA10	0.79144	7.69E-04
C6orf48 /// SNORD48	-0.838211347	2.56E-04	YY1	0.791292	1.58E-04
TXNDC15	-0.838115294	1.00E-04	IFIT2	0.78996	7.91E-05
RAB38	-0.837416341	4.53E-05	ADIRF	0.789793	2.05E-04
MRPL2	-0.835492857	4.65E-04	CDC37L1	0.789207	8.17E-05
PFN1	-0.835201915	7.72E-05	MBNL2	0.787056	1.08E-04
TMX2	-0.835165265	4.60E-05	ZFHX4	0.78598	1.19E-04
DHRS7	-0.834264176	1.49E-04	KITLG	0.784523	1.40E-04
DNAJC18	-0.834119562	1.43E-03	DAPK3 /// MIR637	0.784513	4.80E-03
ST3GAL5	-0.834118841	5.21E-05	SP100	0.78391	1.18E-04
BARD1	-0.834023557	4.66E-05	ZCCHC2	0.783444	4.79E-04
NGFR	-0.832265611	5.56E-05	IFI44L	0.782914	6.48E-05
ZNF611	-0.83226137	5.74E-04	TRIM6	0.78158	1.72E-04
BDKRB1	-0.831471385	5.42E-05	CEP97	0.781145	8.70E-04
MAP3K2	-0.830482717	8.59E-05	DHRS12	0.780735	5.18E-04
CYP4F11	-0.829192171	2.19E-04	RALGPS2	0.780494	3.38E-04
ENTPD3	-0.828852336	5.00E-05	NCOR1	0.779165	2.46E-04
SERINC2	-0.827430242	1.14E-04	FAM91A1	0.778338	2.63E-04
KRT6A	-0.825017642	6.04E-05	DZIP3	0.778308	2.60E-04
PTDSS1	-0.824371334	9.38E-05	PDCL	0.777834	1.57E-04
SMAD1	-0.824340965	1.28E-04	RAB11A	0.777413	1.14E-04
NOS1	-0.823639291	5.59E-05	CNOT6	0.77731	7.97E-05
AGA	-0.823332509	1.48E-04	CLDN1	0.777059	1.28E-04
WFDC5	-0.822393809	1.96E-03	OSR2	0.775398	1.65E-04
RPN2	-0.822310379	5.26E-05	SRSF6	0.775331	1.58E-04
CBS	-0.82177196	8.70E-05	PRPS2	0.775185	3.33E-04
APOL6	-0.820151821	1.83E-04	DEPDC1	0.775176	3.46E-04
GLB1	-0.820148029	5.37E-05	FAM91A1 /// LINC00869	0.774043	7.79E-05
EIF4A2 /// MIR1248 /// SNORA4	-0.819782461	1.07E-04	IER5L	0.773273	8.63E-05
P4HB	-0.819706794	1.29E-04	CDK19	0.770241	1.65E-03
DLL1	-0.819264434	1.27E-04	KMT2E	0.769508	1.12E-04
TGFB1	-0.819192004	2.77E-04	LYRM1	0.769128	1.52E-04
EIF3F	-0.819088268	1.24E-04	AKAP5	0.768051	2.34E-03
VKORC1L1	-0.818049125	2.18E-04	DEK	0.767785	9.68E-05
TACC1	-0.81782127	9.83E-05	ID3	0.767583	8.38E-05
TMEM245	-0.81770575	1.29E-04	NFAT5	0.767526	1.76E-04
SERPINF1	-0.817680532	1.00E-04	ZNF197	0.766757	9.68E-05
CLOCK	-0.81761639	3.94E-04	OXR1	0.766755	1.27E-04

SLC36A1	-0.814619386	6.91E-04	COQ10B	0.766588	1.79E-04
SRPX	-0.814409201	1.60E-04	STRBP	0.765329	2.91E-04
RSBN1	-0.809580117	6.51E-05	EPS15	0.764626	1.30E-04
KIAA0040	-0.809200761	8.12E-05	NSUN6	0.764278	9.18E-05
TIMP3	-0.808134379	1.55E-04	ALPP	0.762864	1.32E-04
PCMT1	-0.80746678	8.94E-05	FUT8	0.762325	7.85E-05
M6PR	-0.807261124	5.70E-05	PTEN	0.76179	1.68E-04
ATP6V0E1	-0.806456408	1.19E-04	ACTR2	0.760859	8.40E-05
RYR1	-0.806282762	1.85E-03	FGF2	0.757686	2.80E-04
CCNI	-0.805575708	6.47E-05	RBM27	0.756754	1.12E-04
HYOU1	-0.803990057	3.50E-04	IQCK	0.754206	1.58E-04
PIGH	-0.802973332	1.95E-04	CAAP1	0.753882	2.28E-04
HMGXB4	-0.801397061	7.26E-05	CFI	0.753829	2.86E-04
SAR1B	-0.801064993	1.87E-04	GTF3A	0.75353	1.55E-04
TMEM154	-0.800928684	6.90E-05	TFDP1	0.752647	2.05E-04
CISD1	-0.80083751	1.59E-04	SFR1	0.752123	2.62E-04
ARFGAP2	-0.800563367	2.10E-04	MTBP	0.752023	1.67E-04
TM2D2	-0.799756261	1.80E-04	ARAP2	0.75191	5.40E-04
CTSL1	-0.797342532	6.40E-05	SUB1	0.751902	6.12E-04
ESYT1	-0.795408242	6.53E-05	ZNF217	0.750037	8.29E-05
CCDC47	-0.795194454	7.10E-05	PARD6B	0.749421	7.57E-04
GPR153	-0.794189333	3.91E-04	PCBP2	0.748353	2.17E-04
GFOD1	-0.793142921	7.92E-05	PPHLN1	0.747654	1.03E-04
HEG1	-0.791811462	1.34E-04	RBKS	0.747621	1.94E-04
RNF170	-0.789477869	1.03E-04	MMD	0.747266	1.64E-04
SCARNA14	-0.788736291	7.03E-03	MXRA5	0.746664	3.24E-04
RAB31	-0.787835601	1.42E-04	LSM5	0.745559	4.06E-04
FERMT1	-0.78411577	1.10E-04	IDNK	0.745219	1.31E-04
AKR1C2 /// LOC101060798	-0.782110231	1.14E-04	USP43	0.744772	1.40E-04
PLLP	-0.781266035	9.47E-05	METRNL	0.743789	6.54E-04
CLN5	-0.780041989	7.98E-05	BSPRY	0.743752	9.39E-05
PMEPA1	-0.779318065	7.80E-05	SKIL	0.742984	1.22E-04
WAC	-0.779222334	4.24E-04	SLC27A2	0.742604	3.31E-03
PTPLAD1	-0.779004085	1.09E-04	SNAPC3	0.741118	2.61E-04
ZNF275	-0.777508151	1.68E-04	IFT74	0.740728	1.25E-04
SRPR	-0.776155953	1.39E-04	RHNO1	0.740153	2.12E-04
PERP	-0.775423473	9.52E-05	NSRP1	0.73947	1.02E-04
NAV3	-0.774716148	2.57E-04	RSRC1	0.739023	3.16E-04
CERS2	-0.773827137	1.50E-04	PRIM2	0.738578	3.96E-04
CTU2 /// PRKAR2A	-0.773315068	1.43E-04	NBR1	0.73852	1.02E-04
PAK2	-0.772577515	1.15E-04	CDC73	0.736434	8.73E-04

RAB7L1	-0.772048338	1.53E-04	SAMHD1	0.736143	4.24E-04
FLVCR1	-0.771640448	1.23E-04	TIA1	0.734798	1.33E-03
SIRPA	-0.770938052	5.17E-04	PPP3CA	0.734077	2.09E-04
TFRC	-0.770039878	8.37E-05	ACTG1P4 /// AMY2A /// AMY2B	0.73291	2.51E-04
ATAD1	-0.767512878	1.65E-04	C9orf41	0.732671	1.11E-02
FKBP9	-0.766912571	3.21E-04	PHF11	0.732051	4.23E-04
GANAB	-0.766848156	2.08E-04	NMD3	0.731401	1.02E-04
ARL1	-0.766550826	3.57E-04	IL8	0.730393	1.16E-04
TSPAN7	-0.765994533	1.07E-03	SH3RF3	0.728795	1.19E-04
MYH9	-0.765136689	1.19E-04	CCNG2	0.727382	1.53E-04
NCSTN	-0.764628591	9.00E-05	ZNF765	0.727362	1.25E-04
SLC35G2	-0.764584895	1.40E-04	EGR1	0.726582	3.92E-04
SPRY4	-0.763842503	6.78E-04	WWTR1	0.726274	1.54E-03
MPHOSPH8	-0.763342399	7.48E-05	SRI	0.726201	5.42E-04
FKBP11	-0.763335723	8.04E-05	CMPK1	0.72545	3.14E-04
DAB2IP	-0.76259252	9.59E-05	EIF2AK1	0.725286	4.38E-04
AP1G1	-0.762474872	5.24E-04	CDC42EP3	0.724679	1.93E-04
HSP90B2P	-0.76179101	1.08E-03	IFIT1	0.723899	1.26E-04
JUP /// KRT17	-0.761508029	8.05E-05	METTL7A	0.72382	1.19E-04
SOX4	-0.761283122	1.44E-04	GPCPD1	0.722589	7.55E-04
ARHGAP25	-0.760869917	2.13E-04	VPS36	0.7224	1.05E-04
EFEMP1	-0.760745544	8.71E-05	ABCA10	0.72237	4.23E-03
LAMB3	-0.759367715	9.71E-05	SIAH1	0.722231	2.13E-04
PPP4R4	-0.758618984	1.10E-04	FAM46A	0.721742	1.04E-04
HOMER1	-0.758509857	2.10E-04	JAK2	0.721339	3.87E-04
DSC3	-0.757950215	9.04E-05	MIS18A	0.720846	2.67E-04
GRN	-0.755781827	8.01E-05	ARID5B	0.720138	1.99E-04
PLAC1	-0.755655122	9.17E-05	AZIN1	0.720079	1.22E-04
ATF6	-0.754603731	8.58E-05	ZNF140	0.719683	1.50E-04
VPS52	-0.754479992	3.15E-04	CKAP2	0.719474	8.74E-04
SSR2	-0.753408208	1.08E-04	KAZN	0.718184	2.91E-03
AQP3	-0.753037349	1.38E-04	ZNF33B	0.717898	8.20E-04
MED6	-0.752637103	9.02E-05	TMF1	0.717429	2.23E-04
KLF7	-0.752135193	8.67E-05	TNS3	0.71695	2.18E-04
SERPINB3	-0.750952515	7.43E-04	FAM102B	0.716637	2.69E-04
POGZ	-0.750670188	6.36E-04	C3	0.716327	1.23E-04
TNFRSF10B	-0.749539434	3.67E-04	GBP4	0.715466	2.77E-04
ADARB1	-0.748886533	1.38E-04	EID1	0.714577	2.86E-04
CRCT1	-0.748518436	2.51E-03	VRK2	0.714029	1.72E-04
RPL23 /// SNORA21	-0.74843897	7.55E-04	C21orf91	0.713644	4.99E-04

