Strukturelle und funktionelle Untersuchung der p53-Familie, im Besonderen p63

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The greatest glory in living lies not in never falling, but in rising every time we fall.

Nelson Mandela

List of abbreviations

2XYT – Expression media
°C – Degrees Celcius
μ - Micro
AA - Amino acid
ADULT – Acro-Dermato-Ungual-Lacrimal-Tooth malformation
AEC – Hay-Wells syndrome or Ankyloblepharon
ATM – Ataxia Teleangiectasia Mutated
AU - arbitary / absorption unit
BSA - Bovine serum albumin
Chk – Checkpoint dependent kinases
CUSP – Chronic ulcerative stomatitis protein
DBD – DNA binding domain
Dd – Double destilled
DMEM – Dulbecco's modified Eagle's media
DNA - Deoxyribonucleic acid
DNA-PK – DNA dependent protein kinase
E.coli - *Escherichia coli*
EDTA – Ethylenediaminetetraacetic acid
EEC – Ectrodactyly Ectodermal-Dysplasia-Clefting syndrome
EtBr - Ethidiumbromide
FBS – Fetal bovine serum albumin
FTL – Amino acids F605/T606/L607 (mutated to alanine)
g - Gram
GAPDH – Glyceraldehyde-3-Phosphate Dehydrogenase
His-tag - Histidine tag
h - Hour
hER – Human estrogen receptor
HRP – Horseradish peroxidase
IKKα – IκB kinase-α
kDa – Kilo Dalton
K – Lysine of the sumoylation site IKEE (mutated to leucine)
KET – Keratinocyte transcription factor
l - litres
LB – Lysogeny broth
LMS – Limb-Mammary syndrome
m - Milli
min - Minute
M-PER – Mammalian Protein Extraction reagent
Myc – Myc tag containing amino acids MEQKLISEED
n - Nano
Ni-NTA - Nickel-nitrilotriacetic acid
dNTP - desoxyribonucleotide triphosphate
OD – Oligomerzation domain
P73L – Tumor protein p73 like
PBS – Phosphate buffered saline
PCR - Polymerase chain reaction
PVDF – Polyvinylidene fluoride
QC - Quickchange

QP – Glutamine and proline rich region
RNA - Ribonucleic acid
RT-PCR – Reverse transcriptase PCR
s - Second
SAM – Sterile alpha domain
SDS - Sodium-dodecyl-sulfate
SEC - Size exclusion chromatography
SHFM – Split-Hand/Foot Malformations
SOC – Super optimal broth + glucose medium
SUMO-1 – Small ubiquitin-like modifier 1
TA – Transactivation
TBS – Tris buffered saline
TID – Transactivation inhibitory domain
TP63 – Transformation related protein 63
Tween20 - Polyoxyethylene-sorbitan-monolaurate 20
U - Units
VDR – Vitamin-D receptor
VEGF – Vascular endothelial growth factor
WB – Western blot
wt – Wild type

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1. Summary

The transcription factor p63 is part of the p53 protein family, which consists of three members, p53, p63 and p73. P63 shares structural similarity with all family members, but is associated to different biological functions than p53 or p73. While p53 is mainly linked to tumor suppression and p73 is connected with neuronal development, p63 has been connected to critical biological roles within ectodermal development and skin stem cell biology as well as supervision of the genetic stability of oocytes. Due to its gene structure p63 is expressed as at least six different isoforms, three of them containing a N-terminal transactivation domain. The isoforms that are of biological relevance both have a C-terminal inhibitory domain that negatively regulates the transcriptional activity. This inhibitory domain is supposed to contain two individual components of which one is internally binding and masking the transactivation domain while the other one can be sumoylated. To further investigate this domain a mutational analysis with the help of transactivation assays in SAOS2 cells was carried out to identify the critical amino acids within the inhibitory domain and the impact on transcriptional activity of TAp63α, the p63-isoform which is essential for the integrity of the female germline. The results of these experiments show that a stretch of approximately 13 amino acids seems to be important for the regulation of transcriptional activity in TAp63α, due to the increased transcriptional activity occurring in this region after mutation. Additional experiments showed that this mechanism is distinct from sumoylation, which seems to have only implications for the intracellular level of TAp63α. As a conclusion, the C-terminus of the Tap63α is essential for two different mechanisms, which control the transcriptional activity of the protein. Both regulatory elements are independent from each other and can now be restricted to certain amino acids. Activation of the wild type protein might take place in the identified region via post-translational modification. Furthermore an inhibition assay was carried out to test if the same region might have implications on the second biological relevant isoform ΔNp63α. The results show that the same amino acids which show an impact on transcriptional activity in Tap63α lead to a significant change in functional behaviour of ΔNp63α. There is a possibility that both proteins are regulated with

opposite effects via the same mechanisms, based at the C-terminus of the p63α-isoforms. In both cases a modification of these residues could lead to a more opened conformation of the protein with consequences on promoter binding, which can be even important for ΔNp63α with respect to promoter squelching. Both α-isoforms seem to be regulated via the C-terminus and to elucidate if that is also the case for TAp63γ a deletion analysis was carried out. The results show that there are also amino acids within the C-terminus of TAp63γ, which have implications on the transcriptional activity of the protein. Therefore the C-terminus seems to play a major role for regulation of diverse p63 isoforms.

2. Zusammenfassung

Der Transkriptionsfaktor p63 ist Mitglied der p53-Familie, zu welcher auch die Transkriptionsfaktoren p53 und p73 gehören. Die Proteine üben unterschiedliche Aufgaben innerhalb der Zelle aus, wobei sie in bestimmten Fällen miteinander in biologischen Prozessen involviert sind oder die Funktion des jeweils anderen Proteins übernehmen können. P53, welches in ungefähr 70 % aller menschlichen Tumore direkt oder indirekt bezüglich seiner Funktionalität, Struktur oder Regulation betroffen ist, gilt als essentiell für die Tumorsuppression. Im Gegensatz zu p63 und p73 ist p53 auch ein häufiges Ziel von Onkoviren. Auch zeigt sich anhand der unterschiedlichen Phänotypen der verschiedenen *knockout* Mäuse, dass p53, p63 und p73 in unterschiedlichen biologischen Vorgängen involviert sind. *Knockout* Mäuse für *p53* zeigen eine normale Entwicklung, neigen jedoch im Vergleich zu Wildtyp-Mäusen relativ früh zu vermehrter Tumorbildung, während eine Deletion von *p73* schwere Folgen für die Entwicklung, besonders des Nervensystems, hat. Eine Deletion von *p63* führt zu anderen schweren Entwicklungsschäden, vor allem des Epithelgewebes, und führt zum Tod binnen weniger Stunden nach der Geburt durch Herzinfarkt.
Fehlbildungen von Extremitäten oder Epithelstrukturen treten auch bei mehreren menschlichen Krankheiten auf (*ectodactylyl ectodermal dysplasia-clefting; limb mammary syndrome; ankyloblepharon-ectodermal dysplasia-clefting syndrome; split hand/foot malformation*). Diese hängen mit heterozygoten Mutationen in der für p63 codierenden Genregion zusammen, wobei diese Mutationen Auswirkungen auf die Funktion und Struktur wichtiger Sekundärstrukturelemente haben. In Sekundärstrukturelementen bestehen strukturelle Homologien innerhalb der p53-Familie. Es ist jedoch zu beachten, dass für p63 und p73 aufgrund der Genstrukturen viele verschiedene Isoformen möglich sind. Für p63 sind daher mindestens sechs verschiedene Isoformen möglich, wobei die längste Isoform TAp63α aus insgesamt sechs Domänen besteht. Diese Domänen, vom N-terminus angefangen, sind die Transaktivierungsdomäne (TA), die DNA-Binde-Domäne (DBD), die Oligomerisierungsdomäne (OD), die SAM- (*Sterile alpha motif)* Domäne, die QP- (*Glutamine and Proline rich region*) Domäne und die Transaktivierungs-inhibierende Domäne (TID). Weiterhin gibt es am C-terminus

von TAp63α noch eine Sumoylierungsstelle. Die verschiedenen Isoformen können über die Oligomerisierungsdomäne miteinander wechselwirken. In durchgeführten Studien bildeten isolierte Oligomerisierungsdomänen von p63 Tetramere (Dimer von Dimeren). Daher wird ein Vorliegen von p63 in der Zelle als Tetramer, wobei sowohl Homo-Tetramere einer einzigen Isoform als auch Hetero-Tetramere zweier unterschiedlicher Isoformen möglich sind.
Zwei der Isoformen von p63 sind in wichtige biologische Vorgänge involviert. TAp63α, die längste Isoform, ist wichtig für die genetische Integrität von Oocyten in Mäusen und nimmt dort eine vergleichbare Rolle wie p53 in somatischen Zellen wahr. Die andere wichtige Isoform ist ΔNp63α. Diese Isoform verfügt nicht über die N-terminale Transaktivierungsdomäne (TA) und ist in basalem Epithelgewebe sehr stark exprimiert. Dort ist sie für die Erhaltung des dortigen Stammzellenpooles essentiell. Beide α-Isoformen verfügen C-terminal über die SAM-Domäne und die TI-Domäne. Besonders die TI-Domäne spielt für die Regulation des Proteins eine wichtige Rolle, wie in der Vergangenheit anhand von Deletionsstudien gezeigt werden konnte. Diese zeigten auch, dass in dieser Region wohl zwei unterschiedliche Funktionen lokalisiert sind. In TAp63α scheint die TI-Domäne intramolekular die TA-Domäne zu binden und damit zu inaktivieren, während ΔNp63α mit Hilfe der freien TI-Domäne intermolekular inhibierend wirken kann. Da angenommen wird, dass p63 als Tetramer in der Zelle vorliegt, findet die inhibierende Interaktion entweder innerhalb des Dimers oder zwischen zwei Dimeren statt.
Wie bereits erwähnt ist am C-terminus der p63α-Isoformen eine konservierte Sumoylierungssequenz lokalisiert. Sumoylierung ist ein analoger Prozess zu Ubiquitinierung und kann verschiedene Auswirkungen haben. Es kann stabilisierend, sowie destabilisierend auf das Ziel-Protein wirken. Außerdem kann Sumoylierung zu einer Konformationsänderung führen, wodurch Interaktionen mit anderen Proteinen entweder ermöglicht oder verhindert werden. Für TAp73α wurde gezeigt, dass Sumoylierung destabilisierend wirkt. Ebenfalls wurde Sumoylierung von TAp63α nachgewiesen.
Diese Doktorarbeit befasst sich hauptsächlich mit der Analyse des C-terminus in den α-Isoformen von p63. Hierfür wurde eine umfangreiche Mutationsanalyse in der TI-Domäne von TAp63α durchgeführt, wobei die Auswirkungen auf die

transkriptionelle Aktivität von TAp63α und die inhibitorische Fähigkeit von ΔNp63α experimentell bestimmt wurden. Es wurden Reporterassays in SAOS2-Zellen durchgeführt, wobei auf zwei unterschiedlichen Promotoren (*p21* und *bax*) die transkriptionelle Aktivität von TAp63α-Konstrukten bestimmt wurde. Hierbei wurde eine Region innerhalb der TID identifiziert, in welcher eine zum Wildtyp erhöhte transkriptionelle Aktivität festzustellen war. Diese Region, in der sich auch mehrere konservierte Aminosäuren befinden, beginnt mit der Aminosäure F605. Durch Bestimmung der intrazellulären Proteinkonzentration für jedes analysierte Konstrukt konnten die transkriptionellen Aktivitäten um Konzentrationseffekte bereinigt und damit normalisiert werden. Die daraus resultierenden Ergebnisse bestätigten bzw. verstärkten den zuvor erkennbaren Trend aus den relativen Aktivitäten ohne Berücksichtigung der Proteinkonzentration. In diesen hatte eine Folge von 13 Aminosäuren, beginnend mit der Aminosäure F605, Auswirkungen auf die transkriptionelle Aktivität des Proteins. Durch Berücksichtigung der Proteinkonzentration wurde außerdem deutlich das Mutationen, welche die Sumoylierungsstelle betreffen, nur einen Einfluss auf eben jene Proteinkonzentration haben, jedoch nicht auf die intrinsische Aktivität des Proteins. Um diesen Punkt weiter zu überprüfen, wurde eine weitere Mutationsreihe durchgeführt, in welcher die zuvor untersuchten Konstrukte zusätzlich eine Mutation an der Sumoylierungsstelle tragen. Die nicht auf die Proteinkonzentration normalisierten Aktivitäten auf dem *p21*-Promoter waren bei diesen Konstrukten meist erhöht. Nach der Normalisierung waren die Aktivitäten jedoch auf einem ähnlichen Niveau wie die der Mutationsreihe im vorherigen Experiment ohne Mutation an der Sumoylierungsstelle. Hierdurch werden zwei voneinander getrennte Möglichkeiten der Regulation von TAp63α deutlich. Zum einen ein intrinsischer Mechanismus (TA-TI), welcher wahrscheinlich über post-translationale Modifikationen reguliert wird. Zum anderen die Sumoylierung, welche die Konzentration des Proteins beeinflusst.
Dieselben Experimente wurden auch auf dem *bax*-Promoter durchgeführt, wobei die zusätzliche Mutation der Sumoylierungsstelle meist keinen Effekt bei den nicht normalisierten Aktivitäten hatte. Durch Normalisierung ergab sich jedoch erneut ein ähnliches Bild der transkriptionellen Aktivitäten wie auf dem *p21*-Promoter. Regulation durch Sumoylierung scheint daher Promoter-abhängig zu

sein. Durchgeführte Lokalisationsstudien mittels Immunfluoreszenz zeigten, dass sowohl aktive als auch inaktive p63 Varianten im Zellkern lokalisiert sind, eventuelle Aktivitätsunterschiede also nicht durch Translokalisation zustande kommen.