HS3ST1	-0.748068297	8.55E-05	GNA13	0.713407	1.11E-04
CEP164	-0.746737544	1.20E-04	AGPS	0.712256	1.58E-04
APOOL	-0.746501545	1.48E-04	TNRC6A	0.712004	2.37E-04
DDB1	-0.746475718	1.58E-04	ERBB2IP	0.711842	2.69E-04
CFLAR	-0.745936749	7.76E-04	MDM1	0.711069	1.10E-04
CD58	-0.744055512	1.65E-04	GLTSCR1L	0.710985	2.89E-04
TMED10	-0.743657256	9.74E-05	PLA2G16	0.710935	6.00E-04
TIMM23 /// TIMM23B	-0.742798419	3.00E-04	CCP110	0.710783	1.33E-04
RABAC1	-0.742475	1.51E-04	VTA1	0.710393	1.52E-04
VEGFA	-0.74239997	9.97E-05	HMGB2	0.707503	1.49E-04
SEMA3C	-0.742082056	1.89E-04	DLG1	0.707139	1.36E-04
HBEGF	-0.741608154	1.90E-04	FOXO3 /// FOXO3B	0.706872	1.37E-04
ABCB6 /// ATG9A	-0.741335487	9.55E-05	IFIT5	0.706071	1.88E-04
TACC2	-0.740998651	2.41E-04	ZNF571	0.705744	5.53E-04
SLC29A1	-0.740164332	2.51E-04	CCNG1	0.705503	1.21E-04
PRADC1	-0.740002653	1.75E-04	UGDH	0.705201	1.22E-04
PHF20L1	-0.73905423	4.12E-04	RBM47	0.705099	1.55E-04
TOR1A	-0.738927921	1.31E-04	OAS2	0.705071	7.00E-04
DRAM2	-0.738647921	1.88E-04	SLITRK6	0.704642	5.95E-04
LEPREL1	-0.738347002	1.18E-04	SEC16B	0.704609	5.20E-03
NOTCH2	-0.736220615	1.22E-04	PLSCR1	0.704307	1.39E-04
CLCA4	-0.734984121	4.71E-03	PTPRH	0.703574	2.36E-02
SDF2	-0.734878081	1.80E-04	MTMR11	0.703557	1.65E-04
THSD4	-0.734686113	1.52E-04	SSBP2	0.702936	5.57E-03
MAGI1	-0.733392184	1.60E-04	HMGN3	0.702862	1.18E-04
PGF	-0.730412171	4.50E-04	ZMIZ2	0.702735	1.79E-04
WISP3	-0.730018135	8.60E-04	MDM2	0.702207	1.36E-04
GPNMB	-0.72909428	1.02E-04	CSNK1G3	0.701975	1.47E-04
CLSTN1	-0.728250371	1.74E-04	LTBP3	0.70165	3.39E-04
PTGS2	-0.72733138	1.63E-04	SAFB	0.701578	1.75E-03
LINS	-0.727113834	2.74E-04	DCK	0.701327	1.76E-04
LTV1	-0.72693196	1.85E-04	C7orf60	0.700472	1.52E-03
SYPL1	-0.726076535	1.56E-04	ZNF564	0.700264	1.63E-03
GPR89A /// GPR89B /// GPR89C	-0.725717751	1.02E-04	MTIF3	0.699141	1.38E-04
EDNRA	-0.725415023	1.04E-04	MX2	0.698625	1.25E-04
LEPROTL1	-0.724976607	1.49E-04	NAA50	0.698549	1.22E-03
PSME3	-0.724872529	2.52E-04	SUDS3	0.698166	4.40E-04
ST7 /// ST7-OT3	-0.724309384	3.64E-04	FBXO22	0.698004	1.05E-03
TSHZ3	-0.724010115	1.62E-04	LOC729020 /// RPE	0.697998	1.51E-04

LY96	-0.723695758	1.21E-04	SLC37A2	0.697979	1.39E-04
CLCA2	-0.723223904	1.42E-04	ALDH8A1	0.697868	1.07E-02
AMIGO2	-0.722170447	2.09E-04	RRAS2	0.697252	1.23E-04
CDH13	-0.722043884	1.42E-04	SAMD9	0.697145	1.80E-04
LEPRE1	-0.721244493	2.34E-04	SHOC2	0.696233	1.85E-04
RPL4 /// SNORD16 /// SNORD18A	-0.7209579	1.68E-03	GMCL1 /// GMCL1P1	0.693693	2.12E-03
LTBP2	-0.720956734	1.57E-04	MND1	0.693098	2.36E-04
HELLPAR /// HERC2P4	-0.720552264	9.13E-03	BAG2	0.692189	2.21E-04
LPP	-0.719397684	2.46E-04	AEBP2	0.691855	5.09E-04
LRRC8C	-0.719180108	1.13E-04	CEP57	0.691499	3.13E-04
SURF4	-0.718767424	1.47E-04	CPNE3	0.690896	1.71E-03
ERGIC3	-0.718056965	1.58E-04	TSC22D2	0.689362	1.34E-04
CD68	-0.71803117	1.91E-04	GSAP	0.689333	7.43E-04
TMEM59	-0.717237071	1.92E-04	RAB27B	0.689162	1.21E-03
SFT2D1	-0.717080038	2.56E-04	NEK4	0.688479	1.36E-04
C7orf10	-0.715661635	6.92E-04	ACTB	0.688442	1.45E-04
MMP13	-0.715580928	1.46E-04	OTUD6B	0.688341	1.47E-04
BAG5	-0.71354012	1.34E-04	IST1	0.688218	6.10E-03
CYP2S1	-0.712979381	2.00E-04	ZNF480	0.68718	1.53E-04
AFAP1L1	-0.71210908	1.50E-04	SMS	0.686703	1.35E-04
SCAMP3	-0.711661312	2.09E-04	KLF3	0.686448	1.37E-04
B3GALTL	-0.711107849	4.93E-04	PLS1	0.685064	3.77E-04
PTHLH	-0.710984051	1.13E-04	MEIS2	0.684457	1.43E-04
PRSS23	-0.710075682	1.97E-04	SPG20	0.684318	2.08E-04
GRHL3	-0.710048141	7.46E-04	TROVE2	0.682259	1.83E-04
UBAP2	-0.709980101	1.83E-04	TMC5	0.681533	4.27E-04
AQR	-0.709317535	1.67E-03	SLC3A2	0.681503	3.12E-04
DPF1	-0.708431374	1.34E-04	GPR110	0.681232	2.36E-04
CLMP	-0.70821646	8.54E-03	DTX4	0.681228	8.26E-04
TGFA	-0.707755059	1.18E-04	SRSF7	0.681018	9.55E-04
FOXD1	-0.70735333	4.89E-04	ELAVL2	0.680496	2.89E-04
HEATR3	-0.707271686	3.21E-04	ZNF230	0.679904	9.34E-04
UGCG	-0.706328005	4.46E-04	RBM43	0.679634	1.46E-03
FADS1 /// MIR1908	-0.704806949	2.14E-04	HSBP1L1	0.679398	1.53E-04
RPS6KA5	-0.703714949	3.11E-04	SAV1	0.679136	1.56E-04
XBP1	-0.703569747	2.06E-04	SCLT1	0.679134	7.12E-04
TM4SF19 /// TM4SF19-TCTEX1D2	-0.703388259	9.88E-04	MAP4K4	0.677325	1.90E-04
MESDC2	-0.703315181	6.46E-04	CASP4	0.677037	2.31E-04
CD82	-0.70142547	1.93E-04	TNKS	0.676907	8.24E-04
METTL3	-0.701401852	1.16E-03	DNAAF2	0.676501	1.59E-03

MYCL1	-0.701108143	8.83E-04	CD2AP	0.675681	2.97E-04
LMAN2L	-0.700572577	1.24E-04	DHFR	0.675324	6.75E-03
ASNA1	-0.699530419	1.78E-04	LARP7	0.67528	1.48E-04
TCTN3	-0.699313815	1.99E-04	C5orf15	0.675062	3.76E-04
MICALCL	-0.698434885	1.61E-03	ATAD5	0.673819	2.08E-04
CYB5R1	-0.698327925	2.50E-04	RB1CC1	0.673734	4.60E-04
XRN2	-0.698205607	1.86E-04	LMNB1	0.673663	4.29E-04
TSPAN6	-0.698091768	1.27E-03	KLK6	0.673583	3.24E-04
SUGP2	-0.697814975	9.95E-04	HEXIM2	0.673536	2.89E-04
FGD6	-0.697030797	1.38E-04	SLC39A2	0.673511	1.67E-03
MRPS10	-0.696087743	1.29E-04	CHMP2B	0.673505	3.34E-04
MGLL	-0.695970671	2.15E-04	SNX8	0.672567	3.62E-04
C1GALT1C1	-0.695965917	2.49E-04	KRCC1	0.672383	4.69E-04
PUM1	-0.695818272	3.52E-04	XIAP	0.670946	5.33E-04
ACSL3	-0.69576479	1.61E-04	ZNF772	0.670935	5.69E-04
IL17RA	-0.695708457	5.50E-04	MTHFD2L	0.67087	1.42E-03
HSPA5	-0.695687038	3.09E-04	SP4	0.670644	3.73E-04
DNAJC3	-0.69457486	1.35E-04	CRIPT	0.6697	2.13E-03
UQCRC1	-0.693431328	1.56E-04	JAZF1	0.669193	1.17E-02
CAMKK2	-0.692681416	3.87E-04	KRT18	0.669113	1.99E-04
ARPC5L	-0.692669222	2.35E-04	SMIM15	0.668986	2.87E-04
AMPD3	-0.691155261	5.59E-04	IDS	0.668808	1.66E-04
TGOLN2	-0.690953629	1.31E-04	GSN	0.668679	5.90E-04
ZKSCAN1	-0.690756731	1.68E-04	USP46	0.668234	2.93E-04
C1R	-0.68974624	1.47E-04	MZT1	0.667176	1.71E-04
KDELR2	-0.689560596	1.43E-04	XPO1	0.666235	2.65E-04
C1orf43	-0.689543869	1.68E-04	KIAA1143	0.666066	8.83E-04
ABLIM1	-0.689396964	6.34E-04	PRDX6	0.665977	3.60E-04
RHBDL2	-0.689055896	1.09E-03	UBE2L6	0.664875	2.19E-04
TLL2	-0.687936411	1.03E-03	EPN3	0.664806	2.74E-04
FAM83C	-0.687748613	2.74E-03	MAP4K3	0.664466	4.08E-04
CKLF /// CKLF-CMTM1	-0.687695253	2.01E-04	CTDSPL2	0.664284	5.22E-04
PCYOX1	-0.687425698	1.49E-04	RABGAP1L	0.664095	1.40E-03
ANTXR1	-0.686340078	2.69E-04	ANLN	0.663769	1.93E-04
CKMT1A /// CKMT1B	-0.68593183	1.61E-04	TIPRL	0.662932	2.64E-04
HIST2H2AA3 /// HIST2H2AA4	-0.682993292	2.35E-04	LLGL2	0.662416	3.45E-04
C10orf32	-0.682834405	6.85E-03	VPS26A	0.661635	5.14E-04
TGFBI	-0.682692618	3.75E-04	GAN	0.661156	5.27E-04
TSPAN14	-0.681586841	3.27E-04	NR2F2	0.660806	7.84E-04
EIF3H	-0.681517886	1.52E-04	NCCRP1	0.660806	2.64E-03
ERLIN2	-0.681485789	1.40E-04	FAM111B	0.660217	3.63E-04

SLC2A1	-0.680321278	1.96E-04	ZRANB2	0.65948	1.88E-04
RNF43	-0.67922099	1.33E-02	EIF4E3	0.659282	4.04E-04
SLC35B1	-0.679200839	4.64E-04	EIF5	0.659186	5.43E-04
MFSD2A	-0.678964305	2.18E-04	PTBP3	0.658991	7.11E-04
SLC31A2	-0.678151521	1.58E-04	CRBN	0.657958	2.91E-04
DHRS9	-0.677865252	2.83E-04	PBRM1	0.657913	3.85E-04
HLA-C	-0.67773805	1.50E-04	PPP3R1	0.657662	4.84E-04
TMEM50A	-0.677717263	4.39E-04	CEP44	0.657515	3.83E-04
TMEM43	-0.677174845	2.00E-04	U2SURP	0.657392	1.25E-03
HSPH1	-0.676690623	6.49E-04	ARL5A	0.656794	1.88E-04
SNORA12	-0.675881852	1.30E-03	AHI1	0.656732	4.88E-04
GINS1	-0.67324142	2.96E-04	OSBPL11	0.656062	4.38E-04
PGM2L1	-0.672991115	1.08E-02	MAP4K5	0.655936	5.40E-04
ANKDD1A	-0.67295315	1.64E-04	PLOD2	0.655882	1.77E-04
PLAUR	-0.672808915	2.02E-04	OAS1	0.65565	2.99E-04
DUSP6	-0.672311057	1.85E-04	LAMA5	0.654267	2.04E-04
HIC2	-0.67224056	3.75E-04	DBF4	0.653568	7.13E-04
CARD10	-0.67150635	1.97E-04	PCGF5	0.653408	2.48E-04
MMP10	-0.671009912	4.11E-04	ERI2	0.653349	8.56E-04
EGFL8 /// PPT2	-0.669735462	2.83E-04	FAM199X	0.652612	6.13E-04
ZXDC	-0.669318239	1.74E-04	FAM198B	0.652124	6.14E-04
CRTAP /// LOC100653071	-0.668893107	1.54E-03	NBR2	0.651759	7.91E-04
KIF1B	-0.668351487	1.56E-03	BNIP3L	0.651725	2.67E-04
STAR	-0.668136619	1.37E-02	PPP1R1C	0.65168	7.91E-04
BDKRB2	-0.667759285	1.35E-03	CHEK1	0.651543	4.32E-04
MRPS31P5 /// THSD1	-0.667602983	1.81E-04	SMEK2	0.650935	3.39E-04
MIB1	-0.66730436	4.04E-04	C18orf54	0.650087	8.28E-04
ADRM1	-0.667202358	9.99E-04	DCAF10	0.649555	1.14E-03
CYR61	-0.666962897	2.49E-04	C5orf38	0.64922	2.20E-04
LTBP1	-0.666503358	2.02E-04	RAD51L3-RFFL /// RFFL	0.648836	5.75E-04
LRRC8B	-0.66446589	5.63E-04	ZNF165	0.648502	3.12E-04
EBLN2	-0.66438796	1.96E-04	LITAF	0.647981	4.08E-04
ABHD13	-0.664009203	1.34E-03	METTL10	0.647942	3.06E-04
HTRA1	-0.662716279	2.90E-03	SMIM14	0.6479	2.36E-04
PSMD7	-0.662433311	3.90E-04	TUBB8 /// TUBBP5	0.647814	1.99E-03
FAM98B	-0.661805046	4.15E-04	BRIP1	0.647665	7.23E-03
MYCBP2	-0.661396041	4.06E-04	CLDN22 /// WWC2	0.647572	1.56E-03
LOC101060541 /// RBM8A	-0.661196255	3.44E-04	AP1S2	0.647567	3.90E-04
EBP	-0.660948877	2.51E-04	ZNF17	0.646592	8.00E-04

TSEN2	-0.660722704	2.10E-03	SUZ12	0.64634	2.06E-04
SMPDL3A	-0.660397352	2.21E-04	NMI	0.645619	2.03E-04
NT5E	-0.659142534	1.31E-03	TRIM34 /// TRIM6-TRIM34	0.645288	1.09E-03
IGFBP2	-0.658988012	2.57E-04	ZMYM2	0.645195	7.17E-04
HLA-A	-0.658460343	1.90E-04	CFL2	0.644969	2.54E-04
DUSP5	-0.658351601	1.97E-04	KDM3B	0.644811	1.88E-03
SLC26A11	-0.657741071	3.40E-03	DNAJC21	0.644706	2.37E-04
AGTRAP	-0.657667608	3.26E-04	STOX1	0.644576	1.99E-04
STIM2	-0.656694684	8.78E-04	SPIN1	0.644354	2.15E-04
TXNDC17	-0.656377647	5.71E-04	PXK	0.644332	9.45E-04
KCNK6	-0.655901229	1.82E-04	DNAJB6	0.643567	7.04E-04
PAG1	-0.655811241	4.55E-04	PPP6C	0.643215	2.83E-04
NDUFC2 /// NDUFC2-KCTD14	-0.655792365	1.89E-04	TTK	0.643051	4.11E-04
TMEM138	-0.655011141	4.12E-04	FAM103A1	0.642722	2.38E-04
SLC35E1	-0.654917482	3.80E-04	S100A4	0.642332	2.06E-04
KCNJ15	-0.654532	2.40E-04	FAM46B	0.641863	9.75E-04
AP2M1	-0.653769582	2.37E-04	NBN	0.640172	2.35E-04
PC	-0.653298187	3.52E-04	MST4	0.638879	2.77E-04
GOLPH3L	-0.651634746	3.05E-04	KLHL13	0.638591	3.39E-04
STEAP1B	-0.651538732	3.55E-04	Sep-07	0.638559	2.09E-04
MET	-0.651183765	2.01E-04	PPP2CA	0.638382	2.33E-04
NFE2L1	-0.650753021	3.07E-04	PPP2R5E	0.638376	5.82E-04
MFAP2	-0.649980295	3.08E-04	CD164	0.63764	2.76E-04
LARP1	-0.649923488	5.94E-04	PPIP5K2	0.636153	5.91E-03
JAG1	-0.649049355	2.47E-04	LRIF1	0.635614	2.75E-04
TP63	-0.648706615	2.10E-04	CCDC176	0.635271	1.75E-03
EBNA1BP2	-0.648602106	2.45E-04	ERI1	0.634702	4.04E-04
OCIAD2	-0.648278654	2.60E-04	SIX1	0.634477	1.23E-03
TOR3A	-0.648247537	6.72E-04	FEM1C	0.634333	2.32E-04
ATP6V0C	-0.647692119	7.38E-04	LOC100129961	0.633856	1.93E-03
RTN3	-0.647690047	2.15E-04	SASS6	0.633738	5.08E-04
ABCA1	-0.647547085	5.81E-04	DCLRE1A	0.633658	7.28E-04
CERS6	-0.646220438	9.57E-04	ANAPC16	0.633445	3.73E-04
FAF2	-0.646095707	3.00E-04	ZMYM4	0.633424	8.34E-04
KLK11	-0.645883191	1.04E-03	RSF1	0.633345	6.82E-04
NDUFB9	-0.645486049	3.60E-04	ZNF148	0.633162	3.18E-04
PLP2	-0.645436086	2.09E-04	CGN	0.631814	1.14E-03
FAM25A /// FAM25B	-0.645239773	8.65E-04	FOSL2	0.631684	7.19E-04
RSBN1L	-0.645066953	1.15E-03	ARFIP1	0.630332	9.02E-04
CNPY2	-0.644879927	4.30E-04	SPAG9	0.629947	5.92E-04

CBLB	-0.644719121	3.74E-03	FBXO5	0.629684	4.24E-04
MMGT1	-0.644516066	1.51E-03	ODF2L	0.628978	3.46E-04
GPR155	-0.644492689	3.27E-03	GSK3B	0.628889	2.92E-04
KIAA1161	-0.644444888	2.84E-04	RBM38	0.628758	1.28E-03
VHL	-0.643811939	9.53E-04	NR3C1	0.628743	3.42E-04
WASF2	-0.642173989	3.96E-04	USP9X	0.627705	4.31E-04
MAB21L1	-0.642049417	4.31E-04	ZNF83	0.627463	3.72E-04
KDSR	-0.641671024	1.95E-04	PPDPF	0.62742	6.13E-04
CISD2	-0.641286817	5.58E-04	CSNK1A1	0.627363	3.40E-04
ATRN	-0.640460173	1.97E-04	IRX3	0.626864	2.06E-03
ATP2C1	-0.639471731	2.29E-04	CCDC6	0.626752	4.62E-04
GPR98	-0.639203526	5.53E-04	KYNU	0.626727	2.44E-03
APMAP	-0.638724555	2.26E-04	RIF1	0.626629	4.46E-04
CLN8	-0.638609711	5.57E-04	SNN	0.626499	2.67E-04
UNC50	-0.638553963	2.93E-04	ANKIB1	0.625956	4.43E-04
NOP16	-0.638145956	2.54E-04	DR1	0.625506	3.20E-04
SLC38A5	-0.637634131	2.10E-04	NUCKS1	0.625323	3.62E-04
PRCP	-0.637212246	2.36E-04	MYADM	0.625203	2.99E-04
MRPL43	-0.636333492	2.41E-04	PSPC1	0.625126	4.78E-04
RBBP6	-0.636270063	5.57E-04	DHX58	0.625056	3.19E-04
MRPS5	-0.635865354	5.26E-04	ETV7	0.624839	5.17E-04
HES1	-0.635401435	3.25E-04	NARG2	0.624818	3.09E-04
CGNL1	-0.635364687	3.30E-04	ZNF510	0.62414	5.42E-04
LOC100507412	-0.635173656	2.08E-04	USP10	0.623572	4.54E-04
TYW1	-0.63514836	2.71E-04	BBX	0.623442	2.39E-03
ANG	-0.634968213	5.39E-04	DPH6	0.623018	2.44E-03
LRPAP1	-0.633186992	2.19E-04	DNAJB4	0.622764	4.23E-04
SLC20A2	-0.633174578	6.39E-04	PRKACB	0.621924	1.48E-03
SRSF11	-0.632238415	2.15E-04	IRF2	0.620031	5.74E-04
FGFBP1	-0.632222517	2.20E-04	YBX3	0.620005	3.08E-04
JKAMP	-0.631769986	7.22E-04	ELL2	0.619662	5.85E-04
NOMO1 /// NOMO2 /// NOMO3	-0.63130034	2.60E-04	APPL1	0.61916	3.06E-04
DSP	-0.630951589	2.99E-04	PARP9	0.618544	3.43E-04
SOAT1	-0.630432443	3.24E-04	ZNF562	0.618444	5.81E-04
CD83	-0.630278902	3.02E-04	MKRN2	0.618424	5.27E-04
EXOC4	-0.628514823	3.03E-03	CAPZA2	0.618358	8.40E-04
INSR	-0.628326713	6.03E-04	ZNF367	0.617943	3.73E-04
PLGRKT	-0.627590281	8.30E-04	SOX2	0.617875	2.46E-04
NRCAM	-0.627537187	3.71E-03	APITD1 /// APITD1-CORT	0.617051	4.86E-04
FHL1	-0.627472994	7.13E-04	ZNF506	0.616969	3.36E-03

TBRG1	-0.625373338	4.26E-04	FGFR1OP	0.616678	3.97E-04
KLHL28	-0.625360681	4.56E-04	FAM149B1	0.616489	4.85E-04
VIM	-0.625153476	2.34E-04	EFCAB7	0.616455	9.98E-04
TRIM63	-0.624374823	3.22E-03	SMNDC1	0.616078	2.23E-03
SDC1	-0.624354775	3.75E-04	SNORD49A /// SNORD49B /// SNORD65	0.615309	3.98E-04
GLA	-0.6241364	4.21E-04	LGALS8	0.614635	6.24E-04
C1S	-0.623995844	1.11E-03	RAD23B	0.614567	4.75E-04
APH1B	-0.623764179	5.72E-04	ZMYM6	0.614304	3.91E-04
PAR-SN /// SNRPN /// SNURF	-0.62373335	2.40E-04	SSX2IP	0.614235	4.25E-04
FN1	-0.623409948	8.47E-04	TNFSF9	0.614208	1.50E-03
ZNF750	-0.623289216	3.52E-04	LEPR	0.614018	2.50E-04
ANGPTL4	-0.623059619	2.35E-04	IRF1	0.613226	3.64E-04
F11R	-0.622889543	2.40E-04	NAA15	0.612897	8.10E-04
FLRT3	-0.622445806	1.31E-03	DNAJC6	0.612815	5.48E-04
CXCL11	-0.621151357	1.28E-03	ALG13	0.612639	3.06E-04
EPT1	-0.618954976	2.75E-04	GNAQ	0.612639	7.13E-04
ASPHD2	-0.618786654	4.85E-04	ZC3H12C	0.612021	1.61E-03
FSIP1	-0.618591526	1.29E-03	SGOL2	0.611887	3.77E-04
FAM20A	-0.618051124	3.19E-03	GSR	0.611823	3.14E-04
PHF14	-0.617877735	4.90E-04	GCOM1 /// POLR2M	0.611536	3.48E-04
P2RX5	-0.617792273	2.89E-03	GATAD1	0.611213	3.92E-04
PVR	-0.617609528	1.23E-03	CDC42SE2	0.611161	4.74E-04
PGRMC1	-0.615817375	3.71E-04	PPP1R15B	0.611139	1.52E-03
KAT6A	-0.615239362	1.46E-03	GALNT7	0.610765	3.95E-04
TMEM136	-0.614474038	1.13E-02	THNSL1	0.61027	4.10E-04
HFE	-0.613144705	7.96E-04	UBE2J1	0.610202	4.21E-04
ADAM17	-0.613115338	5.01E-04	PPM1D	0.610085	3.45E-04
LYRM7	-0.61276038	5.77E-04	TJP1	0.609873	4.17E-04
GNPDA2	-0.612732603	9.92E-04	MVP	0.609774	6.61E-04
PCDHB14	-0.612614898	5.37E-04	TIFA	0.609587	1.01E-03
ATP6V1G2-DDX39B /// DDX39B /// SNORD84	-0.611859369	1.87E-03	NEK2	0.609324	3.16E-04
SDHD	-0.611853545	3.69E-03	B3GNT7	0.609066	3.15E-04
SERINC3	-0.611596693	3.94E-04	GPBP1	0.608793	7.32E-04
GALNT2	-0.61099215	5.12E-04	GABPA	0.608297	3.60E-04
MARS	-0.610966781	2.58E-04	MAP3K8	0.608294	3.00E-04
PWP1	-0.61074832	1.62E-03	ARID2	0.608128	7.79E-04
DDX21	-0.610670652	2.84E-04	UBE2D2	0.607557	1.93E-03
ANO6	-0.61046459	1.02E-03	TTC39C	0.606934	4.44E-04