Ein weiteres Experiment sollte klären, ob geringe Proteinkonzentrationen im Zusammenhang mit erhöhter transkriptioneller Aktivität stehen. Hierzu wurde ein aktives Konstrukt, welches in den Zellen in niedriger Konzentration vorliegt, mit einer zusätzlichen Mutation in der DBD (*DNA binding domain*) versehen. Diese inaktive Variante ist in einer ähnlichen Konzentration wie der Wildtyp von TAp63α nachweisbar. Eine hohe transkriptionelle Aktivität hängt im Fall der durchgeführten Experimente als auch in anderen Fällen mit einer niedrigen Proteinkonzentration in der Zelle zusammen und Regulation findet wohl statt, wenn ein aktives Protein an einen Promoter bindet bzw. an einem späteren Punkt wie z.B. der Transaktivierung.

Die durchgeführten Ergebnisse zeigen, dass für die Autoinhibition von TAp63α eine Folge von Aminosäuren eine wichtige Rolle spielt. Hierbei hat besonders die Mutation der drei Aminosäuren, F605, T606 und L607 zu Alanin signifikante Auswirkungen. Das Threonin innerhalb dieser drei Aminosäuren ist zwischen Spezies unterschiedlicher evolutionärer Entwicklungsschritte vollständig konserviert. Diese Aminosäure dient womöglich als Akzeptor für Phosphorylierung oder anderer Modifikationen. Ein Experiment, in welchen das Threonin zu einem Glutamat mutiert wurde um eine Phosphorylierung zu simulieren, brachte jedoch kein signifikantes Ergebnis. Dies kann verschiedene Ursachen haben u.a. ist es wahrscheinlich, dass Glutamat kein gutes Phosphorylierungsanaloga im Fall von Threonin darstellt.

Die identifizierten Reste scheinen wichtig für die Funktion bzw. die Struktur des Proteins zu sein, da in biophysikalischen Experimenten ebenso deutliche Auswirkungen auf die intramolekularen Wechselwirkungen zwischen TA- und TI-Domäne durch analoge Mutationen gezeigt werden konnten. Für TAp63α ist daher eine Signifikanz dieser Ergebnisse bezüglich der Regulation und möglicherweise der Struktur anzunehmen. Über Modifikationen an den identifizierten Resten könnte das Protein eine offene und damit aktive Konformation einnehmen. Auslöser könnte ein Signalweg sein, bei welchem eine

bestimmte Kinase aktiviert wird. Dieser mögliche Signalweg könnte beispielsweise in Oocyten der Maus bei DNA-Doppelstrangbrüchen aktiviert werden, da in diesem Fall TAp63α phosphoryliert wird.

Die vorgestellten Ergebnisse geben einen Hinweis auf welche Weise TAp63α aktiviert werden könnte. Um zu analysieren, ob die gleichen Aminosäuren Auswirkungen auf die Funktion von ΔNp63α haben, wurde eine analoge Mutagenese der erwähnten Aminosäuren FTL in ΔNp63α durchgeführt. Da ΔNp63α auf dem *p21*-Promoter inaktiv ist, wurde ein Inhibierungsexperiment durchgeführt, um eventuelle Auswirkungen festzustellen. Auf die transkriptionelle Aktivität der aktivsten Isoform TAp63γ wirkt ΔNp63α inhibierend. Dies geschieht zum einen durch direkte Inhibition über die Bildung von Hetero-Tetrameren und der daraus resultierenden Interaktion der freien TA-Domänen (TAp63γ) und der freien TI-Domänen (ΔNp63α).

Außerdem findet eine indirekte Inhibition statt, indem Homo-Tetramere von ΔNp63α an den Zielpromoter binden und so für andere aktive Tetramere blockieren (*Promoter squelching*). In einem Reporterassay wurden zunächst SAOS2- Zellen mit unterschiedlichen Plasmidkonzentrationen von ΔNp63α und einer jeweils immer gleichen Menge TAp63γ–Plasmid co-transfiziert. Die Proteinkonzentrationen wurden mittels *Western Blot* ermittelt.

Es zeigte sich, dass der größte Teil der Inhibierung der transkriptionellen Aktivität von TAp63γ durch ΔNp63α über direkte Inhibition (TA-TI-Interaktion) zustande kommt. Diese direkten inhibitorischen Fähigkeiten von ΔNp63α zeigten erhebliche Einbußen, wenn die Aminosäuren FTL in der TI-Domäne zu Alanin mutiert wurden, was auch auf TAp63α signifikante Auswirkungen hatte. Jedoch gibt es einen entgegen gesetzten Effekt bei der indirekten Inhibition, da sich die Fähigkeit zur DNA-Bindung anscheinend durch die Mutation von FTL verbessert, was auf eine offenere Konformation des Proteins hindeuten könnte. Ähnliches lässt sich auch im Fall von TAp63α vermuten. Dies deutet auf die Wichtigkeit jener in dieser Doktorarbeit identifizierten Aminosäuren FTL für die Regulation und Struktur beider Isoformen hin. Die Mutation dieser Aminosäuren führt vermutlich zu einem Aufbrechen von Interaktionen und damit zu der erwähnten offeneren Konformation. Aufgrund der Inhibierungsassays lässt sich auch vermuten, dass eine Tetramerisierung eventuell für die volle transkriptionelle

Funktionalität notwendig ist. Bei einem Konzentrationsüberschuss von ΔNp63α ist anzunehmen, dass TAp63γ vornehmlich in gemischten Tetrameren zwischen TAp63γ und ΔNp63α, vorliegt. Eine Doppelmutation (DBD + TID) in ΔNp63α führte im Inhibierungsexperiment dazu, dass diese inaktive Isoform nicht mehr inhibierend über DNA-Bindung oder TA-TI Interaktion wirken kann, sondern nur noch über die Bildung von Hetero-Oligomeren. Diese haben die gleiche Aktivität wie ein TAp63γ-Konstrukt, welches nur noch Dimere bilden kann. Dies deutet auf die Wichtigkeit des Oligomerisierungszustandes hin.

Es besteht die Möglichkeit die Wildtypen von TAp63γ und ΔNp63α durch gegenseitige Expression und der daraus resultierenden Bildung von Hetero-Oligomeren zu regulieren.

Im Fall von TAp63α und ΔNp63α wäre die Regulation über Modifikation in der TI-Domäne oder über Sumoylierung unabhängig von anderen Isoformen. Da TAp63γ weder über die TI-Domäne noch über eine bekannte Sumoylierungsstelle verfügt sind diese Regulationsmechanismen der α-Isoformen für TAp63γ ausgeschlossen. Jedoch könnte, analog zur Regulation von TAp63α, die Aktivität von TAp63γ über posttranslationale Modifikationen zu steuern. Daher wurde für TAp63γ, auch wenn noch keine biologische Funktion feststeht, eine Deletionsstudie des C-terminus durchgeführt.

Auch in diesem Fall wurden Aminosäuren identifiziert, die die transkriptionelle Aktivität des Proteins beeinflussen. Hierbei handelt es sich um ein Threonin und ein Serin, die, wenn sie fehlen oder zu Alanin mutiert sind, einen signifikanten Abfall der transkriptionellen Aktivität des Proteins verursachen. Eine Mutation führt in jedem Fall zur Inaktivität, während die Inaktivität nach Deletion Einschränkungen unterworfen ist. Diese Ergebnisse müssen nicht zwangsläufig bedeuten, dass auch am C-terminus von TAp63γ ein Kontrollelement lokalisiert ist, sondern können nur darauf hinweisen, dass diese Aminosäuren essentiell für die Funktion des Proteins sind. Eventuell liegen die erwähnten Aminosäuren in der Zelle phosphoryliert und damit TAp63γ im aktiven Zustand vor. Dies würde auch erklären, warum eine Mutation dieser Aminosäuren zu Inaktivität des Proteins führt. In jedem Fall hat eine Modifikation am C-terminus von TAp63γ Auswirkungen auf die Aktivität und diese Implikationen werden durch zwei Aminosäuren verursacht. Somit gibt es am C-terminus beider untersuchten TA-

Formen Aminosäuren, die Einfluss auf die transkriptionelle Aktivität der jeweiligen Isoform haben. Des Weiteren sind dieselben Aminosäuren, welche die transkriptionelle Aktivität von TAp63α beeinflussen, wichtig für die Fähigkeit von ΔNp63α die aktivste Isoform TAp63γ zu inhibieren.

Wie bereits erwähnt, spielt ΔNp63α eine wichtige Rolle für die Entwicklung von Epithelgewebe. Wie sich in einem Experiment zur Genexpression verschiedener Gene zeigte, welche in der Osteogenese involviert sind, hat die Überexpression von ΔNp63α dort deutliche Auswirkungen. In diesem Fall scheint es wahrscheinlich, dass die jeweiligen Promotoren durch ΔNp63α blockiert werden (*Promoter squelching*) und keine direkte Interaktion mit anderen Proteinen stattfindet, was weitere Experimente zeigen könnten. Es gilt vor allem zu beachten, dass ΔNp63α im vorliegenden Fall überexprimiert vorliegt. Unter zellulären Bedingungen wäre die Konzentration von ΔNp63α wohl nicht derart erhöht und für ein vollständiges Blockieren der Ziel Promotoren vermutlich nicht ausreichend. Jedoch ist es denkbar ein Blockieren der Ziel-Promotoren bei niedrigerer Proteinkonzentration über eine erhöhte Affinität von ΔNp63α zu DNA zu ereichen. Für eine Kontrolle der Genexpression über Promoterbindung von ΔNp63α, würde der mögliche identifizierte Regulationsmechanismus Auswirkungen haben, da eine „aktivierte“ Form von ΔNp63α eventuell eine höhere Affinität zur DNA besitzt. Dies wäre eine Möglichkeit über die ΔNp63α reguliert werden könnte, unabhängig von der Interaktion mit anderen Isoformen von p63. Experimente könnten diese Hypothese zur Regulation von ΔNp63α bestätigen. Wie auch im Fall von TAp63α könnten weitere Untersuchungen zeigen, ob eine der identifizierten Aminosäuren durch zelluläre Signale modifiziert wird, wie z.B. bei DNA-Schäden in Oocyten.

3. Introduction

3.1 The p53-family

The p53-family consists of three transcription factors, p53, p63 and p73, which share structural [Fig. 1] and to some extend functional similarity [1,2]. Because of this similarity p63 and p73 can regulate p53 target genes and have been implicated in p53-dependent apoptosis [3,4]. The merging of the three proteins to a p53-superfamily of transcription factors started after the identification of the genes for p63 and p73 in 1997 and 1998 [5-8]. The p53 gene was the first tumor suppressor gene to be identified and has been first described in 1979 [9]. The biological role of p53 is tumor-suppression by cell cycle arrest and apoptosis. Approximately 70% of all human cancers are linked to misfunction of p53, either by mutations in the p53 gene or indirect inactivation [9-12]. Mice with a *p53* knockout develop normally, but have a highly increased rate of tumorigenesis [13]. Thus p53 is known as the "guardian of the genome" [14].
P63 and p73 show high sequence homology to p53 and to each other [Fig. 1], especially in certain structural elements, like the DNA binding domain (DBD) or the oligomerization domain (OD), but p63 and p73 both have additional structural elements after the oligomerization domain, which might be important for their structure, functionality and regulation [15,16].

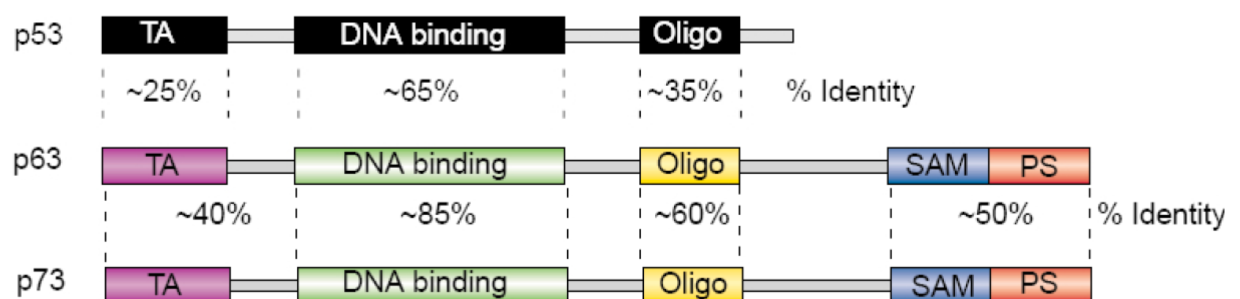

Figure 1: Domain structures of the p53 family members (full length isoforms) and sequence identities. TA, transactivation domain; Oligo, oligomerization domain; SAM, sterile alpha motif domain; PS, post-SAM. [taken from ref. 1, modified].

3.1.1 One family – different functions

The main function of p63 and p73 seems not to be tumor suppression as the knockout mice show totally different phenotypes than the *p53* knockout mice [13,17-20]. Also most tumor virus proteins only target p53 [21-25]. For *p73* the knockout mice show developmental defects, including hippocampal dysgenesis and hydrocephalus due to hypersecretion of cerebrospinal fluid in addition to purulent respiratory infections, also their reproductive and social behaviour is abnormal as a result of dysfunctional pheromone pathways [19,20]. Therefore p73 seems to be essential for the development of the central nervous system. Also it seems to be essential for preventing neurodegeneration [26].
P63, despite the structural similarity to p73, is essential for other biological developmental and differentiation processes than p73. The knockout-mice for *p63* show severe defects including limb truncations and lack of all squamous epithelia and die within a few hours after birth [Fig. 2] [17,18].