HLA-E	-0.609989315	2.82E-04	KIF22	0.606894	1.40E-03
IGSF9	-0.609874645	7.85E-04	HPS3	0.606882	3.94E-04
TMCC3	-0.609813134	1.19E-03	SLC25A24	0.606717	7.70E-03
RRP7A	-0.609791927	1.93E-03	SDCBP	0.606579	2.82E-04
WDR65	-0.609440038	7.83E-04	ATG12	0.605741	2.79E-04
MCOLN2	-0.60875291	6.50E-04	RC3H1	0.605668	4.45E-04
PON2	-0.608534309	3.29E-04	MRP63	0.605329	4.61E-04
QPCT	-0.608509086	3.39E-04	MSL3P1	0.60472	8.31E-04
RAB7A	-0.608334991	3.90E-04	TOP1 /// TOP1P1	0.60454	9.88E-03
ABCG1	-0.608189837	3.15E-04	CALD1	0.603345	4.86E-04
SNHG8 /// SNORA24	-0.607642505	6.69E-04	CEP55	0.603052	3.93E-04
CPT1A	-0.607440856	9.58E-04	KIAA1217	0.602781	3.89E-04
SQLE	-0.607418528	3.13E-04	ZHX1	0.601921	4.05E-04
MFSD1	-0.607058843	3.87E-04	C6orf211	0.601836	3.81E-04
CNIH4	-0.606970989	5.05E-04	SMARCD2	0.601467	1.63E-03
LRRC28	-0.606899148	2.64E-04	CPPED1	0.601222	4.79E-04
TBC1D5	-0.606720349	2.69E-03	TCAIM	0.601153	4.12E-04
CYP51A1 /// LRRD1	-0.60641759	3.81E-04	LRRCC1	0.60108	4.29E-04
LRP4	-0.606278025	3.42E-04	PRRC1	0.600916	4.59E-04
TMEM242	-0.606222809	4.14E-04	SS18	0.600716	3.94E-04
MOB3B	-0.606121843	5.23E-04	ZNF146	0.600371	8.86E-04
CFL1	-0.604665695	3.19E-04	RPS6KB1	0.599902	2.83E-04
GALNT1	-0.604093684	3.89E-03	CLIC4	0.599631	5.31E-04
CSPG4	-0.603911664	1.28E-03	MAT2B	0.599551	5.85E-04
SEC63	-0.603740236	4.64E-04	ZBTB21	0.599506	4.95E-04
MFSD8	-0.603608623	4.91E-04	PHF17	0.59938	7.36E-04
B3GNT1	-0.603367482	3.14E-04	C5orf34	0.599308	4.45E-04
LAMA3	-0.602726095	3.62E-04	DYRK2	0.59863	7.54E-04
TRIM7	-0.602650992	5.13E-04	VPS41	0.598116	1.18E-03
MEF2A	-0.601158624	5.79E-04	KRBOX4	0.597839	1.69E-03
CAMK2G	-0.60113945	2.89E-04	PTPN2	0.59749	4.34E-04
TBL2	-0.601112232	7.90E-04	SPIN4	0.597387	2.96E-04
TMEM66	-0.600912462	2.79E-04	ZNF292	0.597117	1.65E-03
IKBIP	-0.600804784	3.61E-04	TMPO	0.596877	4.30E-04
CD59	-0.600320772	3.02E-04	RUNX2	0.596709	4.57E-04
PBDC1	-0.600264156	4.92E-04	GID8	0.596681	2.31E-03
OSBPL1A	-0.59994519	6.39E-04	TTC30A	0.596573	1.12E-03
C19orf10	-0.59970125	3.01E-04	EIF4EBP2	0.596392	1.13E-03
DNAJB11	-0.599626502	5.98E-04	PGM2	0.596321	3.36E-04
PRDM1	-0.599459399	5.30E-04	HDAC2	0.59608	3.76E-04
ATP6AP2	-0.598918312	3.38E-04	SH2D4A	0.596041	7.09E-04

KIAA0754	-0.598764705	2.79E-02	OGFRL1	0.595539	5.06E-04
ZNF689	-0.598537778	4.85E-04	CTNS	0.595482	4.43E-04
IGHMBP2	-0.598398666	3.55E-04	RBM18	0.594863	8.02E-04
LOC653375 /// WBSCR16	-0.59797547	4.39E-02	VEZF1	0.594755	2.36E-03
TMEM39A	-0.597766864	1.91E-03	RIOK3	0.594636	4.94E-04
MICA	-0.59694735	3.92E-04	CGGBP1	0.594324	3.36E-04
KIF26B	-0.59669761	5.85E-04	CA13 /// LOC100507258	0.593996	7.23E-04
TRAPPC10	-0.596422756	1.84E-03	KIAA1586	0.593789	1.76E-03
AKAP1	-0.596052532	4.42E-04	FAM49B	0.59365	4.57E-04
TRIM37	-0.595849337	3.13E-04	GPRC5A	0.593418	6.99E-04
SOBP	-0.594987088	6.32E-04	UBE2E3	0.593259	8.38E-04
MED27	-0.594804057	3.06E-04	ELAC1	0.593017	9.88E-03
EPPK1	-0.59468502	8.39E-04	CNKSR3	0.592834	3.66E-04
FXYD5	-0.594552589	3.10E-04	L2HGDH	0.592172	4.37E-04
ABCC9	-0.594465384	6.80E-04	MSRB1	0.591933	4.00E-04
CLU	-0.594407363	3.34E-04	FAM72A /// FAM72B /// FAM72C	0.591824	4.70E-04
RMDN3	-0.594105943	3.21E-04	SLC16A3	0.591483	1.48E-03
FAM69A	-0.593864565	7.15E-04	HDGFRP3	0.591462	3.47E-04
RHOB	-0.593724831	4.47E-04	EPB41L1	0.591155	5.85E-04
PTGR1	-0.593630116	4.63E-04	TULP4	0.59064	1.09E-03
DFNA5	-0.59359853	7.06E-04	NDRG3	0.590555	2.62E-03
C10orf54	-0.593061345	6.86E-04	TBK1	0.590321	4.01E-04
GPR176	-0.592860303	1.34E-03	AP2B1	0.590208	6.00E-04
CD68 /// SNORA67	-0.592793532	1.71E-03	CCNC	0.58916	4.58E-04
SET /// SETP4	-0.592620407	3.83E-04	LHFPL2	0.589126	4.11E-04
KLHL42	-0.592537982	2.66E-03	ZNF350	0.588698	1.16E-03
SULT2B1	-0.592456597	3.20E-04	PACRGL	0.588525	6.18E-04
GPX8	-0.592378975	5.77E-04	CYB5B	0.588361	3.16E-04
GAL3ST4	-0.592217436	1.06E-03	ZNF134	0.58808	2.40E-03
MYLK	-0.592023749	3.07E-04	TBC1D8	0.587925	3.69E-04
MANF	-0.591503124	4.80E-04	HOXA7	0.587624	8.05E-03
PSMB5	-0.590898195	3.71E-04	STX7	0.587199	3.71E-03
TTL	-0.590292767	6.08E-04	MAP2K6	0.587178	5.83E-03
TRPS1	-0.590077112	5.59E-04	PACSIN3	0.586804	1.01E-03
PDPR	-0.589770948	3.42E-04	TTC26	0.586199	1.79E-03
ORMDL2	-0.589686193	3.59E-04	AGGF1	0.586186	8.17E-04
SGCE	-0.589383296	3.74E-04	HNRNPH3	0.585858	3.57E-04
UBE2N	-0.589250799	3.72E-04	ZNF181 /// ZNF302	0.585673	3.21E-04
TIPARP	-0.588618903	1.54E-02	NAA30	0.585636	1.03E-03

COL7A1	-0.588281232	5.42E-04	NAMPT	0.585346	9.27E-04
CKMT1B	-0.587956821	1.00E-02	ZNF204P	0.585052	1.66E-03
RAC1	-0.587953828	6.90E-04	NCOA3	0.58479	3.58E-04
SMAD7	-0.587890747	7.60E-04	ALDH1A3	0.584465	5.16E-04
EMC7	-0.587646123	6.25E-04	GAGE1 /// GAGE13 /// GAGE2A	0.584222	1.76E-03
SLC2A9	-0.587113556	3.17E-03	RMI2	0.584054	4.38E-04
PEX3	-0.586841852	7.32E-04	FOXP1	0.583627	7.47E-04
SDR16C5	-0.586758973	9.79E-03	GRAMD3	0.583601	3.93E-04
SPATS2	-0.586737208	8.53E-04	MIR4680 /// PDCD4	0.582827	6.30E-04
GJA1	-0.586423808	4.15E-04	AFF1	0.582656	8.81E-04
C10orf32 /// C10orf32-AS3MT	-0.586305319	1.76E-03	KIAA0146 /// LOC100996383	0.582326	3.29E-04
FAM83A	-0.586162298	3.31E-04	TAPBP	0.581432	3.47E-03
ADAM8	-0.584827723	5.83E-04	LMNB1 /// PCIF1	0.581331	5.09E-04
CREBRF	-0.584804252	7.52E-03	SERPINB13	0.581286	7.26E-04
PTX3	-0.58439596	9.27E-03	PDZD2	0.581185	7.88E-04
CPM	-0.583964944	5.38E-04	WWOX	0.580956	5.39E-04
NUBPL	-0.583738741	6.79E-04	ANXA9	0.580763	1.52E-03
HOXD10 /// HOXD11	-0.583021071	3.62E-03	PEX2	0.580721	6.52E-04
TMEM101	-0.582609973	5.46E-04	ZNF114	0.580697	1.94E-03
TM2D3	-0.582372233	4.50E-04	FGFR1OP2	0.580121	6.82E-04
CES2	-0.582347081	6.03E-04	ZNF280D	0.579919	8.45E-04
YWHAE	-0.582009212	1.11E-03	STIL	0.579502	2.69E-03
PRKAR2A	-0.581695042	4.53E-04	NT5C2	0.579086	3.74E-04
MAP2K7	-0.581647897	8.45E-04	ZBTB41	0.578978	5.57E-04
SMARCC1	-0.581301896	5.40E-04	WISP2	0.578789	2.44E-03
ATP5J2	-0.581183984	3.06E-03	CCDC90B	0.578607	3.65E-04
TMEM206	-0.580935753	4.57E-04	TIMP2	0.578499	5.10E-04
CELF1	-0.58065046	3.60E-04	EXOC5	0.578264	7.32E-04
DMKN	-0.580575707	6.45E-04	HELLS	0.578199	6.60E-04
YIF1A	-0.580176923	8.75E-04	ESCO2	0.578177	6.18E-04
GJB5	-0.579972522	5.93E-04	MIER3	0.577914	6.95E-04
MRPS28 /// TPD52	-0.579803718	3.94E-04	WDR60	0.577796	7.31E-04
YIF1B	-0.5795384	3.50E-04	UFM1	0.577462	4.62E-04
CD86	-0.579536585	1.31E-03	KIAA1841	0.577302	2.28E-03
ELOVL1	-0.579297993	3.56E-04	LOC100130872 /// SPON2	0.577171	3.52E-04
PIGL	-0.578953083	2.22E-03	GNE	0.577055	4.58E-03
CDC42EP1	-0.578649104	3.60E-04	SNX4	0.576796	3.63E-04
LOC100996628 ///	-0.578594929	5.14E-04	IFT80	0.576455	4.30E-04

SHROOM3					
SLC4A7	-0.578207261	4.48E-04	DDX58	0.576366	1.13E-03
IL23A	-0.578130475	1.51E-03	MX1	0.576255	4.42E-04
MICAL2	-0.577696844	1.55E-03	CBFB	0.576245	6.65E-04
EIF3K	-0.577216351	7.75E-04	CTBS	0.575975	6.68E-03
FLNA	-0.577069286	3.90E-04	ZNF138	0.575878	7.95E-04
KDELR3	-0.57694251	7.80E-04	SNRPD1	0.574936	4.61E-04
SPDYE2B	-0.575873922	5.75E-04	ASAP2	0.574647	6.67E-04
RBPJ	-0.575692139	8.13E-03	CLIP1	0.574183	6.07E-04
SERPINA1	-0.574859365	3.68E-04	KIAA1432	0.57408	8.24E-04
SNORA76	-0.574421029	8.14E-04	AP5M1	0.574012	4.06E-04
WHSC1	-0.573865316	6.04E-04	PIBF1	0.573893	3.65E-04
TEX2	-0.573799039	3.98E-04	UACA	0.573808	6.57E-04
PTAFR	-0.573690046	4.03E-04	BIRC2	0.573751	4.49E-04
TRABD2A	-0.573131073	6.04E-04	TES	0.573729	1.26E-03
ZNF24	-0.572977288	3.61E-04	NT5DC1	0.573674	1.17E-03
KDELC1	-0.572777658	1.93E-03	CEP57L1	0.57304	8.97E-04
IL20RB	-0.572734016	1.35E-03	LYPLAL1	0.57302	5.17E-04
SLC25A37	-0.572126792	4.05E-04	HIST1H2BD	0.572929	9.24E-04
ETV4	-0.571743525	6.17E-04	RAP2C	0.572856	4.24E-04
IGFBP4	-0.571688832	4.77E-04	MAL2	0.572169	5.66E-04
CNPY3	-0.571311675	5.88E-04	CLINT1	0.572074	9.27E-04
MTRF1L	-0.571069842	9.92E-04	TSPAN2	0.572072	4.94E-03
VKORC1	-0.570954548	3.69E-04	CCNE2	0.572069	3.94E-04
REXO2	-0.570717475	5.78E-04	EIF4G3	0.571909	3.44E-03
TMBIM1	-0.570680629	3.94E-04	OSBPL7	0.57181	6.14E-04
HTATSF1	-0.570222453	2.01E-03	ZNF706	0.571753	4.07E-04
			ATF1	0.571564	3.81E-04
			ZNF273	0.570823	4.14E-04
			NETO2	0.570494	7.21E-04
			CYP1B1	0.570278	5.53E-04
			ARHGAP32	0.570257	3.72E-04
			VAPA	0.570179	5.76E-04

Annexure III: Association of survivin 3B with differentiation and correlation in expression of survivin isoforms.

Association of Survivin 3B expression with differentiation status.

s3bNrC * differentiation status Crosstabulation

			differentiation status			
			moderately	well differentiated	poorly differentiated	Total
s3bNrC	1	Count	39	9	7	55
		% within s3bNrC	70.9%	16.4%	12.7%	100.0%
		% within differentiation status	90.7%	69.2%	63.6%	82.1%
		% of Total	58.2%	13.4%	10.4%	82.1%
	2	Count	4	4	4	12
		% within s3bNrC	33.3%	33.3%	33.3%	100.0%
		% within differentiation status	9.3%	30.8%	36.4%	17.9%
		% of Total	6.0%	6.0%	6.0%	17.9%
Total		Count	43	13	11	67
		% within s3bNrC	64.2%	19.4%	16.4%	100.0%
		% within differentiation status	100.0%	100.0%	100.0%	100.0%
		% of Total	64.2%	19.4%	16.4%	100.0%

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	6.177[a]	2	.046
Likelihood Ratio	5.900	2	.052
Linear-by-Linear Association	5.685	1	.017
N of Valid Cases	67		

a. 2 cells (33.3%) have expected count less than 5. The minimum expected count is 1.97.

Spearman Correlation of survivin isoform expression and fold change of expression.

Survivin transcript expression				
Variables	Survivin 3B	Survivin 2B	Survivin 2α	Survivin ΔEx3
Survivin wt	0.408**	0.691**	0.460**	0.667**
Survivin 3B		0.375**	0.794**	0.87
Survivin 2B			0.480**	0.445**
Survivin 2α				0.387*
Fold change of Survivin isoforms				
Survivin wt	0.393**	0.821**	0.340*	0.749**
Survivin 3B		0.425**	0.911**	0.242*
Survivin 2B			0.416**	0.634**
Survivin 2α				0.323**

Ratio of Survivin isoforms			
	Survivin 2B/wt	Survivin 2α/wt	Survivin ΔEx3/wt
Survivin 3B/wt	0.482**	0.886**	0.134
Survivin 2B/wt		0.463**	0.083
Survivin 2α/wt			0.234*

High expression of survivin and its splice variants survivin ΔEx3 and survivin 2 B in oral cancers

Rupa Mishra, MSc,[a] Vinayak Palve, PhD,[b] Sadhana Kannan, MSc,[c] Sagar Pawar, MSc,[a] and Tanuja Teni, PhD[a]
Tata Memorial Centre, Navi Mumbai, and Institute of Bioinformatics and Applied Biotechnology (IBAB), Bangalore, India

Objectives. We have previously reported inactivation of p53 in 46% of Indian patients with oral cancer. Survivin, a p53 target gene and an inhibitor of apoptosis protein (IAP), is overexpressed in several cancers, including oral cancers. Studies assessing the role of survivin and its splice variants in oral cancers are, however, rare.

Materials and Methods. The expression of 6 survivin isoforms in 4 oral cancer cell lines (AW8507, AW13516, UPCI-SCC040, UPCI-SCC029 B), a dysplastic oral cell line (DOK), 75 paired oral tumor and adjacent normal tissues, and 12 normal oral tissue samples from healthy individuals was analyzed by real-time PCR. The expression was correlated with clinicopathologic parameters, which included age, sex, tumor-node-metastasis (TNM) staging, tobacco and/or alcohol consumption, site, and differentiation status of tumor.

Results. This is the first study to find overexpression of the 6 characterized survivin isoforms in oral cancers compared with normal tissues ($P < .05$). Additionally, a significant ($P < .05$) correlation among the fold changes of all 6 survivin isoforms was observed. Survivin wild type (wt) was the predominantly expressed isoform in oral cell lines and tumor tissues versus normal tissues ($P < .05$). Among the minor isoforms, survivin ΔEx3 and survivin 2 B were dominantly expressed, whereas survivin 2 α and survivin 3 α overexpression was found for the first time. Further high survivin 3 B expression exhibited a significant association ($P < .05$) with poorly differentiated tumors. Interestingly the combined expression of the antiapoptotic survivin isoforms, survivin wt, survivin ΔEx3, and survivin 3 B, exhibited a significant association with TNM staging of the tumor.

Conclusions. Our studies thus indicate that oral cancers overexpress the antiapoptotic survivin variants, which exhibit an association with advanced tumor stage, implying a role for these variants in oral tumorigenesis. (Oral Surg Oral Med Oral Pathol Oral Radiol 2015;120:497-507)

Oral squamous cell carcinomas are the most common cancers in India and Southeast Asia, with a majority arising from premalignant lesions such as submucous fibrosis and leukoplakias.[1] Lifestyle factors such as chewing tobacco, a prevalent habit in the country, and alcohol consumption are known to predispose to oral cancers, and the association of human papillomavirus (HPV) has recently emerged.[2] The role of tobacco carcinogens and HPV E6/E7 proteins in p53 and Rb inactivation is well documented.[3,4] Previous reports from our laboratory have found p53 inactivation in 46% Indian oral cancers,[5] whereas Rb inactivation has been reported in a subset of oral cancers.[6] Further studies by Raj et al. show that p53 and Rb (via E2F) repress target gene survivin expression by direct binding to survivin promoter, and their knockdown causes transcriptional upregulation of survivin in human melanocytes.[7] Hence inactivation of these tumor suppressors in oral cancers may lead to upregulation of survivin. Additionally, studies also indicate that HPV E6 can transcriptionally upregulate survivin promoter activity in HeLa cells.[8] Interestingly, several studies have reported high expression of survivin in oral malignant and premalignant tissues.[9-11]

Survivin, the smallest member of the inhibitor of apoptosis protein (IAP) family, contains the evolutionarily conserved baculovirus IAP repeat (BIR) domain and is a dual mediator of apoptosis resistance as well as cell division.[12] Genetic, epigenetic, and post-translational mechanisms of gene regulation have been reported for survivin in cells.[7,13,14] Survivin is reported to be overexpressed in different benign and

Statement of Clinical Relevance

The overexpression of survivin isoforms and association of the antiapoptotic forms with TNM staging was found in oral cancers. Our results suggest that the balance between the proapoptotic and antiapoptotic isoforms may play a role in oral pathogenesis.

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[a]Teni Lab, Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Centre, Kharghar, Navi Mumbai, India.
[b]Genomics Applications and Information Technology (GANIT) Labs, Institute of Bioinformatics and Applied Biotechnology (IBAB), Bangalore, India.
[c]Epidemiology and Clinical Trial Unit (ECTU), Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Centre, Kharghar, Navi Mumbai, India.

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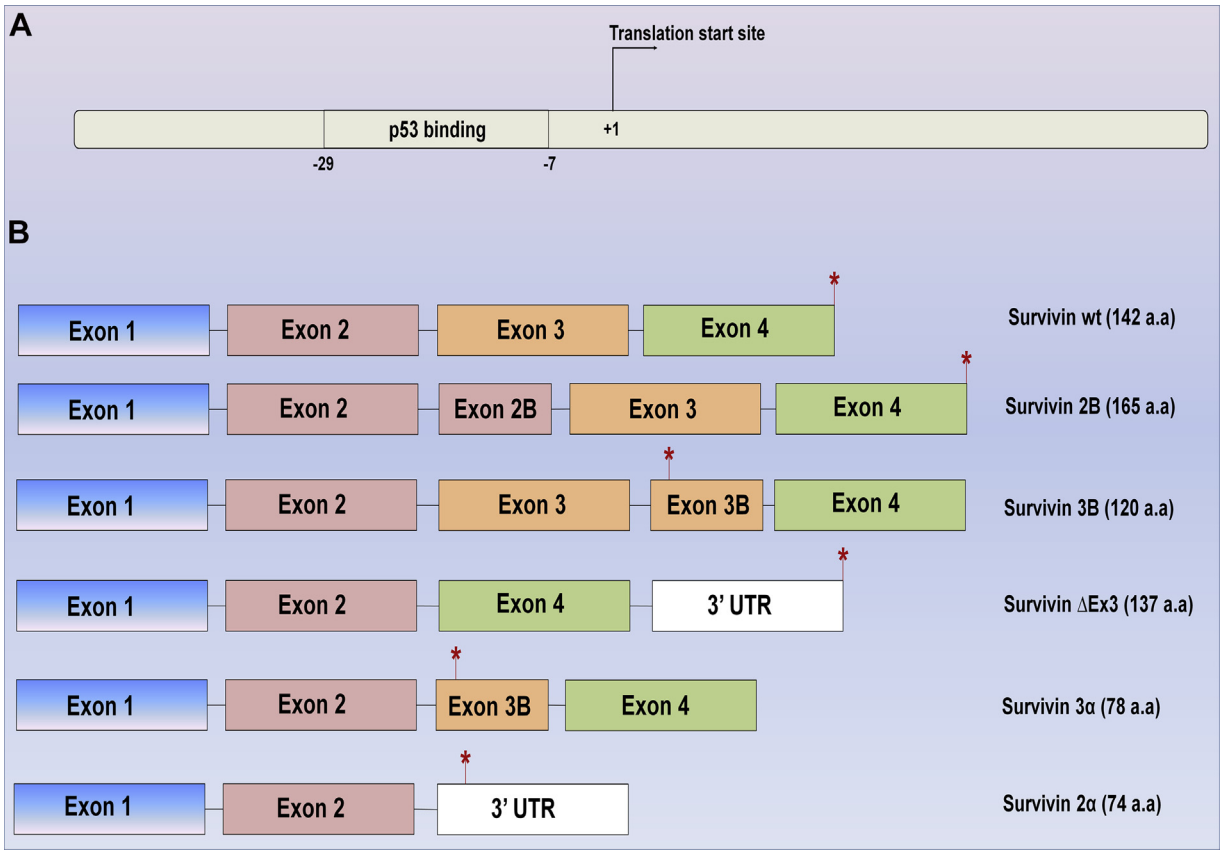