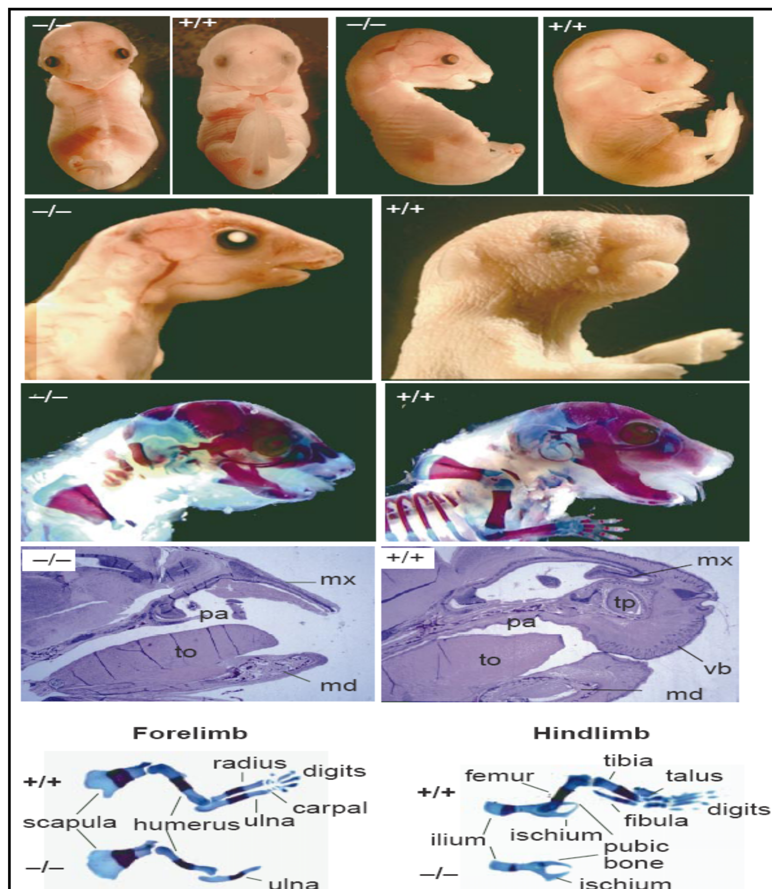

Figure 2: Comparison of a *p63*-knockout mouse (-/-) with a wt mouse (+/+). The knockout mouse shows severe developmental defects, especially in extremities and epithelial structures. [taken from ref. 17]

Mice heterozygous for *p63* develop normally and in contrast to *p53* are not prone to spontaneous or chemically induced tumors [27]. Therefore p63 has been assigned a crucial role in epithelial stem cell biology [17,18,28-31]. Recent findings also show that p63 is essential for the surveillance of the genetic stability of murine Oocytes [32]. Also there is emerging evidence that p63 is regulating neuronal cell survival together with p53 and p73 [33]. Taken together p63 seems to be important in various biological pathways and its function is different to the functions of p53 and p73. Nevertheless these proteins might act together in some pathways or might be able to substitute each other in some cases.

3.2 p63

3.2.1. Nomenclature

The main topic of this thesis is p63. Different names has been assigned to this protein throughout the years. It is also known as transformation related protein 63 (TP63), tumor protein p73 like (P73L), p51, p40, Keratinocyte transcription factor KET or Chronic ulcerative stomatitis protein CUSP.

3.2.2 Gene structure and isoforms

The gene of p63 has been identified and characterized in 1997 and 1998 [6, 17, 18]. Due to the exon structure of the p63 gene [Fig. 3] six different splicing variants of p63 exist [6].

Figure 3: Exon structure of the p63 gene (coding sequences in black). The two promoters are indicated as arrows. Splicing combinations are indicated as beta and gamma, alpha is the full length isoform. [taken from ref. 6]

The gene structure with two promoters combined with three alternative splicing variants leads to six different isoforms [Fig. 4].

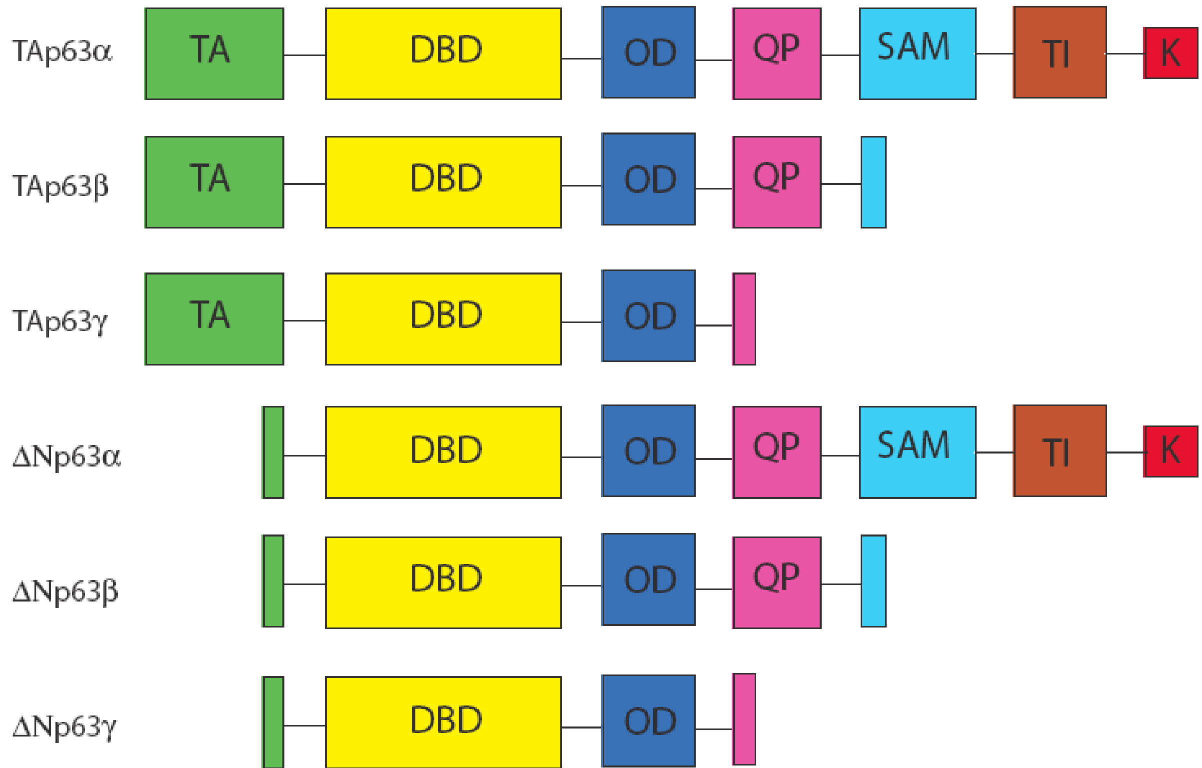

Figure 4: Domain structure of the p63 isoforms. (TA, transactivation domain; DBD, DNA binding domain; OD, oligomerization domain; QP, glutamine and proline rich domain; SAM, sterile alpha motif; TI, Transactivation inhibitory domain; K, sumoylation site IKEE).

The transcriptional activities of the six isoforms in cell culture experiments are different. The ΔN isoforms show no transcriptional activity on most promoters, the three isoforms with the transactivation domain TAp63β and TAp63γ are constitutively active on promoters like *p21*, *mdm2* and *bax* while TAp63α is inactive, which is achieved by an auto-inhibitory element located at the C-terminus (TI domain) [6,15]. The same element is responsible for the strong inhibitory potential of ΔNp63α [6].

3.2.3 Biological relevance

The two α-isoforms have been assigned to critical biological functions [17,18,32].

TAp63α, the largest isoform, is expressed in murine Oocytes, where it is essential for a p53 independent manner of apoptosis induced by DNA damage and therefore monitors the integrity of the female germ line [32]. Oocytes stay arrested in the prophase of meiosis I (dictyate arrest) during oogenesis until they are recruited for ovulation, a period which can last up to several decades in humans. In the arrested cells Tap63α is highly expressed and mice experiments showed that it is transcriptionally inhibited [32]. After an exposure to ionizing radiation the binding affinity of p63 to DNA increased ~20 fold and this increase was accompanied by p63-phosphorylation and death of the irradiated oocytes. Therefore TAp63α seems to be crucial for the integrity of the female germline.
The other biologically important isoform ΔNp63α which lacks the N-terminal transactivation domain is highly expressed in the basal layer of epithelial tissue [17]. It seems to be involved in processes maintaining the regenerative potential of epithelial stem cells explaining the defects in p63 knockout mice as loss of regenerative potential of cells.
Both isoforms essential for these biological processes, ΔNp63α and TAp63α, have a non-truncated C-terminus (α-isoforms). The domains localized there are the SAM-Domain (SAM, sterile alpha motif), the TI- (transactivation inhibitory) domain and the sumoylation site (amino acid sequence IKEE).
TAp63 is assumed to be a tetrameric protein, the OD for itself forms dimers of dimers [16]. Therefore the inhibiting interaction between the TI and TA domain could take place between two monomers within a dimer or between two dimers within a tetramere. The oligomeric state might be important for the regulation of the protein. In TAp63α the C-terminal domain and its interaction within the protein or between two proteins might lead to a closed conformation of the protein and therefore less ability to bind DNA. A previous study mapped the TID to the last ~70 amino acids and showed that the domain contains of two different sub-domains which both contribute to inhibition in a different manner, one of them located in the first 45 amino acids of the TID and the second in the last 25 amino acids [15]. These regions are further investigated in this thesis.
Besides the lethal effect of a deletion of the p63 gene in mice mentioned before, there are also human syndromes associated with germline mutations of *p63* causing limb defects and ectodermal dysplasia, such as Ectrodactyly Ectodermal-

Dysplasia-Clefting Syndrome (EEC), Split-Hand/Foot Malformations (SHFM), Limb-Mammary Syndrome (LMS), Hay-Wells syndrome or Ankyloblepharon (AEC), Acro-Dermato-Ungual-Lacrimal-Tooth malformation (ADULT) and Rapp-Hodgkin Syndrome [34,35]. The occurred mutations are heterozygous and might act as gain of function or dominant-negative allele rather than by haploinsuffiency. For EEC mostly all mutations were substitutions of amino acids in the DNA binding domain [36]. Only 10% of SHFM patients carried mutations with variable effects on DNA binding, frameshift mutations were located in exon 13 and 14 in the case of LMS, which leads to a truncated protein lacking the TID and partially the SAM domain exclusively in the α-isoforms [37]. The Hay-Wells syndrome on the other hand is caused by missense mutations in the SAM domain, which might affect protein-protein interactions [38]. The ADULT syndrome seems to be caused by mutations which affect only the ΔN isoforms, but one patient was found, which had an mutation in the region of the DBD [39, 40]. Taken together, the mutations found in these syndromes are localized in the DBD or SAM domain. A lot of these mutations can be also found in inactive p53 mutants [41].

3.3. Regulation

3.3.1 Regulation of p53

Transcription factors can be regulated by post-translational changes i.e. phosphorylation, acetylation, glycosylation, ubiquitination or lipid modification [42]. One regulation mechanism for the family member p53 is via the interaction with the ubiquitin E3 ligase Mdm2 [Fig 5] which leads to a short half life of the protein between 20 min and 2 hours [9,43,44]. Ubiquitin, a 76 AA polypeptide, is covalently linked to lysine residues on target proteins by an enzymatic cascade, which targets the protein for degradation in the proteosomal pathway [45]. Mdm2 binds to the transactivation domain of p53 which leads to poly-ubiquitination of the protein and proteosomal degradation [42,43,46,47]. The regulation of p53 is achieved by the ratio of the concentrations of Mdm2 and p53, which is modulated by the Mdm2 homologue Mdm4 [43,48]. If cellular stress such as DNA-double

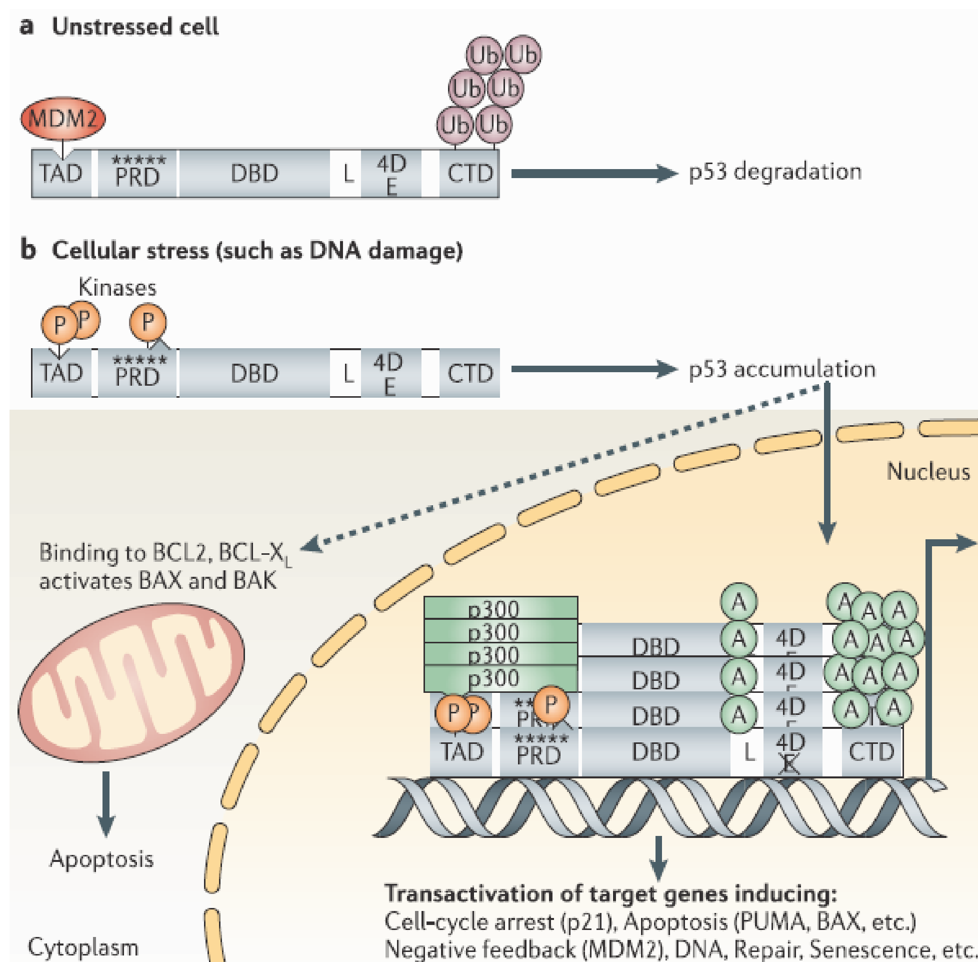

Figure 5: Regulation of p53 [taken from ref. 43]. In an unstressed cell p53 gets ubiquitinated and degraded, while cellular stress leads to phosphorylation of p53 leading to a transcription of target genes.

strand brakes i.e. after ionising radiation occur several kinases like DNA-PK (DNA dependent protein kinase), ATM (Ataxia Teleangiectasia Mutated) or Checkpoint-dependent kinases (Chk) phosphorylate p53 at the sites crucial for the Mdm2 interaction which leads to an accumulation and activation of p53 [43,44,49,50]. Also involved in p53 regulation is the prolyl isomerase Pin1, which induces a conformational change in p53 as response to stress signals and also seems to be involved in the regulation of p73 [51,52].

3.3.2 The TID in p63α

Even though the p63-dependent apoptotic pathway in murine oocytes might be

similar to the p53-dependent in somatic cells the regulation of p63 seems to be more complicated due to the different isoforms. As mentioned before the C-terminus of the α isoforms acts as an transactivation inhibitory domain [15]. Therefore the full length isoform TAp63α is inactive because of the intramolecular inhibition. The second isoform harbouring the TI domain, ΔNp63α, is capable of inhibiting other isoforms [6]. The TI-domain and its conserved amino acids are shown in Fig. 6.

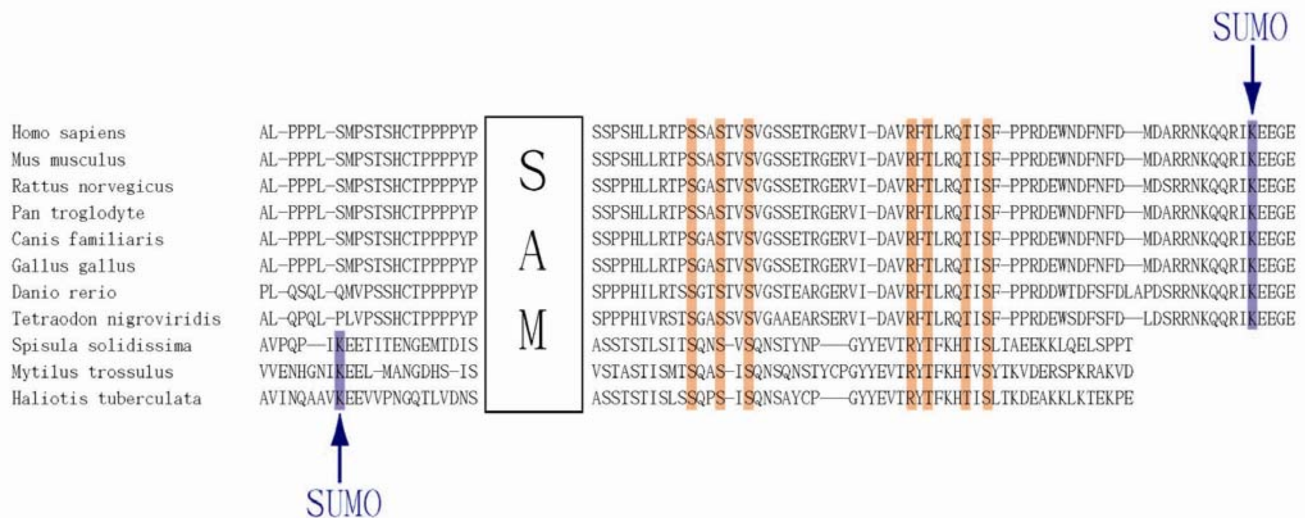

Figure 6: Sequence alignment of C-terminal p63α sequences of various vertebrate and invertebrate species. Sequences N-terminal to the SAM domain to the end of the protein are shown. The sequences of the SAM domains themselves are not displayed. Strictly conserved amino acids are labelled red. The conserved KEE sumoylation motif is labelled in blue. This sumoylation sequence is located N-terminal to the SAM domain in invertebrate species [taken from ref. 53].

Even though the possible function of the TID has been shown, the important amino acids of this function were unknown. In this thesis data is presented about the amino acids in the TI domain, which seem to be important for the function of the TID and its inhibitory interaction. A region is identified, where mutations have significant effects on transcriptional activity of TAp63α. Additionally, If these amino acids are mutated in ΔNp63α it looses its inhibitory abilities. As mentioned before ΔNp63α plays an important role in epithelial development and possible implications on its function might be significant for these biological processes. In the natural state these amino acids might be involved in a control mechanism. It is also possible that transcriptional activity might be controlled by concentrations

of the different isoforms.

3.3.3 Sumoylation

Another regulation mechanism of p63 is sumoylation, which is an ubiquitination like mechanism [54]. As mentioned before p53 protein levels are tightly controlled by Mdm2, which is an ubiquitin ligase and promotes rapid degradation via the proteasome pathway [42,43,47,48,49]. At the C-terminus of human TAp63α a classical sumoylation sequence is located and sumoylation was shown for both TAp63α and TAp73α [54-56]. SUMO-1 (small ubiquitin-like modifier 1] is a ubiquitin-like tagging polypeptide and modifies a large number of proteins. It is attached to the lysine in the sumoylation sequence by a similar enzymatic system like ubiquitin, [57,58]. This can lead to degradation as well as stabilization of the target protein, also it can have effects on interactions of the target protein [Fig. 6] [58-64].

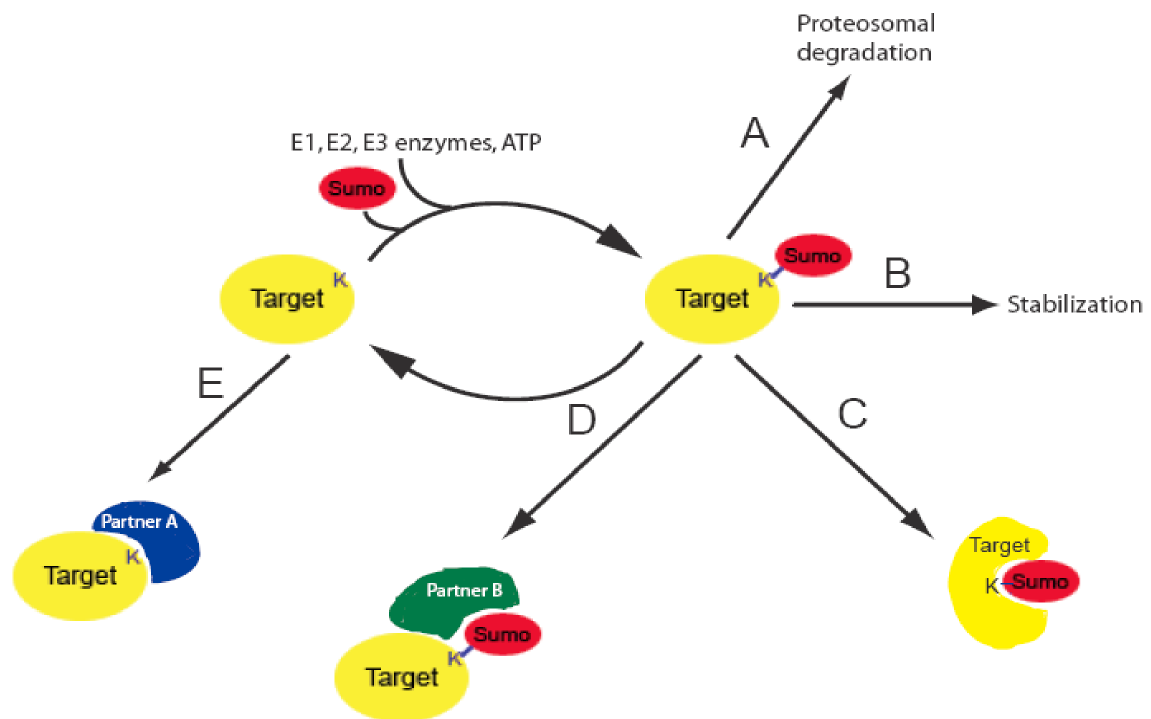

Figure 7: Consequences of SUMO-1 attachment (detachment). A: Degradation; B: Stabilization; C: Conformational change; D, (E): Providing binding site.

Modification of p73α by SUMO-1 seems to destabilize the protein [33]. The sumoylation site in TAp63α or its homologues is conserved throughout various organisms as well as some amino acids within the TID [Fig 6]. The sumoylation site is located at different positions in vertebrates and invertebrates but remained conserved.
Additional to the extensive mutational analysis that was carried out throughout the whole TI domain to identify the important amino acids, the implications of a mutation of the sumoylation site was analyzed and if both sites are independent from each other.

3.3.4 Oligomerization state and regulation of TAp63γ

Another important question regarding the function and regulation of p63 is its oligomerization state. The oligomerization domains of human p63, as well as p73 and p53 can form stable homo tetrameres or the different splicing forms can interact with each other via the oligomerization domain and form hetero tetrameres [65, 66]. Interestingly the amino acids in the oligomerization domain are not as highly conserved as i.e. in the DNA binding domain. Some stabilizing structural elements, like the SAM domain, might be necessary to keep it in its tetrameric state [16]. An isoform without the SAM and the TI domain and with a different C-terminus than TAp63α is TAp63γ, which is also the most active isoform of p63 [6,15]. Its structural organization looks similar to that of p53. Its regulation has to be different than that of TAp63α due to the missing TID, but at the C-terminus of TAp63γ there might also be a control element. The C-terminus of TAp63γ is also a part of this thesis as well as consequences of the oligomeric state on the transcriptional activity.

3.2.4 Target genes of p63

Many potential target genes for p63 involved in different cellular actions have been identified by various techniques [67]. Two of the identified target genes are

bax and *p21* [68,69]. Therefore the promoters of these genes have been used for analysis of the transcriptional activity of p63 in this thesis. Also the promoter of the Vitamin-D receptor (VDR) gene was identified as a target of p63 [70]. The VDR as a ligand dependent transcription factor is important for differentiation of keratinocytes and osetoblasts [71,72]. In this thesis data is shown about the implications of the different p63 isoforms on genes involved in osteogenesis (*VDR*, *Osteopontin*, *Osteocalcin*, *Collagene*). It has been shown that for several proteins, like VEGF (Vascular endothelial growth factor), the ΔN and TA forms of p63 have different or opposite effects on expression of the proteins [73,74]. There are also other reported target genes, which seem to act together with p63 in one pathway. One of them is the *IκB kinase-α* (IKK-α), which seems to be a direct downstream target of p63 [75]. As mentioned before p63 plays a major role in the development of epidermal tissue [17,18,28-31]. The cells responsible for regeneration of this multi-layered, stratified epithelium are terminally keratinocytes, which are continuously differentiating [76-78]. IKK-α is essential for the formation of the epidermis and the knockout mice for *IKK-α* look very similar to those of p63 and show very similar syndromes [79-82]. The important question is, how p63 is regulated to activate or inactivate genes involved in various biological pathways and which isoforms are regulated in which way.

4. Materials

4.1 Reagents

Reagents were purchased by Carl Roth GmbH. Exceptions are listed below.

Bovine serum albumin (BSA)	Sigma
Complete mini EDTA-free protease inhibitors	Roche
Coomassie Brillant Blue R250	Sigma
Desoxynucleosid-5‘-triphosphate (dNTPs)	NEB
Ethylenediaminetetraacetc acid (EDTA / 100 X)	Pierce
Halt™ Protease inhibitor cocktail (100 X)	Pierce
Hoechst fluorochrome 33278	Sigma
Mammalian Protein Extraction Reagent (M-PER)	Pierce
Non-fat dry milk powder	Fluka
Trypsin-EDTA	PAA
Tween20	Sigma

4.2 Enzymes

Accutase	PAA
DNAse I	Sigma
Lyzozyme	Sigma
Phusion DNA Polymerase	Finnzymes
Proteinase K	Invitrogen
Restriction enzymes	NEB
RNase A	Sigma
T4-DNA-ligase	NEB
Tev Proteinase	Institute
Turbo Pfu DNA Polymerase	Stratagene
Vent DNA Polymerase	NEB

4.3 Media + additives

Ampicillin	Carl Roth
Chloramphenicol	Carl Roth
Dulbecco's modified Eagle's media (DMEM)	PAA
Epression Media (2XYT)	Institute
Fetal bovine serum (FBS)	PAA
Kanamycin	Carl Roth
LB (Lysogeny broth)	Institute
LB plates	Institute
L-Glutamine (200 mM)	PAA
Penicillin-streptomycin (100U/ml and 100μg/ml)	PAA
SOC (Super Optimal Broth + Glucose media)	Institute

4.4 Ladders

Gene Ruler 1 kb DNA Ladder	Fermentas
Kaleidoscope Protein Ladder Prestained	Bio-Rad
Benchmark Protein Ladder	Invitrogen

4.5 Antibodies

Primary antibodies:	
Anti-myc 4A6	Millipore
Anti-GAPDH (Glyceraldehyde-3-phosphate dehydrogenase	Millipore
Anti-myc 9E10 Cy3	Sigma
Secondary antibodies:	
Anti-mouse HRP (Horseradish peroxidase)	Sigma

4.6 Materials

Materials were purchased by Carl Roth GmbH. Exceptions are listed below.