Fig. 1. Survivin splice variants. (**A**) The characterized p53 binding site on the survivin promoter from −29 to −7 is indicated. It overlaps the Sp1 binding site on the promoter. (**B**) The formation of the 6 survivin splice variants are depicted in the cartoon diagram. Boxes represent exons; the insertion of intronic nucleotides in Survivin 2 B and Survivin 3 B are represented by exons 2 B and 3 B. The red asterisk indicates the stop codon, whereas the final molecular weight of the protein is stated beside each isoform.

malignant tissue types, but it is undetectable in normal differentiated adult tissues, implying a potential role for survivin in tumorigenesis.[15-17] Survivin is functional as a dimer, and its overexpression has been associated with aggressive tumor features, poor overall survival, and resistance to chemotherapy and radiotherapy.[18,19] In vitro and in vivo knockdown studies of survivin have found reduced proliferation, invasion, and angiogenesis. Several studies have indicated survivin to be a potential therapeutic target in cancers.[20-22]

The molecular mechanisms by which survivin carries out these diverse functions differentially in malignant and normal cells is unclear. This, however, may be partly attributed to the heterodimerization of survivin with its splice variants in tumor cells. The survivin gene contains 4 exons, and alternative splicing of its pre-mRNA yields splice variants, survivin wild type (wt), survivin ΔEx3, survivin 2B, survivin 3B, and survivin 2α, with opposing apoptotic functions[23-26] (Figure 1). Survivin wt, survivin ΔEx3, and survivin 3B are antiapoptotic, whereas survivin 2B and survivin 2α are implied to have a proapoptotic function. The role of survivin 3α has not yet been elucidated.[27,28] Studies by Zhu et al. reported that the differential modulation of survivin variants may be one of the mechanisms of apoptosis induction by p53.[29] In different cancers, some of these splice variants are differentially expressed and have been reported to correlate with clinical parameters, including outcome.[14,27,30,31] Studies indicate that survivin 2B may naturally antagonize the function of antiapoptotic survivin wt and/or survivin ΔEx3.[25] Thus the relative expression of survivin splice variants may help in fine tuning the outcome. In diverse malignancies survivin ΔEx3 has been associated with advanced tumor stage, high tumor aggressiveness, and poor prognosis.[32,33] The biological functions and role of survivin splice variants in cancer progression, however, are still not clear. There is only a single study reporting the expression of 4 survivin variants in oral tumors.[34] To identify the survivin splice variants associated with oral cancers, we assessed the expression of the 6 known survivin splice variants in 4 oral cancer cell lines, a dysplastic oral cell line, 75 paired tumor and adjacent normal tissues, and 12 normal tissues from healthy individuals. Such a study analyzing the levels of all 6 survivin variants in oral

tumors and additionally in oral cell lines has not been reported earlier.

MATERIALS AND METHODS

Cell culture

The tongue cancer cell lines used in the study included AW8507, AW13516 (established by and obtained from the Immunology and Cell Biology Division at Tata Memorial Centre, India)[35] and UPCI:SCC040 and the buccal mucosa cancer cell line UPCI:SCC029 B (kind gift from Dr. Susanne Gollin, University of Pittsburgh, PA, USA).[36] The dysplastic oral keratinocyte cell line (DOK) (kind gift from Dr. Ken Parkinson, Queen Mary's School of Medicine and Dentistry, London, UK)[37] was also used to compare the expression of the cancer cell lines. The cells were authenticated by short tandem repeat (STR) profiling using the PowerPlex 21 System (Promega Corp., Madison, WI, USA). Cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) or Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), standard antibiotic mixture, in 5% CO_2 at 37°C as described in earlier reports.[38,39]

Patient samples

The study was approved by the Institutional Review Board of Tata Memorial Centre and Sharad Pawar Dental College and written informed consents were obtained from all the patients. Seventy-five oral tumor and adjacent normal samples were obtained from the Tumor Tissue Repository, Tata Memorial Centre, Mumbai, India. The normal samples were obtained from the Sharad Pawar Dental College from patients with no detectable lesions who were undergoing minor or major surgical procedures such as removal of impacted third molar, mucogingival surgery, or reconstruction surgery for cleft lip and cleft palate. Hematoxylin-eosin (H&E) analysis of the normal and tumor samples was carried out to verify the pathology of the tissues, and tumor tissues showing >80% tumor content were included in the study. The clinicopathologic characteristics of the patients in the study are shown in Table I. The median age of the patients was 55 with a mean of 51.49 ± 13.31 and 70% of the patients were men.

RNA isolation

Total RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. The isolated RNA was converted to cDNA using RevertAid cDNA Synthesis Kit (MBI Fermentas, Amherst, NY, USA) according to the manufacturer's instructions.

Table I. Clinicopathologic characteristics

Characteristics	*No (%)*
Age	
<45	24/75 (32)
>45	51/75 (68)
Sex	
Male	52/75 (69.3)
Female	23/75 (30.7)
Site of lesion	
Alveolus	25/75 (33.3)
Buccal mucosa	30/75 (40.0)
Mandible	3/75 (4.0)
Tongue	16/75 (21.3)
Missing	1/75 (1.3)
Tumor (T) status	
T1-T2	9/75 (12.0)
T3-T4	63/75 (84.0)
Missing	3/80 (4.0)
Nodal (N) status	
N0-N1	49/75 (65.3)
N2-N3	25/75 (33.3)
Missing	1/75 (1.3)
Differentiation status	
Poorly differentiated	11/75 (14.7)
Moderately differentiated	43/75 (57.3)
Well differentiated	13/75 (17.3)
Missing	8/75 (10.7)
Habits	
Tobacco	48/75 (64)
Alcohol + tobacco	10/75 (13.3)
No habits	4/75 (5.4)
Missing	13/75 (17.3)

Real-time PCR

Real-time PCR analysis of the survivin isoforms was performed on ABI QuantStudio 12 K Flex Sequence detection system (Applied Biosystems, Foster City, CA, USA). The primers used for survivin isoforms were as reported earlier.[40,41] SYBR Green chemistry (MBI Fermentas, Amherst, NY, USA) was used for the PCR, and all experiments were run in duplicates using RPS13 as the internal control for cell lines and GAPDH as the internal control for tissues. The results were analyzed by relative quantification using the ΔCt method. Fold change of expression was calculated as reported earlier.[42] To determine the extent of upregulation of the isoforms, the expression in tumors was categorized using the median expression of adjacent normal and using the median of normal tissues from healthy individuals. Besides determining the association of clinicopathologic parameters with expression of the individual isoforms, the combined expression was also categorized based on upregulation of at least 1 antiapoptotic isoform and downregulation of the proapoptotic isoforms. The tumor-node-metastasis (TNM) staging was also categorized into 4 stages for oral cancer cases as described earlier.[43]

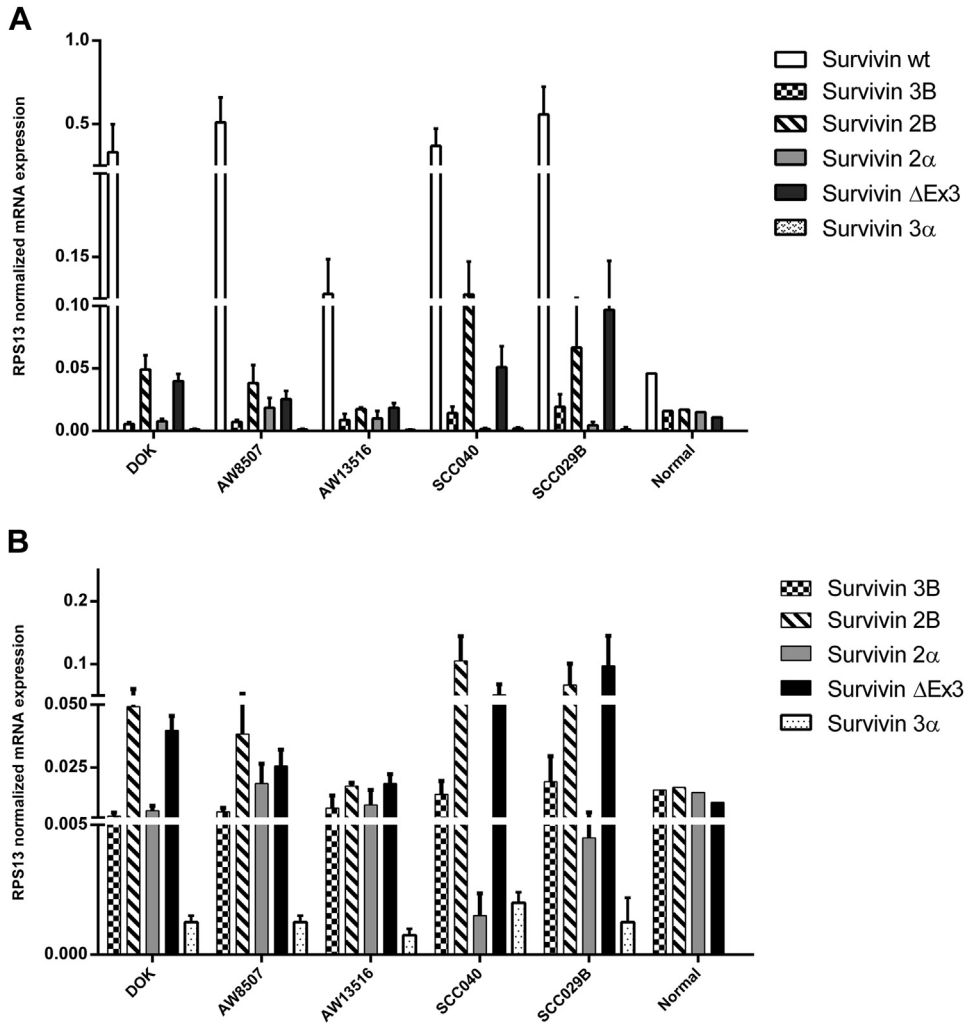

Fig. 2. Survivin isoform expression in cell lines compared with normal tissues. (**A**) The normalized mRNA expression of the 6 survivin isoforms in oral cancer cell lines and dysplastic cell line and in normal tissues from healthy individuals. The expression was normalized using RPS13 as an internal control and the error bars represent the standard error of mean. Survivin wt was found to be the predominant isoform in cell lines compared with other isoforms. Among the minor isoforms, survivin ΔEx3 and survivin 2 B were predominant compared with the other forms. (**B**) The normalized expression of the minor isoforms in the cell lines and normal tissues is presented. As is observed, expression of survivin 3 α is the least in the cell lines as well as normal tissues.

Statistical analysis

Statistical analysis was performed on SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Normality distribution was determined using the Shapiro-Wilk test. Associations were determined using χ^2 for categorical variables and using the Student's *t* test for numerical variables. Correlation between the expression of the various isoforms was determined using Pearson correlation for normally distributed samples and Spearman correlation for non-Gaussian distribution.