Coverslips	VWR
Cryotuber	Nalgene
Culture flasks	Greiner Bio One
Gel blotting paper	VWR
Glaswares und Glaspipettes	Fisher Scientific
Nitrile Gloves Safeskin	Kimberly-Clark
PVDF membrane	Millipore
Parafilm	American National Can
Pipette tips 20 µl, 200 µl, 1000 µl	Omnitip
Novex gels	Invitrogen
Well plates (black)	Greiner Bio One
Well plates (Luminescence)	PAA
Plastic ware	Sarstedt, Greiner
Ni^{+}-NTA-column	GE
Gelfiltration column Superdex 200	GE

4.7 Kits

Dual-Glo Luciferase Assay	Promega
Effectene® Transfection reagent	Qiagen
Endofree® Plasmid Buffer Set + Tip 100	Qiagen
Mycosplasma PCR Test Kit	Applichem
Nucleospin® Plasmid	Macherey Nagel
QIAquick Gel Extraction Kit	Qiagen
QIAquick PCR Purification Kit	Qiagen
QuikChange® Site-Directed Mutagenesis Kit	Stratagene
RT-PCR Kit Cellsdirect™	Invitrogen

4.8 Centrifuges

HERAEUS Biofuge 13

HERAEUS Minifuge GL

EPPENDORF Centrifuge 5417 R

EPPENDORF Centrifuge 5810 R

Sorvall Biofuge 13 Evolution RC

Sorvall Biofuge 13 RC-5B

4.9 Instruments

Äkta-Protein purification system	Amersham Biosciences
Analytical balance	Sartorius
Autoclave	Getinge
Axiocam MRm	Zeiss
Balance	Mettler Toledo
Clean Bench Herasafe	Kendro
Counting chamber	Carl Roth
Fluorescence microscope Axiovert 200	Zeiss
Freezer –20°C Profiline	Liebherr
Freezer –80°C Profiline	Liebherr
Gel Imager	Biometra
Heatable magnetic stirrer Ikamak	IKA-Labortechnik
Heating block	VWR
Incubator Heracell	Heraeus
Incubator model 400	Memmert GmbH
Light microscope Axiovert 40 CFL	Zeiss
Lumi Imager P1	Roche
Luminescence Reader Genios Pro	Tecan
Microwave 600 W	Alaska
Multipipettes	Eppendorf
N_2 Dewer GT40	L’Air Liquide
Nanodrop 100	peqlab

NuPAGE Novex Gel system	Invitrogen
pH-meter PHM 210	Radiometer
Pipettes	Eppendorf, Abimed
Power supply	Biometra
Pump	HLL
SDS-Gelelektrophoresis chamber	Biorad
Shaker	Heidolph
Shaking incubator	Innova AG
Shaking incubator	New Brunswick
Sonifier Labsonic U	B. Braun
Thermocycler	Bio-Rad, Eppendorf
Vakuum pump	Abm
Vortexer Reax 2000	Heidolph
Waterbath	Haake
Water treatment system Milli-Q	Millipore

4.10 Buffers

Buffers were adjusted to final volume with ddH_20.

10 x Protein Sample Buffer	10 % Glycerol
	5 % ß-Mercaptoethanole
	2 % (v/v) SDS
	0,05 % (w/v) Bromphenoleblue
	1,25 M Tris-HCl, pH 6,8
2 X Protein Sample buffer	20 % 10X Protein Sample Buffer
	19 % ß-Mercaptoethanole
Buffer D	50 mM Tris-HCl, pH 8.0
	20 mM NaCl
	1 mM EDTA
	1% SDS

Coommassie brilliant blue solution	0.1% (w/v) Coomassie R250 40% Methanol 10% Acetic acid
Destaining solution	40% Methanol 10% Acetic acid
DNA-Samplebuffer	50 % Glycerol 0,1% (w/v) Bromphenoleblue 100 mM Tris-HCl, pH 8,0
ECL1	100 mM Tris-HCl, pH 8.5 2.5 mM Luminol 0.4 mM p-cumaric acid
ECL 2	100 mM Tris-HCl, pH 8.5 0.0183% H2O2
Lysis-, Dialysisbuffer	250 mM NaCl 250 mM KCl 50 mM Tris-HCl, pH 8,0
Mammalian cells lysis buffer	98% M-PER 1% Protease inhibitor cocktail 100 X (Halt™) 1 % EDTA 100 X 100 U DNAse I
MOPS SDS running buffer	50 mM MOPS 1 mM EDTA 0.1 % SDS 50 mM Tris-HCl, pH 7,7

Mounting media	90% Glycerol 100mM Tris pH 8.0
PCR (QC) Buffers	10 X or 5 X buffers delivered by manufacturer
PBS	10mM NaH_2PO_4 10mM Na_2HPO_4 150mM NaCl
PBST	PBS + 0.2 % TritonX-100
SDS-PAGE running buffer (10x)	1.92M Glycine 0.25M Tris pH8.3
T_4-ligase buffer	10 X buffer delivered by manufacturer
TAE (50x)	2M Tris-acetate 100mM EDTA
TBS (1x)	50mM Tris 150mM NaCl
TBST (1x)	1x TBS + 0.1% Tween20
TE buffer	10mM Tris pH8 1mM EDTA
Western Blot Transfer Buffer	25 mM Bicine 1 mM EDTA 25 mM Bis-Tris, pH 7,7

4.11 Cells

Bacterial strains

***E. coli* DH5:**	*rec*A1, *end*A1, *rel*A1, *gyr*A96, *thi*-1, *sup*E44, U169, *deo*R, *hsd*R17 (rK-, mK+), λ-, F-, Φ80, *lac*ZΔM15, Δ(*lac*ZYA-*arg*F)
***E. coli* BL21 (DE3) simple**	B, F-, *dcm* *omp*T, *hsd*S (rB- mB-) *gal* λ (DE3)
***E. coli* BL21 lac rare**	F-, *omp*T, *hsd*SB (rB-mB-), *gal* *dcm* (DE3)

Mammalian cells

Osteosarcoma cells (SAOS2) obtained from DSMZ

4.12 Software

Adobe Illustrator CS 2
Adobe Photoshop CS 2
AxioVision AC rel 4.5, Zeiss
BioDocAnalyze 2.0, Biometra GmbH
Cn3D 4.1, NCBI
Clustal X Multiple Sequence Alignment 1.83
Lumianalyst, Roche
Magellan 5, Tecan
MS Excel 2003, Microsoft
MS Word 2003, Microsoft
pDraw 32 1.0, Acaclone Software
Pymol 2005, DeLano Scientific LLC
SAPO Trace Viewer

4.13 Oligodeoxyribonucleotides

The Oligonucleotides listed in table 1 were either ordered at Biospring or MWG.

Table 1: Used Oligonucleotides

Oligonucleotide-name	Oligonucleotide sequence	Purpose
p21outNhe_f	TTT TTT GCT AGC ACT CTT GTC CCC CAG GCT GAG CCT CCC	pGL3-Insert
p21outXho_r	CCC CCC CTC GAG GTC TCC TGT CTC CTA CCA TCC CCT TCC	pGL3-Insert
FEL_f	CGA TGC CGT GCG CTT TGA GCT CCG CCA GAC CAT CTC	Quickchange
FEL_r	GAG ATG GTC TGG CGG AGC TCA AAG CGC ACG GCA TCG	Quickchange
FTL->AAA_f	GTG ATC GAT GCC GTG CGC GCT GCA GCC CGC CAG ACC ATC TCT TTT	Quickchange
FTL->AAA_r	GAA AAG AGA TGG TCT GGC GGG CTG CAG CGC GCA CGG CAT CGA TCA C	Quickchange
R279H_f	CTG CGT CGG AGG AAT GAA CCA CCG TCC AAT TTT AAT C	Quickchange
R279H_r	GAT TAA AAT TGG ACG GTG GTT CAT TCC TCC GAC GCA G	Quickchange
MI->QR_f	CGT GAG ACG TAC GAG CAG TTG CTG AAG CGC AAA GAG TCA CTG GAG	Quickchange
MI->QR_r	CTC CAG TGA CTC TTT GCG CTT CAG CAA CTG CTC GTA CGT CTC ACG	Quickchange
gammaDEL_PN_f	CAT TCC AAC CCC CCA AAC TAG TCC GTG TAC CCA TAG	Quickchange
gammaDEL_PN_r	CTA TGG GTA CAC GGA CTA GTT TGG GGG GTT GGA ATG	Quickchange
gammaDEL_RG_f	GAG CTT GTG GAG CCC TAG GGA GAA GCT CCG ACA CAG	Quickchange
gammaDEL_RG_r	CTG TGT CGG AGC TTC TCC CTA GGG CTC CAC AAG CTC	Quickchange
gammaDEL_RH_f	GAC GTC TTC TTT AGA CAT TAG AAC CCC CCA AAC CAC TCC	Quickchange
gammaDEL_RH_r	GGA GTG GTT TGG GGG GTT CTA ATG TCT AAA GAA GAC GTC	Quickchange
gammaDEL_TQ_f	GGA GAA GCT CCG ACA CAG TAG GAC GTC TTC TTT AGA C	Quickchange
gammaDEL_TQ_r	GTC TAA AGA AGA CGT CCT ACT GTG TCG GAG CTT CTC C	Quickchange
gamma_PTQS >PAQA_f	CGG GGA GAA GCT CCG GCA CAG GCT GAC GTC TTC TTT AGA	Quickchange
gamma_PTQS >PAQA_r	TCT AAA GAA GAC GTC AGC CTG TGC CGG AGC TTC TCC CCG	Quickchange
Tagamma wo Helix_f	CAT GCA GTA CCT CCC TCA GCA CTG CTT CAG GAA TGA GCT TGT G	Quickchange
Tagamma wo Helix_r	CAC AAG CTC ATT CCT GAA GCA GTG CTG AGG GAG GTA CTG CAT	Quickchange
RT-Osteoc_f	GGG GGG ATG AGA GCC CTC ACA CTC CTC	RT-PCR
RT-Osteoc_r	TTT TTT GCC GTA GAA GCG CCG ATA GGC	RT-PCR
RT-Coll_f	TTT TTT GGA CAC AAT GGA TTG CAA GG	RT-PCR
RT-Coll_r	GGG GGG TAA CCA CTG CTC CAC TCT GG	RT-PCR
RT-Osteop_f	GGG GGG CAT CTC AGA AGC AGA ATC TCC	RT-PCR
RT-Osteop_r	TTT TTT CCA TAA ACC ACA CTA TCA CCT	RT-PCR
RT_VDR_f	TTT TTT CAG TAA CAG GTT GCG ACG GAG	RT-PCR
RT_VDR_r	TTT TTT GAT GAT TAT AGG TGC GGA TAC	RT-PCR
RT_p21_f	TTT TTT AGG CGC CAT GTC AGA ACC GGC	RT-PCR
RT_p21_r	TTT TTT GGA AGG TAG AGC TTG GGC AGG	RT-PCR

4.14 Vectors

4.14.1 pBH4

The pBH4-Vektor was used for the expression of the ODΔQPSAM-construct. It is an expression vector with a T7-Promotor, an ampicillin resitance gene and a N-terminal His-Tag.

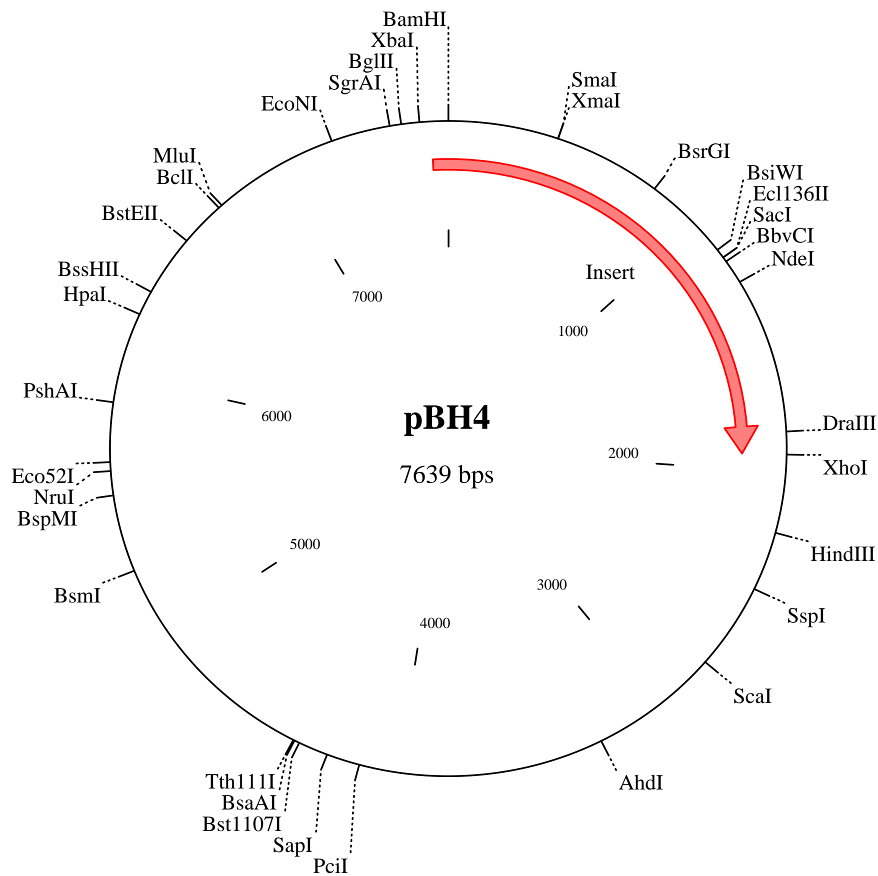

Figure 8: pBH4-Vector

The gene for the expressed ODΔQPSAM construct has been inserted between the restriction sites for *BamH*I and *Xho*I. For expression *E.coli* BL21 were used.

4.14.2 pcDNA3

The pcDNA3-vector is a vector for expression in mammalian cells. A myc-tag is encoded N-terminal to the inserted protein.

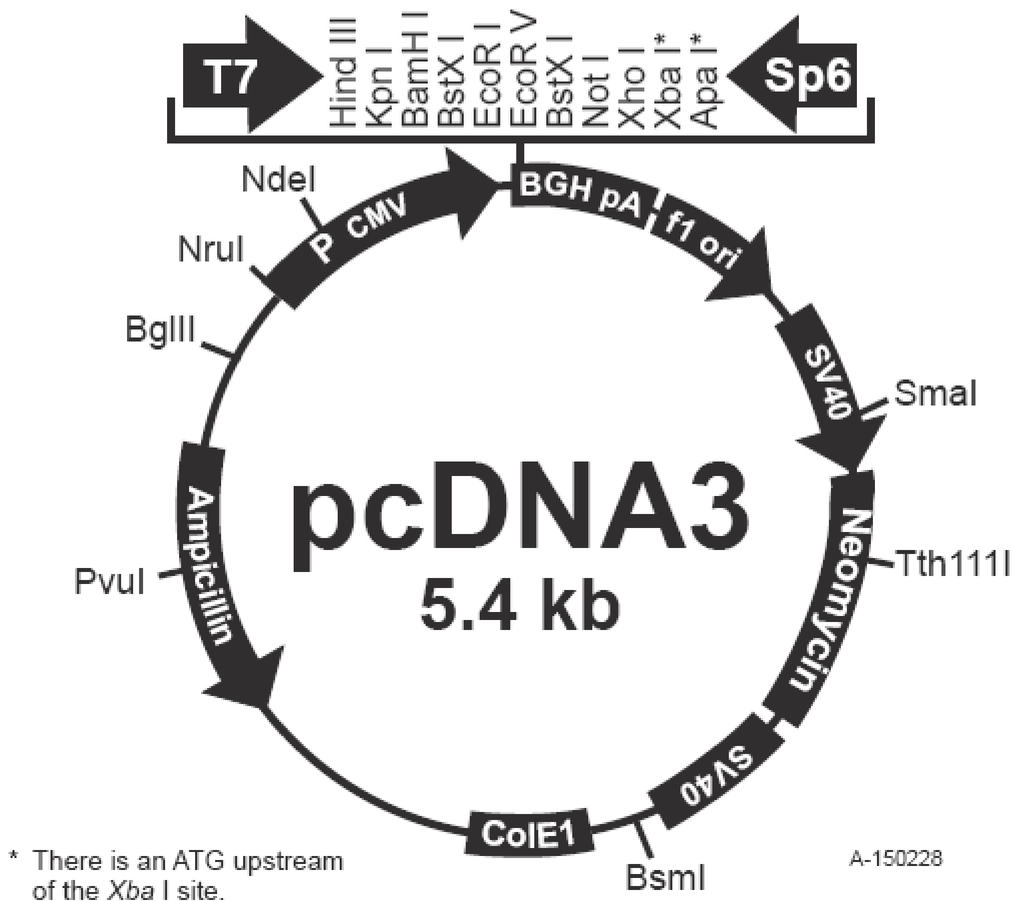

Figure 9: pcDNA3-Vector [taken from ref. 83]

The genes for the p63 constructs were inserted between the restriction sites *Xho*I and *Xba*I. The pcDNA3 constructs, which were not cloned with the Oligonucleotides (the FTL->AAA primer listed was used for the ΔNp63α construct) listed in 4.13 were cloned by Dr. Wesley McGinn-Straub.

4.14.3 pGL3

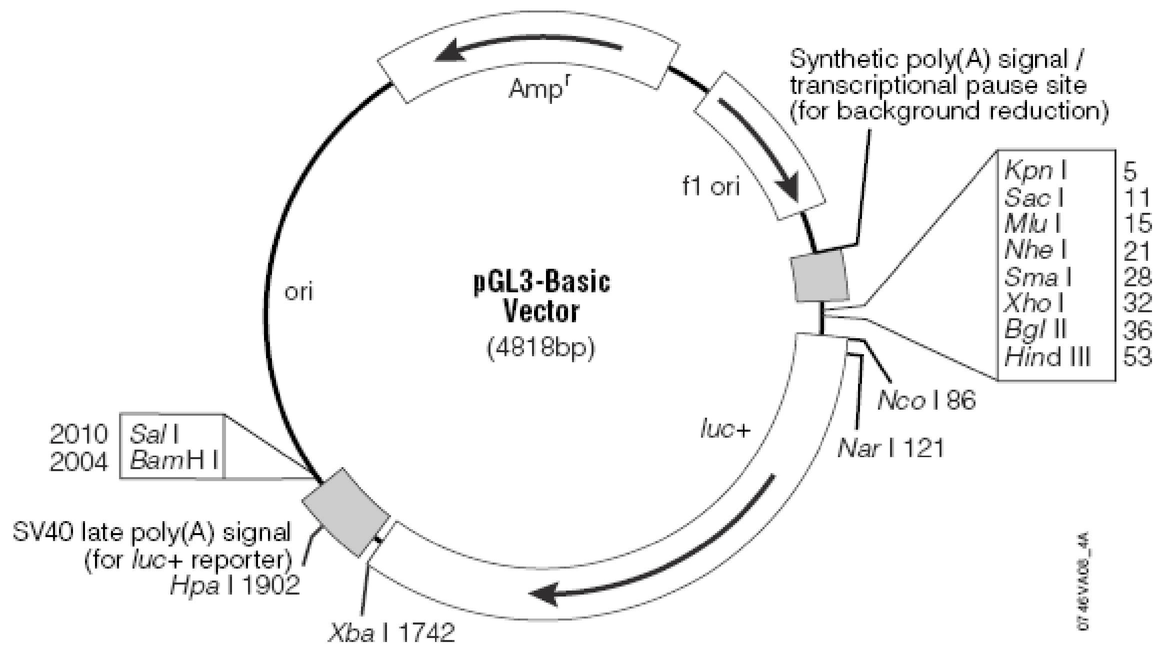

Figure 10: pGL3-Vector [taken from ref. 84]

The promoters of *p21* and *bax* have been inserted between the restriction sites *Nhe*I (*p21*) / *Sma*I (*bax*) and *Xho*I. The pGL3 vector containing the *bax* promoter was cloned by Dr. Horng Der Ou.

4.14.4 pRLCMV

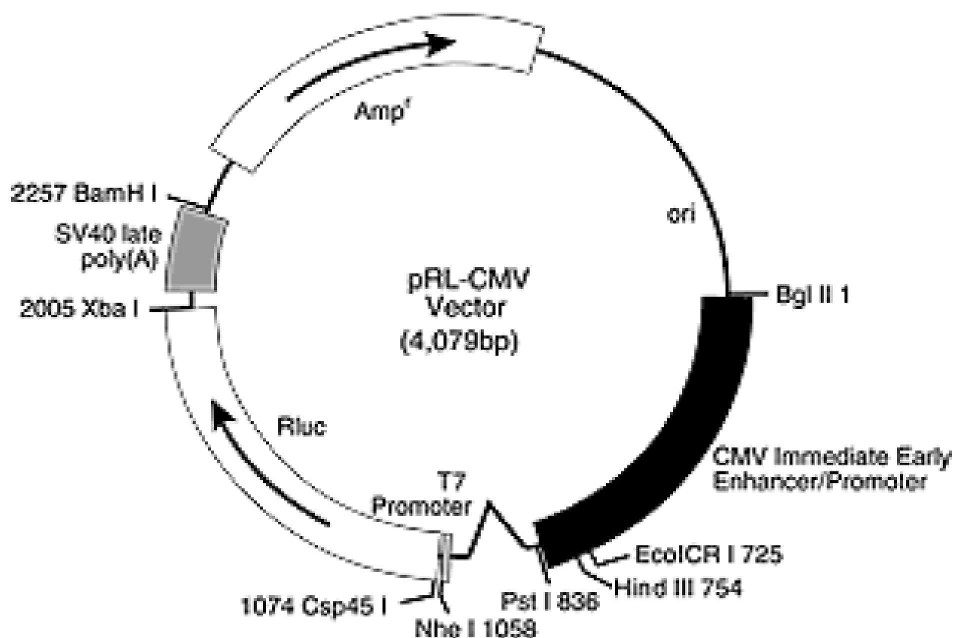

Figure 11: pRLCMV-Vector [taken from ref. 85]

5. Methods

5.1 Molecular biology methods

5.1.1 Obtaining chromosomal DNA from mammalian cells

For the pGL3 construct containing the *p21* promoter sequence the corresponding DNA sequence had to be obtained by PCR out of chromosomal DNA [86]. For this purpose 5 X 10^4 SAOS2 cells were lyzed in 50 µl Buffer D. The mixture was incubated for 55°C for 15 min, vortexed, and incubated again for 15 min at 55°C. The final volume was brought to 500 µl with ddH_20 and the sample was incubated for 5 min at 95°C. 1 µl of this sample was used as template for PCR.

5.1.2 Polymerase Chain reaction (PCR)

Defined DNA-fragments can be amplified by using the PCR. In a first step the DNA double strand (template) is denaturated, to make the second step possible where oligonucleotides as primers can anneal to their complementary sequence within the template. It is possible to use chromosomal DNA, plasmid DNA or short DNA fragments as a template. After annealing of the primers the 3'-OH ends get extended by the thermostable DNA-Polymerase of choice. The Vent Polymerase was used, if the template has been a plasmide, the Phusion Polymerase has been used, if the template has been chromosomal DNA, according to manufacturer's instructions. The length of the newly synthesized DNA double strand depends on the time fort the extension. After the extension time, denaturing of the DNA starts again and the new double strands act as templates in the new cycle. In this way it is possible to get a huge amount of a defined DNA fragment. The used amounts of each component in a standard PCR reaction are listed below.

PCR	
50	µl total volume
1	µl forward primer [10µM]
1	µl reverse primer [10µM]
1	µl DNA Polymerase [2 U / µl]
1	µl template-DNA (50 ng)
1	µl dNTPs [10mM]
5	µl 10x DNA-Polymerase Buffer
40	µl ddH_20

The theoretical annealing temperature of the primers depends on the content of Guanine and Cytosine basepairs and the length and can be calculated by the following equation.

$$T_M = [69,3 + 0,41 \cdot (\%GC)] - \frac{650}{n} \qquad \textbf{(Eq. 1)}$$

$$T_A = \left[\frac{(T_{M1} + T_{M2})}{2} \right] - 3 \qquad \textbf{(Eq. 2)}$$

T_M : Melting temperature of the oligonucleotides [°C]

T_A : Annealing temperature [°C]

%GC : GC-content of the oligonucleotides %

n : Number of nucleotides within the oligonucleotide

The used standard PCR programme is shown below. All steps can differ in time and for the annealing step (below at 62°C) also the temperature can differ.

PCR programme:

°C	sec	
95	60	
95	45	--
62	45	| 35x
72	60	--
72	300	
4	∞	

PCR purification and gel extraction were performed using the Qiagen QIAquick PCR Purification Kit and QIAquick Gel Extraction Kit according to manufacturer's instructions.
The PCR-product can be used in different ways i.e. for ligation.

5.1.3 Agarose gel electrophoresis

This method was used to separate different DNA-fragments by size. 1% (w/v) Agarose were diluted in 1 X TAE-buffer and heated in the microwave until the solution becomes clear. At a temperature of approximately 60°C 2 µl of ethidiumbromide (98 %) were added to 50 ml agarose solution and the solution was put into a gelbox to polymerize. Afterwards the gel was covered with 1 X TAE-buffer in a chamber, the DNA + loading buffer was loaded and the voltage was set to 100 V for 60 min. The DNA now gets separated because of the negative charge and small fragments move faster inside the gel than big ones. The DNA bands get visible by treatment with UV-light because of the ethidium bromide and the bands corresponding to the right products can be cut out of the gel with a scalpel.

5.1.4 Digestion of DNA with restriction endonucleases

Restrictionendonucleases are used for specific cutting of dsDNA and normally cut at a specific palindromic DNA-sequence. Every digestion was carried out with restriction enzymes from New England Biolabs according to the manufacturers instruction.

5.1.5 Ligation

After PCR amplification of the gene of interest with gene-specific primers containing overhanging ends with restriction enzyme recognition sequences, the PCR product was purified and digested with the appropriate restriction enzymes.

The vector backbone was also digested with restriction enzymes. Backbone and PCR products were separated by agarose gel electrophoresis and extracted from the gel with the help of the QIAquick gelextraction kit. PCR product and backbone were ligated. Ligations were performed in 20,5 µl, using the T4-DNA-ligase from NEB according to the manufacturer's instructions. The molar ratio of insert to vector was 1:1 or 2:1 with a maximum amount of 50ng vector DNA. The ligation was performed overnight at 4°C and 10 µl were transformed the next day into bacteria. After overnight incubation at 37°C, colonies were picked, and grown in 6ml liquid culture overnight. The next day, plasmid DNA was purified and analyzed by restriction digest for insertion of the PCR product into the vector backbone. Positive clones were submitted for sequencing.

5.1.6 Mutagenesis

Mutagenesis was carried out with the QuikChange® Site-Directed Mutagenesis Kit from Stratagene. A Quickchange (QC) in general works like a normal PCR, but within the primers there are some mismatching nucleotides to the complementary DNA sequence of the template. Due to this fact the annealing becomes more complicated and the primers for this mutagenesis reaction are normally longer than non mismatching primers. The PCR reaction itself is very similar to the normal PCR-reaction and just has a longer annealing time. And the reaction mixture is digested by *Dpn*I after the PCR is completed to get rid of the methylated wild type template DNA so only the modified DNA gets transformed later on. The used amounts of each component for standard QC are listed below.

Quickchange PCR	
25	µl total volume
0,5	µl forward primer [10 µM}
0,5	µl reverse primer [10 µM]
0,5	µl Pfu Turbo DNA-Polymerase [2.5 U / µl]
0,5	µl template-DNA (25 ng)
2,5	µl 10x Pfu buffer
0,5	µl $MgSO_4$ [100 mM]
0,5	µl dNTPs [10 mM]
19,5	µl ddH_20

The used standard QC-PCR programme is shown below. All steps can differ in time and for the annealing step (below at 62°C) also the temperature can differ even more than for a normal PCR (between 50 and 68 °C).