RESULTS

Expression of survivin isoforms in cell lines

Figure 2 illustrates the expression of the 6 survivin isoforms determined using real-time PCR in DOK and the 4 oral cancer cell lines. A significant predominant expression of survivin wt was observed in all the cell lines compared with the minor isoforms of survivin ($P < .05$). In cancer cell lines, survivin ΔEx3 and survivin 2 B were the predominant minor isoforms. Survivin is known to function as a dimer and 2 of the isoforms have been reported to be capable of binding to survivin wt, hence the ratio of the expression of survivin isoforms was also determined. In the cell lines, the ratio of the isoforms to survivin wt did not show significant difference. Because we could not procure normal oral cell lines, we compared the expression in cell lines and tissues with normal tissues obtained from healthy individuals (n = 12). Survivin wt expression had a 13-fold higher expression in cell lines compared

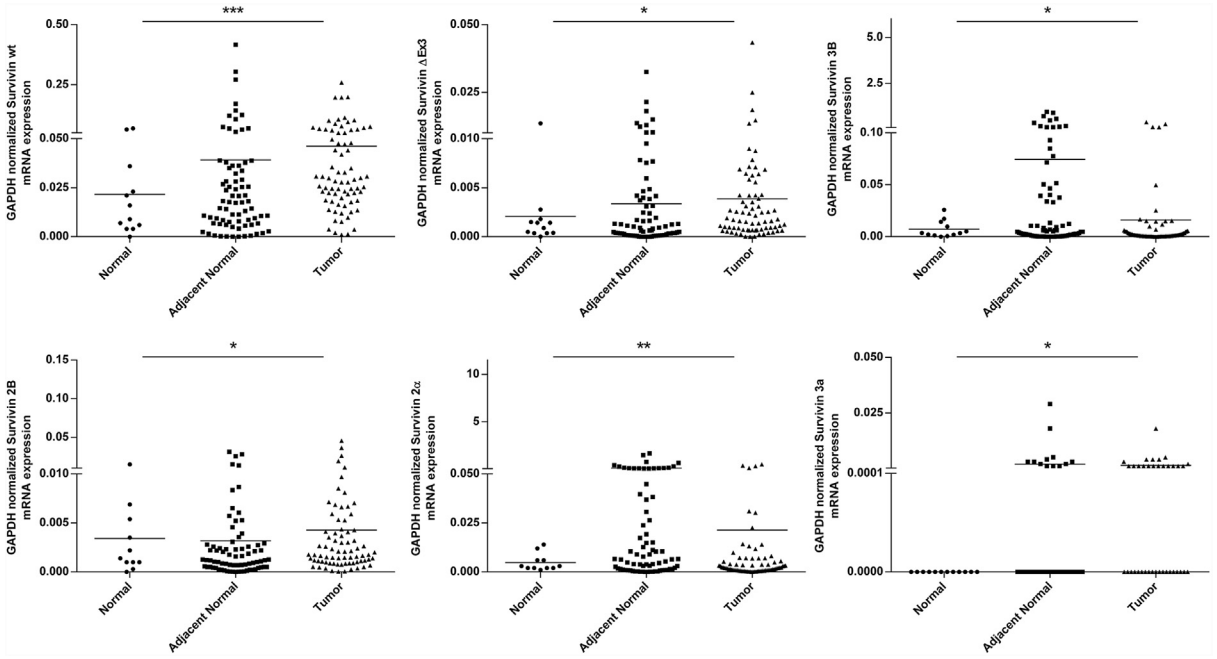

Fig. 3. Expression of survivin isoforms in normal, adjacent normal, and oral tumor tissues. Expression of the 5 survivin isoforms by real-time PCR was determined in 12 normal tissue samples from healthy individuals and 75 tumor and adjacent normal tissue samples. For survivin 3 α the expression was determined in 40 tumor and adjacent normal tissue samples and 12 normal tissue samples. The mRNA expression was normalized using GAPDH as the internal control and the mean values are indicated by the bar. Survivin wt had significant upregulation in tumor tissues compared with normal tissues.

with normal tissues. Survivin 2 B and survivin ΔEx3 had close to 10-fold higher expression in oral cancer cell lines compared with normal tissues.

Expression of survivin transcripts in tumor tissues

The expression of the 5 survivin isoforms was determined by real-time PCR in 75 oral tumor and adjacent normal tissues and 12 normal oral tissues from healthy individuals. Because of lack of material, survivin 3 α expression could be determined in 40 oral tumor and adjacent normal tissues. Figure 3 shows the relative mRNA expression of survivin isoforms in the oral tissues.

Compared with normal tissues from healthy individuals, 88% of oral cancer samples had overexpression of survivin wt, whereas survivin ΔEx3 was upregulated in 64% cases. Survivin 3 B and survivin 2 B had overexpression in 33% and 76% cases, respectively. Overexpression of survivin 2 α was observed in 41% cases and survivin 3 α in 50% cases. This trend was also reflected in fold change, wherein all isoforms had increased expression, with survivin wt showing significant upregulation ($P < .01$) (Figure 4).

As observed with normal tissues, the fold change of survivin wt, survivin 2 B, and survivin ΔEx3 also exhibited upregulation in tumors compared with adjacent normal samples. On the other hand, survivin wt was upregulated in 80% cases and survivin ΔEx3 in 69% cases compared with adjacent normal samples.

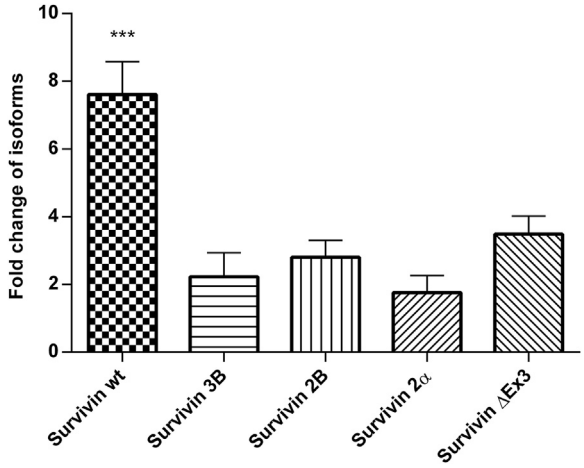

Fig. 4. Fold change of survivin isoform expression in tumor tissues. The fold change of survivin isoform expression in tumor tissues compared with normal was calculated as $2^{-\Delta\Delta Ct}$. In tumor tissues, survivin wt showed an 8-fold upregulation over normal tissues. The minor isoforms of survivin showed <5-fold upregulation in tumor tissues compared with normal tissues.

Survivin 2 B and survivin 3 α were overexpressed in 76% and 50% cases, respectively. Interestingly, however, the average expression of survivin 3 B and survivin 2 α was lower in tumors compared with adjacent normal samples.

Table II. Spearman correlation of survivin transcript expression in 75 oral tumor tissues

Variables	Survivin 3 B	Survivin 2 B	Survivin 2 α	Survivin ΔEx3
Survivin transcript expression				
Survivin wt	0.408*	0.691*	0.460*	0.667*
Survivin 3 B		0.375*	0.794*	0.87
Survivin 2 B			0.480*	0.445*
Survivin 2 α				0.387†
Fold change of survivin isoforms				
Survivin wt	0.393*	0.821*	0.340†	0.749*
Survivin 3 B		0.425*	0.911*	0.242†
Survivin 2 B			0.416*	0.634*
Survivin 2 α				0.323*

	Survivin 2 B/wt	Survivin 2 α/wt	Survivin ΔEx3/wt
Ratio of survivin isoforms			
Survivin 3 B/wt	0.482*	0.886*	0.134
Survivin 2 B/wt		0.463*	0.083
Survivin 2 α/wt			0.234†

*$P < .01$.
†$P < .05$.

Association of survivin isoform expression with clinicopathologic parameters

Statistical analysis indicated a significant association of survivin 3 B with differentiation status of the tumor ($P < .05$), wherein higher survivin 3 B expression correlated with poorly differentiated tumors. No association with clinicopathologic parameters was observed for the other isoforms of survivin. The ratio of the minor isoforms to survivin wt also did not correlate with clinicopathologic parameters. However, because it is the levels of antiapoptotic versus the proapoptotic forms that possibly determines cell fate, we also looked at the association of the expression of the antiapoptotic forms with clinicopathologic parameters. For the analysis, the expression was categorized as higher expression of at least 1 antiapoptotic form (survivin wt, survivin 3 B, and survivin ΔEx3) and lower expression of proapoptotic forms or vice versa. A significant association was observed between overexpression of the antiapoptotic forms and higher TNM stage, with 64% of stage IV cases having overexpression of at least 1 antiapoptotic form.

Correlation between the expression of survivin splice variants

The correlation between the expression of the survivin splice variants is outlined in Table II. The expression of survivin splice variants had significant ($P < .05$) correlation with each other except survivin 3 B expression, which did not correlate with survivin ΔEx3 expression. However, the fold change of expression of all isoforms had significant correlation ($P < .001$, Table II). Table II also shows the correlation between the ratio of the survivin minor isoforms to survivin wt.

DISCUSSION

Survivin, since its discovery in 1997, has been a targetable molecule because of its differential expression in tumors compared with normal differentiated tissues.[21,22,44] Survivin expression has been found to be upregulated in a variety of cancers, although only recently have studies looked at the relative expression of survivin isoforms. Recently few reviews have outlined the expression of survivin isoforms in cancers.[10,11,27] In Table III we have summarized the studies on survivin isoform expression in the past 5 years in various cancers. As is shown, in oral cancer cases only 1 study to date has determined the expression of 4 of the survivin isoforms.[34] Our study is thus the first to determine the expression of the 6 survivin isoforms in oral cancer cases. We determined the expression of survivin isoforms in 75 paired tumor and adjacent normal tissues, 12 normal tissues from healthy individuals, and 5 oral cell lines. In keeping with previous literature, survivin wt was the predominantly expressed isoform in oral cancer cell lines as well as in tissues in our study. We have for the first time found correlation of survivin 3 B expression with differentiation status of the tumor and the overexpression of the antiapoptotic isoforms of survivin with TNM staging of the tumor.

Previously, survivin expression has been reported in oral cell lines by Farnebo et al.; however, the study looked at the overall expression of survivin protein and not the expression of each of the isoforms of survivin.[45] Therefore, the present study is the first to show the expression of the survivin isoforms in oral cancer and dysplastic cell lines. Real-time PCR analysis indicated a 13-fold higher expression of survivin wt in cell lines compared with normal tissues, whereas survivin ΔEx3 and survivin 2 B had a 10-fold increase in expression in cell lines compared with normal tissues. Interestingly, our analysis revealed upregulation of the survivin isoforms even in the dysplastic cell line DOK compared with normal tissues. However, the reasons for this upregulation of survivin in oral cancer cell lines may be attributed to the absence of a functional p53 protein or presence of human papillomavirus (HPV), which also partly acts via degrading wild type p53 by HPV E6 protein. Survivin is known to be repressed by wild type p53, whereas mutant p53 is incapable of regulating survivin.[46] All the cell lines used in the study show the absence of a functional p53 protein,[47,48] which may result in overexpression of survivin in these cells (Table IV). Recently the role of HPV in regulating the expression of survivin has emerged.[49] However, we

Table III. Studies on survivin isoform expression in cancers in the past 5 years

	Function	*Cancer type*	*Up-/downregulation*	*Association*	*References*
Survivin wt	Antiapoptotic, cytoplasmic localization	GBM	+		31
		Breast	+		51, 53
		Colorectal	+	T stage	54
		Bladder	+	In metastatic stages	55, 63
		AML	+	Poor clinical outcome	62
		Oral	+		**34**
Survivin 3 B	Antiapoptotic	Breast	+	Shorter overall survival	61, 66
		Colorectal	+	Survivin/survivin 3 B higher in neoplastic compared with normal tissues	65
Survivin 2 B	Proapoptotic/ antiapoptotic	Breast	+	Tumor grade	52
			+	High-grade ER negative and invasive tumors	61
				Reduction after chemotherapy associated with responsive tumors	66
		Colorectal	+	2 B/survivin wt with ER positivity	51
				Morphologic type, tumor grade	14, 54, 65
		Oral	+	**Tumor grade**	34
		Renal	+	Progressive T stage	63
		AML	+	Poor clinical outcome	62
		Ovarian	+	Overall survival	64
Survivin ΔEx3	Antiapoptotic	GBM	+	Tumor progression and reduced DFS	31
		Thyroid	+		32
		Breast	+	Histologic grade	52, 61
		Oral	+ **(Second highest expression)**	**Decreased in advanced stages and in metastasis cases**	34
		Colorectal	+	T stage	54
		Bladder	+		55
Survivin 2 α	Proapoptotic	Thyroid	+		16
		Breast	+	Low-grade and noninvasive tumors	61
			(Most dominant isoform)		28
		Renal	−		63
Survivin 3 α	−	Astrocytoma	−		13
		Breast	+		28

ER, Estrogen receptor; *DFS*, disease-free survival; *T*, tumor.
Bold values/text represent studies conducted in oral cancers.

have observed high survivin expression in HPV-negative and HPV-positive cancer cell lines and oral tumors (data not shown), indicating that survivin expression in oral cancers possibly may be due to the presence of a nonfunctional p53 (in HPV-negative cell lines) or absence of a functional p53 (in HPV-positive cell lines) rather than the presence of HPV.