QC PCR programme:

°C	**min**	
95	2	
95	1	--
62	2	| 24x (16+1 per mutated nucleotide)
72	16	--
72	10	
4	∞	

After the completion 0,5 µl *Dpn*I [20 U / µl] were added to the reaction and incubated for 1 h. Afterwards transformation into *E.Coli* DH5α-cells was performed.

5.1.7 Transformation

For the synthesis of any plasmide DNA 100 µl of competent *E.Coli* DH5α-cells [Optical density = 11] were transformed with 1 µg DNA (Mini or Midi preparation) or 10 µl Quickchange or ligation reaction mix and incubated for 30 min on ice. Then the cells were incubated for 45 s at 42°C (heatshock) and afterwards kept on ice for 2 min. If the transformed DNA has been out of a Mini or Midipreparation the cells were plated out at this point on agar plated containing an antibiotic corresponding to the transformed plasmid. For QC or ligation DNA 100 µl SOCS media were added now and the cells were incubated for 30 min at 37°C. Then they were centrifuged for 5 min at 5000 g and resuspended in 100 µl LB media. The mixture was plated out on agar plates containing an antibiotic. The plates were incubated for 10-16 h at 37°C. Colonies on the plates were picked for DNA-preparation and analysis and grown in 6 ml LB media containing the antibiotic corresponding to the transformed plasmid i.e. 1 mg ampicillin per ml media.

5.1.8 Isolation of plasmid DNA (Mini-/Midi-Preparation)

For isolation of DNA by Mini preparation 6 ml of an overnight culture of plasmid containing bacteria were harvested by centrifugation at 5000 g for 10 min and the Mini preparation kit of Macherey Nagel was used according to the manufacturers instruction.

Midi preparation was performed by using the Endotoxin-free Qiagen Kit. All steps of the protocol were carried out according to the manufacturers instruction but the used volumes of buffers were modified in the following way.

Resuspension (Step 4) = 4 ml P1
Lysis (Step 5) = 4 ml P2
Neutralization (Step 6) = 4 ml P3
Endotoxin removal (Step 9) = 1 ml ER
Equilibration (Step 10) = 4 ml QBT
Washing (Step 12) = 2 X 10 ml QC
Elution (Step 13) = 5 ml QN
Precipitation (Step 14) = 3.5 ml isopropanole
Washing (Step 15) = 2 ml Ethanol (70 %)

5.1.9 Determination of DNA-concentration

The DNA concentration was determined by measurement at a Nano drop. The purity of the DNA was analyzed by use of the measured spectra and the ratio of the absorption at 260 and 280 nm.

5.1.10 DNA-Sequencing

DNA sequencing was performed by the Research & Development GmbH in Oberursel. Plasmid DNA and primers were submitted at the suggested concentration and volume.

5.1.11 Reverse Transcriptase PCR

Reverse Transcriptase PCR was performed with the Cells-direct RT-PCR Kit (Invitrogen) according to the manufacturers instruction. The primers for PCR reaction are listed in Table X.

5.2 Protein chemistry

5.2.1 Protein expression

For the expression of the ODΔQPSAM construct the pBH4 vector was used. *E.coli* BL21 (DE3 rare) were transformed with 1 µl of a Mini DNA preparation and plated out on an agar plate with ampicillin and chloramphenicol antibiotics (100 µg / ml]. A colony was picked for a 6 ml overnight preculture of LB-Media with the antibiotics [100 µg / ml]. This culture was centrifuged and resuspended in LB Media and put into 500 ml of expression media (2XYT) containing ampicillin [100 µg / ml]. Afterwards the culture was grown at 37°C and 180 rpm to an OD_{600} of 0,8 and induced with 500 µl of 1M IPTG. After incubation over night at 22°C and 180 rpm the culture was centrifuged at 5000 g for 10 min. The cell pellet was resuspended in 20 ml cell lysis buffer (4°C). The cells were incubated with lyzozyme for 15 min on ice and were lysed by sonification (3 x 1 min). Then the lysate was centrifuged at 50.000 g and the supernatant was kept for purification.

5.2.2 Purification by Ni-NTA

The supernatant was filtered and loaded on a Ni-NTA FF column connected to an Äkta system. The Histidine side chains (His-tag) bind to the Nickel resin and proteins were purified by washing with buffer containing an Imidazole concentration up to 100 mM. The elution buffer for the protein contains 250 mM Imidazole. The collected fractions were analyzed by SDS-PAGE and dialyzed to get rid of the Imidazole.

5.2.3 Size exclusion chromatography (Gelfiltration)

Proteins can be separated by size using a gelfiltration column. Also different oligomeric states can be analyzed by gelfiltration. Big molecules move faster to the column than small ones. By calibration with molecules of known sizes even the mass of a molecule can be determined approximately or the oligomeric state. For analysis of the oligomeric state of a wt ODΔQSAM and a mutant ODΔQPSAM construct a Superdex 75 column was used, which had been equilibrated before with two column volumes lysis buffer. The protein samples had a volume of 5 ml, the flow rate was 1 ml/min. The eluate of the peak fractions was analyzed by SDS-PAGE.

5.2.4 Determination of protein concentration

The protein concentration was determined by measurement at a Nano drop. The purity of the protein was analyzed by use of the measured spectra.

5.3 Analytical methods

5.3.1 SDS-Polyacrylamidgelelectrophoresis (SDS-PAGE)

Proteins are separated by size by SDS-Polyacrylamidgelelectrophoresis. The proteins get denaturated and negatively charged because of the detergent Sodiumdodecylsulfat (SDS). Approximately one SDS-molecule binds to 2 amino acids. The protein samples for analysis of the protein expression were analyzed on 12% SDS gels at 200 V for 60 min, stained with Coomasiee, destained and scanned.

For samples obtained from mammalian cells SDS-polyacrylamid-gelelectrophoresis was performed using the NuPAGE Novex Gel system from Invitrogen with purchased NuPAGE 4-12% BisTris gels. Protein samples were

mixed with 20 µl 4X sample buffer containing 20 % β-ME, incubated for 5 min at 95°C, and centrifuged for 1 min at 13000 g. The Gels were run in 1x MOPS buffer (Invitrogen) at 200 V for 50 min.

5.3.2 Western Blot (WB)

Proteins were transferred from the gel to a Polyvinylidene fluoride (PVDF) membrane using a wet-blot method from Invitrogen according to the manufactures instruction. Proteins were transferred at 30V for 90 min at room temperature (transfer buffer 4°C at beginning). After the transfer the membrane was blocked in TBST containing 5% milk for 30 min. After that, the membrane was incubated with TBST containing 5% milk and the primary antibody at the concentration suggested by the manufacturer overnight at 4°C. The membrane was washed 3 times for 10 min with 50 ml TBST and incubated in TBST containing 5% milk and the secondary antibody at the concentration suggested by the manufacturer for 1h at room temperature. The membrane was washed 3 times for 10 min with 50 ml TBST and then the incubated 1 min in 6 ml ECL1 followed by 1 min ECL1 + 6 ml ECL2 for detection of chemiluminescence. The membrane was exposed in a Ilumimeter and the obtained data was analyzed. The strength of the signals was analyzed quantitatively using the BioDocAnalyze 2.0 software.

5.4. Cell biology methods

5.4.1 Cell culture

SAOS2 cells, obtained from the ATCC, were cultured in Dulbecco's modified Eagle's media (DMEM) with 10% fetale bovine serum, 1 % penicillin-streptomycin [100 U / ml] and 1 % L-Glutamine [200 mM], at 37°C under an atmosphere of 5% CO_2. For experiments 96 well plates were used, the cell concentration per ml was

7 X 10^4 / ml cell, if the experiment was performed the next day after seeding, otherwise 5 X 10^4 / ml cells for experiments 2 days after seeding.

5.4.2 Immunofluorescence

For immunofluorescence, SAOS2 cells were plated on 12mm coverslips and grown overnight. 24 h after transfection the media was aspirated and cells were fixed with 3% formaldehyde in PBS for 8 min at room temperature. Then, cells were washed 4 X with PBST (PBS/0.1% Triton-X100) and blocked with 3% milk in PBST for 30 min at room temperature. Cells were incubated with 100 µl 3 % milk in PBST containing the Cy3 c-myc-specific antibody 9E10 (light sensitive) at the concentration suggested by the manufacturer for 1h at room temperature, followed by 3 washing steps with PBST and incubation with secondary antibody for 30 min at room temperature. After 3 additional washing steps the DNA of the cells was stained with fluorochrome Hoechst 33278 diluted in PBST to 1µg/ml for 1 min. The cells were washed again 2 times with PBST and coverslips were mounted onto microscope slides using small amounts of mounting media and sealed with nail polish. Pictures were taken on an Zeiss Axiovert 200 microscope connected with a Zeiss Axiocam MrM. Two different filters were used, one for the Hoechst Stain signal (DAPI) and one for the Cy3 signal (Cy3).

5.4.3 Microscopy

Cells were observed during cultivation and pre-experimental at the light microscope Axiovert 40 CFL.

5.4.4 Transfection

The transactivation experiments were performed in SAOS-2 cells using the Promega Dual-Glo™ Luciferase reporter assay. The used promoter pGL3 vector was created before and carried either a single copy of the *p21* or the *bax* promoter. Cells were transfected with 133 ng DNA per plasmid, using the Effectene system from Qiagen [Fig. 12]. Transfections were carried out in 96 well plates according to the protocol [appendix], cells were grown for 24 hours and assayed for Renilla and Firefly luciferase activities or western blot. In the titration assays, the concentration of *TAp63γ* was kept constant at 62 ng per assay and the amount of *ΔNp63α* plasmid was varied from a ratio of 1:5 to 5:1. The total amount of DNA transfected was kept constant with pCDNA3.1 vector. Experiments were performed as three independent experiments each in triplicate.

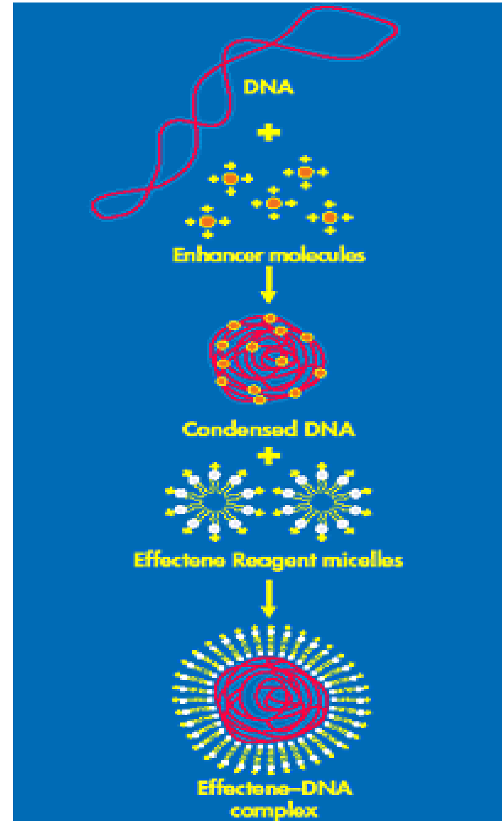

Figure 12: Effectene Transfection [taken from ref. 87]. Plasmid DNA gets condensed by Enhancer molecules and afterwards micelles are formed via Effectene. The formed complexes get into the cells by endocytosis.

5.4.5 Transactivation assays

For the transactivation experiments the Promega Dual-Glo™ Luciferase reporter assay was used according to the manufacturers instruction. The scheme how it works is shown in Fig. 13. The transcriptional activity obtained by these experiments was the relative activity (of the analyzed constructs to TAp63γ).

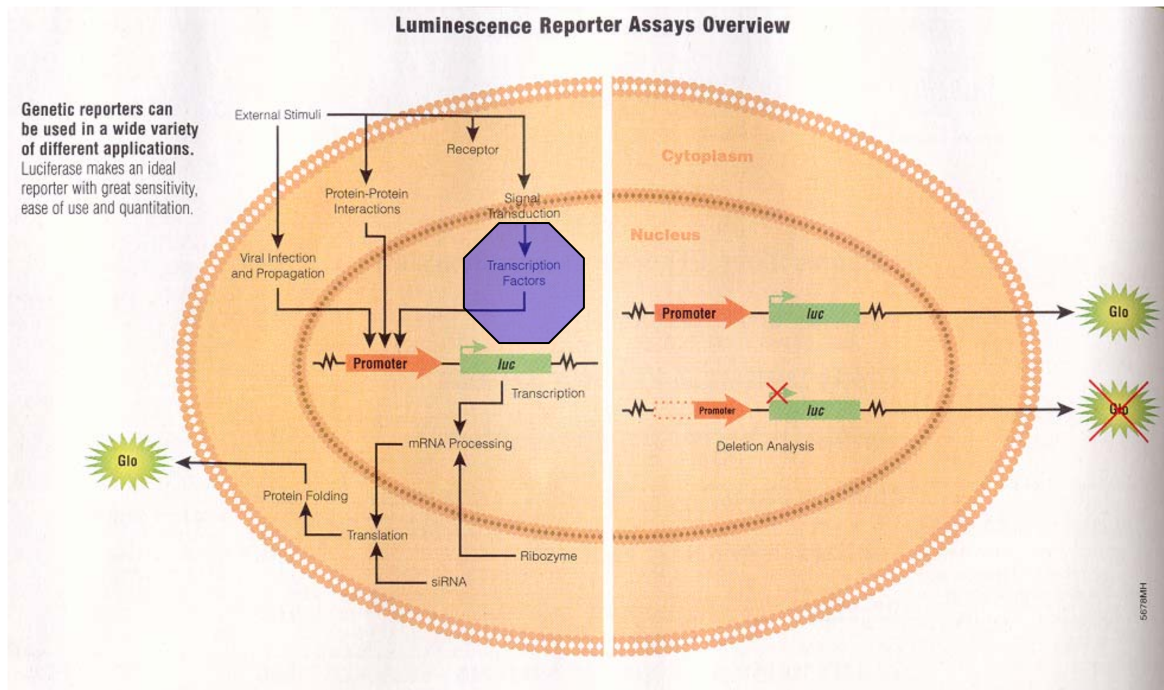

Figure 13: Luminescence Reporter Assays Overview [taken from ref. 84, modified]. In blue the starting point for the carried out experiments is shown. As a transcription factor p63 binds to the promoter sequence in the cotransfected pGL3 vector. The more transcriptional activity the p63 construct has, the more firefly luciferase gets transcripted and the more signal is measured. Background transcriptional activity is obtained by measurement of a second signal obtained by the constitutive transcription of the renilla luciferase gene in the pRLCMV vector.