Although previous reports on the expression of survivin isoforms in dysplastic cell lines are not available, certain studies have determined survivin expression in dysplastic and oral squamous cell carcinoma (OSCC) tissues.[9,50] These reports found survivin protein expression by immunohistochemistry and its overexpression in dysplastic and carcinoma tissues, indicating a role for survivin in the early and late stages of oral carcinogenesis. The expression of the isoforms was, however, not determined in these cases.

As observed in the cell lines, survivin wt was the most predominant isoform and survivin ΔEx3 was the next most overexpressed isoform in oral tumor tissues. Our study found survivin wt to be significantly upregulated in 80-88% oral tumors compared with adjacent normal tissues and normal samples from healthy individuals, respectively, which has also been found in other cancers.[51-55] However, this is the first time we have found that comparison of survivin wt expression with adjacent normal samples might be underestimating the percentage of upregulation, which could be due to field cancerization. Field cancerization has been extensively studied in oral cancers, and studies have reported the presence of molecular changes in the adjacent normal tissues, which may be attributed to continued exposure to the tobacco carcinogens.[56-58] Hence, though we have determined the expression in adjacent normal samples, the expression of survivin isoforms has also been compared with normal oral tissue samples from healthy individuals. The next predominant isoform in oral tissues in our study was survivin ΔEx3, which was also reported by De Maria

Table IV. P53 mutational status of cell lines used in the study

Cell line	*p53 mutations*	*Possible effects of p53 mutation*	*Reference*
DOK	In frame deletion	Destabilizes p53	48
AW8507	R273 H	DNA binding domain	Unpublished
AW13516	R273 H	DNA binding domain	Unpublished
UPCI-SCC040	No mutations detected in p53 exons 5-8	No p53 protein	47
UPCI-SCC029 B	R280 T	DNA binding domain	47

et al.[34] Survivin ΔEx3 has been found to be upregulated in glioblastomas and breast and thyroid cancers.[31,32,52] It was found to be significantly upregulated in thyroid tumors compared with adjacent non-neoplastic and non-tumoral tissues. In breast and thyroid cancers, survivin ΔEx3 has been associated with invasion and malignancy.[53,59] De Maria et al. have reported survivin ΔEx3 to be decreased in advanced stages of oral cancers.[34] In our study survivin ΔEx3 was significantly upregulated in tumors compared with normal tissue samples, though no association with clinicopathologic characteristics was observed. Survivin 3 B interestingly was found to be associated with differentiation status of the tumor, with higher expression correlating with poorly differentiated tumors. The antiapoptotic property of survivin 3 B is reported to be effected by binding to pro caspase 8 and preventing DISC formation.[60] In breast cancers, the expression of survivin 3 B was found to be inversely correlated with 10 proapoptotic genes and 5 antiapoptotic genes.[61] Survivin 3 B overexpression in nontumor cells has been reported to result in tumor formation in nude mice.[60] Besides the 3 antiapoptotic isoforms of survivin, the proapoptotic form, survivin 2 B, also had a significant upregulation in tumors compared with normal tissues. The proapoptotic activity of survivin 2 B is hypothesized to be by modulation of survivin wt and induction of mitochondrial apoptosis.[23] However, in cancers there are discrepancies in the association of survivin 2 B with tumorigenic properties. De Maria et al. have reported an increase in survivin 2 B with tumor grade in oral cancer cases,[34] whereas reduced expression in advanced stages was shown in colorectal cancers.[14] On the other hand, some studies have reported association of survivin 2 B expression with progressive T stage and poor clinical outcome,[62,63] whereas overexpression of survivin 2 B in ovarian cancer cells inhibited cell and tumor growth.[64] In our study no correlation between clinicopathologic parameters and survivin 2 B was observed. The second proapoptotic form, survivin 2 α, was first described by Caldas et al. in medulloblastoma cell lines.[41] Since then very few studies have determined the expression of survivin 2 α in cancer tissues. Our study is the first to find the expression of survivin 2 α in oral cell lines as well as in normal, adjacent normal, and oral tumor tissues. Survivin 2 α caused by the absence of the third coil in the BIR domain has been found to have proapoptotic functions, mainly by attenuation of survivin wt activity.[41] In our study, the expression of survivin 2 α and survivin 3 B was found to be higher in the adjacent normal tissues compared with normal as well as tumor tissues. This has been previously reported in thyroid cancers wherein survivin 2 α expression was highest in adjacent non-neoplastic tissues compared with nontumoral and tumoral samples.[16] In breast cancers as well survivin 2 α expression was higher in low-grade and noninvasive tumors, probably because of its proapoptotic function.[61] Survivin 3 α was detected first in 2006, and since then 2 reports have determined its expression in cancer,[27] though no functional role has as yet been ascribed to it. Although it was not detected in astrocytomas,[13] it was found to be expressed in breast cancer tissues but undetectable in adjacent normal tissues.[28] In our studies, survivin 3 α was undetectable in normal tissues but was expressed in oral tumor tissues.

Interestingly, our studies found an association of the combined expression of the 3 antiapoptotic isoforms of survivin with TNM staging of the tumors, with 64% of stage IV tumors showing overexpression of at least 1 of the antiapoptotic survivin isoforms. Further studies with a higher sample size may help confirm the effect of the isoforms on TNM stage. We also looked at the correlation between the expression of survivin isoforms because the isoforms are predicted to be capable of forming heterodimers. The fold change of survivin wt and the isoforms had significant correlation. Because the ratio of the survivin isoforms may be an important marker in cell fate, we also determined the ratio of the survivin isoforms to survivin wt. Although a significant correlation was observed between the ratio of survivin isoform expression and survivin wt, no association with clinicopathologic characteristics was found. Ratio of survivin ΔEx3 to survivin wt was reported to be higher in advanced Dukes stage in colorectal carcinoma[54] and that of survivin to survivin 3 B was higher in neoplastic colorectal cancer tissues compared with normal tissues[65]; however, no other study has reported on the ratio of survivin isoforms to survivin wt.

We have found for the first time the higher expression of survivin 2 α and survivin 3 α in oral tumor tissues compared with normal tissues. In light of the literature and the results of our study, it appears that the minor survivin isoform profile is different in oral cancers compared with that in other cancers. The isoforms may

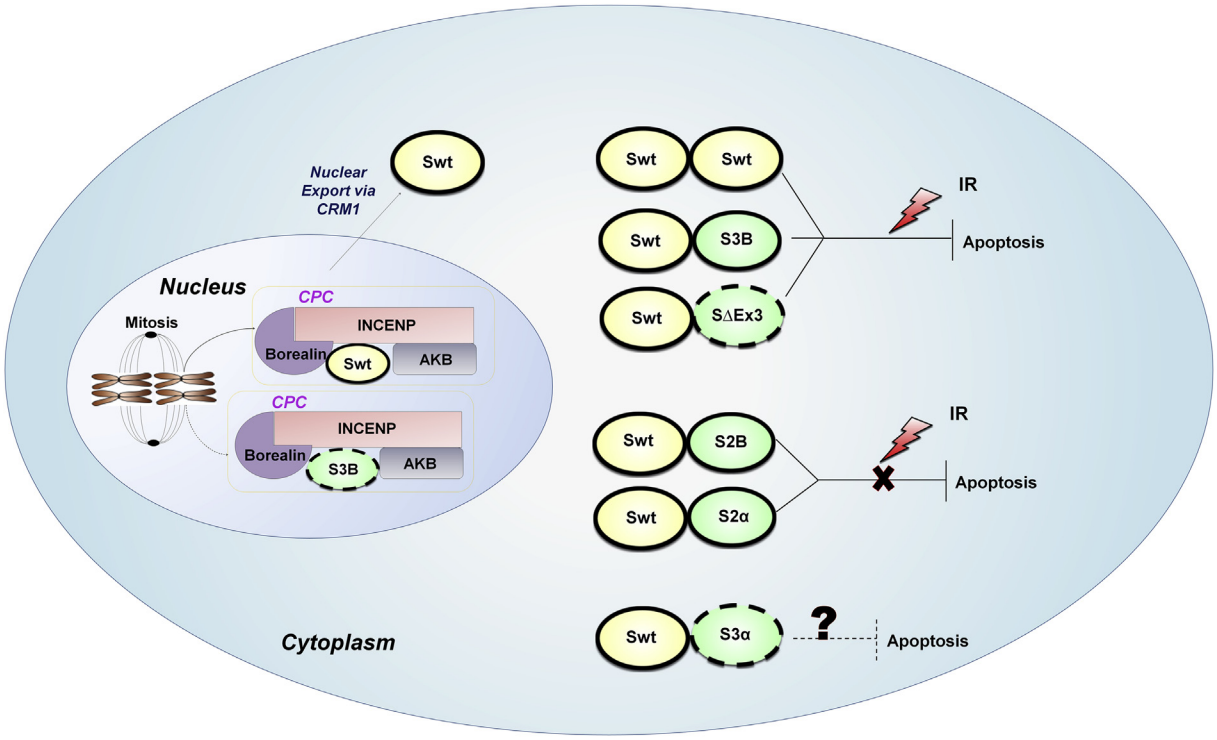

Fig. 5. Interaction between survivin isoforms and their functional importance. Survivin wt as a monomer forms a part of the chromosomal passenger complex and is involved in cell cycle regulation during mitosis. To date only the isoform survivin 3 B has been reported to be capable of binding to the chromosomal passenger complex proteins. When the monomer is exported outside the nucleus using the nuclear export signal (NES) via CRM1, survivin wt homodimerizes and is capable of inhibiting apoptosis in response to radiation. Immunoprecipitation studies have reported that the isoforms survivin 3 B, survivin 2 B, and survivin 2 α may be able to form heterodimers with survivin wt. Survivin 2 B and survivin 2 α, being proapoptotic, attenuate the antiapoptotic function of survivin wt on heterodimerization. The function of survivin 3 α or its ability to heterodimerize with survivin wt has not yet been determined. *Swt*, Survivin wt; *S2 B*, survivin 2 B; *S3 B*, survivin 3 B; *S2 α*, survivin 2 α; *SΔEx3*, survivin ΔEx3; *S3 α*, survivin 3 α; *AKB*, Aurora Kinase B; *CPC*, Chromosomal Passenger Complex; *INCENP*, Inner Centromere Protein; *CRM1*, Chromosomal Maintenance 1.

function differentially in different tissue types. As reported by De Maria et al., we have also not found a significant association of survivin isoforms with clinicopathologic parameters, except survivin 3 B upregulation, which was correlated with poorly differentiated tumors.[34] We have, however, found that on combining the expression of the antiapoptotic forms, an association with TNM staging is observed. Over the years, studies have reported that survivin and its splice variants may be useful as diagnostic or prognostic markers.[10,14,53,66] Our results indicate that along with the expression of survivin wt, the antiapoptotic isoforms survivin ΔEx3 and survivin 3 B may also be involved in the fine tuning of survivin functions during oral tumorigenesis.

Figure 5 is a schematic diagram representing the possible interactions of survivin isoforms and their functions. As depicted in Figure 5, survivin 3 B, survivin 2 B, and survivin 2 α may be capable of binding to survivin wt as shown by immunoprecipitation studies.[25,41] Because survivin 2 B and survivin 2 α may have proapoptotic properties, their dimerization with survivin wt may attenuate the antiapopotic function of survivin wt. Interestingly, besides survivin wt only survivin 3 B has been reported to be capable of localizing and binding to the chromosomal passenger complex proteins,[67] although Noton et al. have reported that the isoforms of survivin wt do not play a role in mitosis.[68] Although no functional role has been ascribed to survivin 3 α, it may resemble survivin 2 α functionally because of structural similarities between the 2 isoforms. The survivin monomer is exported into the cytoplasm via CRM1. In the cytoplasm survivin wt is known to form homodimers and inhibit apoptosis in response to radiation. Depending on the isoforms that dimerize with survivin wt, its antiapoptotic property may be retained or attenuated. However, currently it is not known whether there is a functional effect of the heterodimerization of the isoforms to survivin wt. Further studies on the interaction between the isoforms and their functional roles are warranted to delineate the role of the survivin variants in oral cancers.

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Reprint requests:

Dr. Tanuja Teni, PhD
Advanced Centre for Treatment
Research and Education in Cancer
Tata Memorial Centre
Kharghar
Navi Mumbai 410210
India
Tteni@actrec.gov.in