The vector containing the gene of the protein was a pcDNA3 vector. A pGL3 vector with either a *p21* or *bax* promoter in front of the firefly luciferase gene was used a reporter construct and the background vector has been the pRLCMV vector with a common promoter in front of the renilla luciferase gene.

5.4.6 Harvesting mammalian cells for WB analysis

The cells, which were transfected in the same way as for transactivation assays, were harvested using Accutase (PAA) and centrifuged at 750 g at 4°C. The Cells were lysed using the mammalian protein extraction reagent mixture (Pierce). The supernatant was removed and 23 µl of the reagent was used for lysis of the pellet with 5 min incubation. After that the procedure has been according to 5.3.1.

6. Results

6.1. Functional analysis of the C-terminus of TAp63α

6.1.1 Mutational analysis of the TID

A mutational analysis of the C-terminus of TAp63α was carried out. The transcriptional activity on the *p21* promoter of several constructs with multiple alanine mutations was analyzed by luciferase assays. The results are shown in Fig. 14.

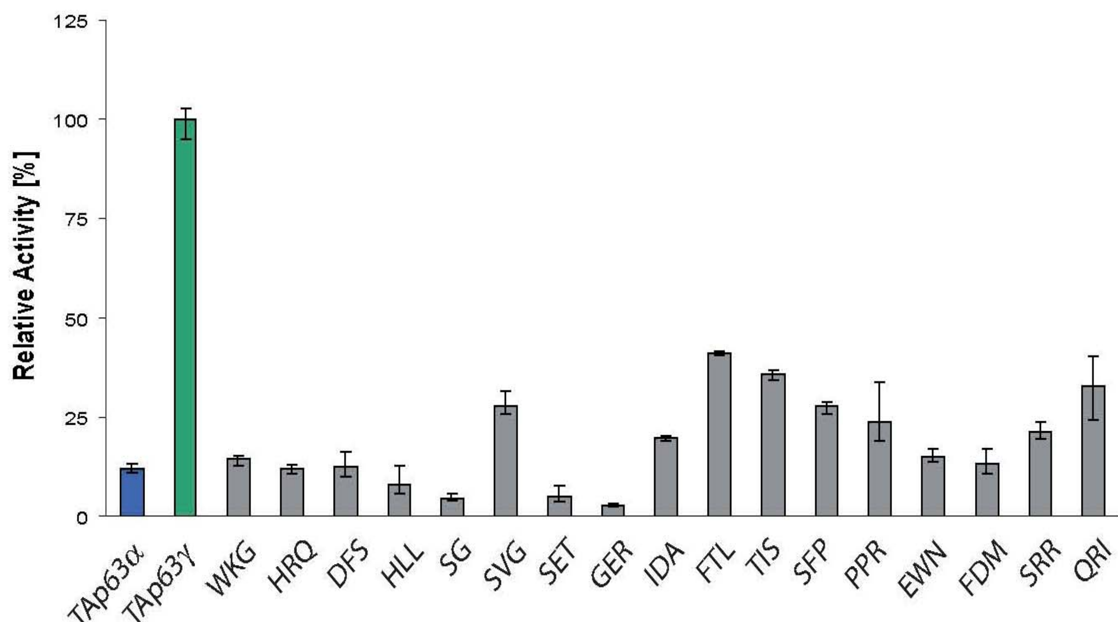

Figure 14: Transcriptional activities on the *p21* promoter of different TAp63α constructs transfected into SAOS2 cells. The activities are shown relative to the wild type of TAp63γ (green), the wt of TAp63α is shown in blue. The activities were measured by luciferase transactivation assays. The indicated amino acids were mutated to alanine [taken from ref. 53].

Most of the constructs with a significantly increased transcriptional activity compared to the wild type TAp63α (TAp63α.wt) in blue are in the region starting with the amino acid F605 and ending with R616. In this region also lies a stretch of conserved amino acids shown in Fig. 6. The other constructs showing significantly increased transcriptional activity are SVG ($TAp63\alpha.^{SVG>AAA}$) and QRI ($TAp63\alpha.^{QRI>AAA}$). The increased activity of QRI is a consequence of affected sumoylation and has been demonstrated before [15, 53]. In the presented

diagram the transcriptional activities are not normalized to the intracellular protein concentration of each construct. To distinguish between protein concentration and other effects, western blot analysis was carried out for each analyzed construct to correlate (normalize) the transcriptional activities to the protein concentrations. The corresponding Western Blots are shown in Fig. 15.

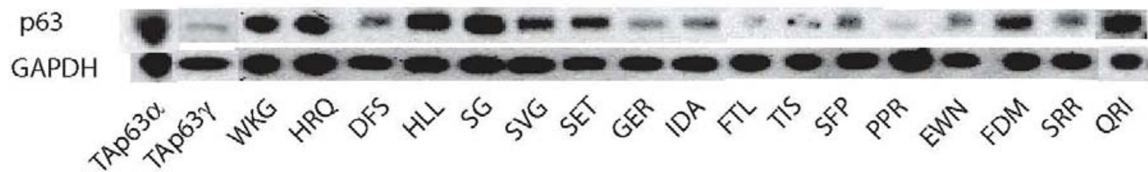

Figure 15: Western Blots of the analyzed TAp63α constructs and TAp63γ. The signal for the Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been used as loading control to compare the p63 signals to each other [taken from ref. 53].

It can be observed (with of SVG and QRI) that the constructs with increased transcriptional activity show low concentrations inside the cells. Normalization of the transcriptional activities by this western blot data leads to the normalized transcriptional activities outlined in Fig. 16.

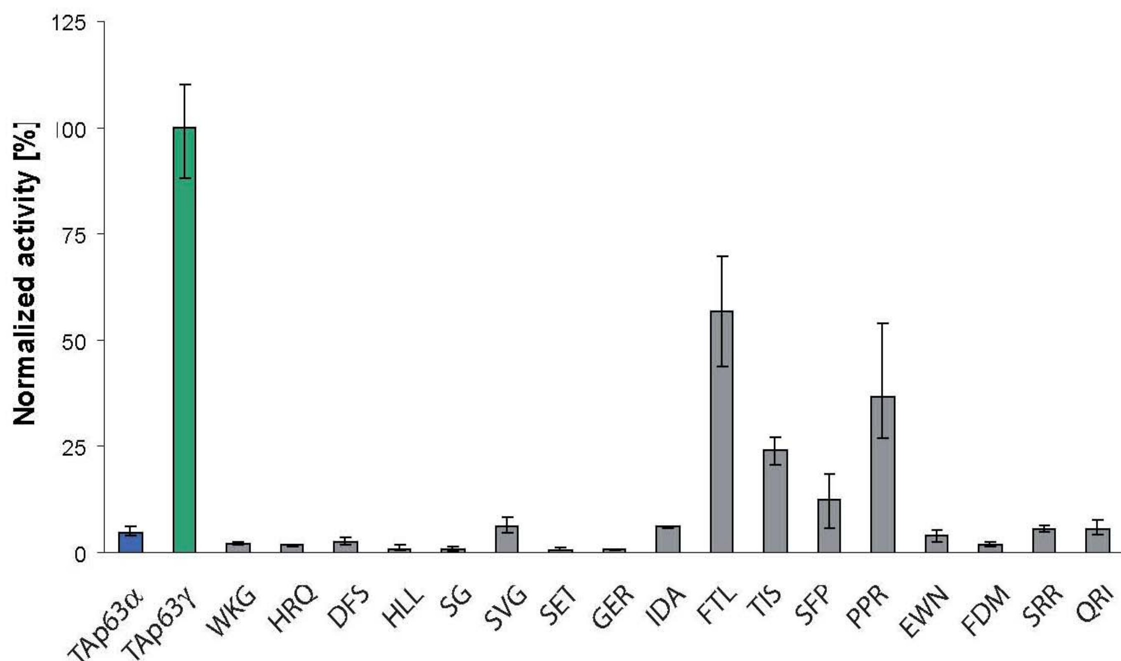

Figure 16: Transcriptional activities of the TAp63α constructs relative to the wt of TAp63γ and normalized to intracellular protein concentration [taken from ref. 53].

It can be seen, that after consideration of the intracellular protein concentrations the constructs with mutated amino acids in the region between F605 and R616 show a significantly higher intrinsic transcriptional activity. Compared to the other regions some amino acids in this region are also highly conserved [Fig. 6]. The other constructs appearing more active in the relative transcriptional activity measurements [Fig. 14] show transcriptional activity levels similar to the wt after normalization. To investigate, if the effect of higher transcriptional activity in the identified stretch of amino acids is independent from regulation mechanisms, like sumoylation, additional experiments were carried out as described below.

6.1.2 Effects of a mutated sumoylation site in TAp63α

Pulldown experiments done previously had reported that the last 25 amino acids, including the sumoylation site are not important for binding of the TA-domain [15]. To further elucidate this sumoylation-independent way of regulating TAp63α and to confirm the sumoylation-independence of the previously identified region the consequences of an affected sumoylation site was investigated in different ways. In one study the acceptor lysine K637 of TAp63α was mutated into leucine (TAp63α.K). In the second study the three amino acids in front of the acceptor lysine have been mutated into alanines (QRI). The consequences of these mutations at the relative and normalized transcriptional activity level on the *p21* promoter are shown in Fig. 17.

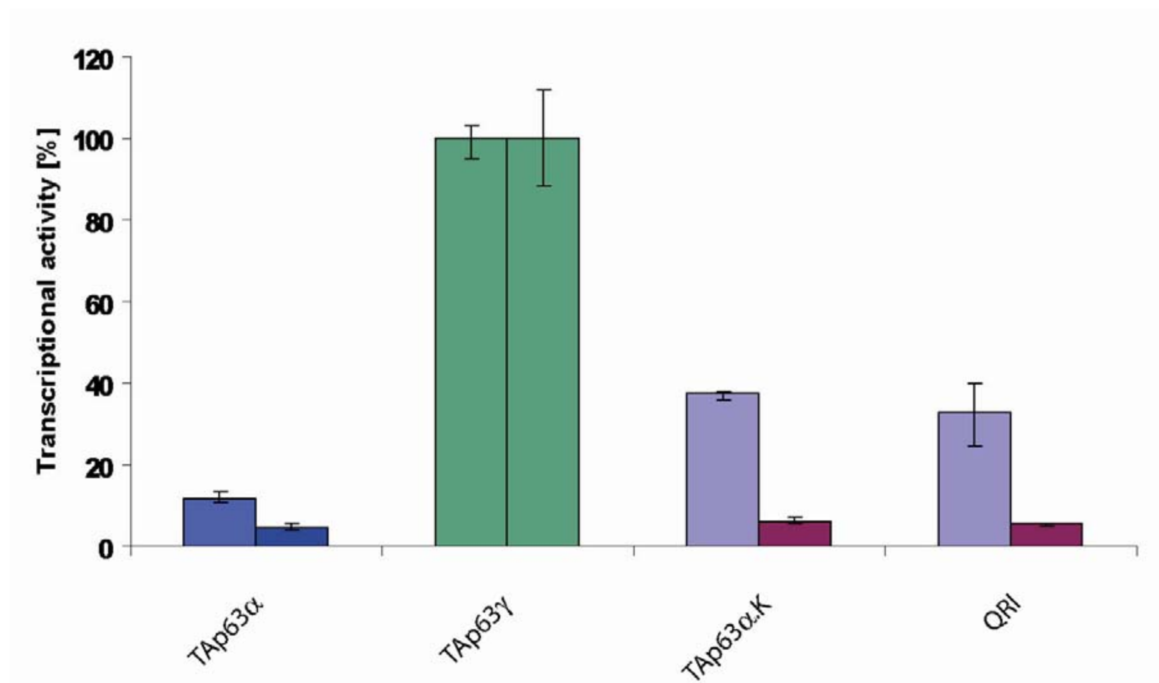

Figure 17: Consequences of an affected sumoylation site in TAp63α compared to TAp63α.wt (dark)blue and TAp63γ.wt (green). The first series shows the relative transcriptional activity measured and the second the normalized transcriptional activity [taken from ref. 53].

Figure 17 shows that both mutations have the same effect on the relative and normalized transcriptional activity of the protein. The higher relative activity compared to the wild type is just due to a higher protein concentration. After normalization the transcriptional activities for both constructs are the same as for the wild type. In the case of TAp63α.wt the mutation of the sumoylation site leads to higher transcriptional activity due to higher intracellular protein concentrations.

6.1.3 Independence of intrinsic and sumoylation dependent activity

To test the effect of higher intracellular protein concentrations and to proof that the two observed implications on transcriptional activity are independent from each other an additional mutational analysis with other constructs was carried out. In these construct an additional mutation was introduced to the TAp63α constructs shown in Fig. 7. Added to the mutation in the TI-domain also the acceptor lysine of the sumoylation site (K637) was mutated to leucine. The relative transcriptional activity on the *p21* promoter for all of these constructs was

measured and the resulting values are shown in Fig. 18.

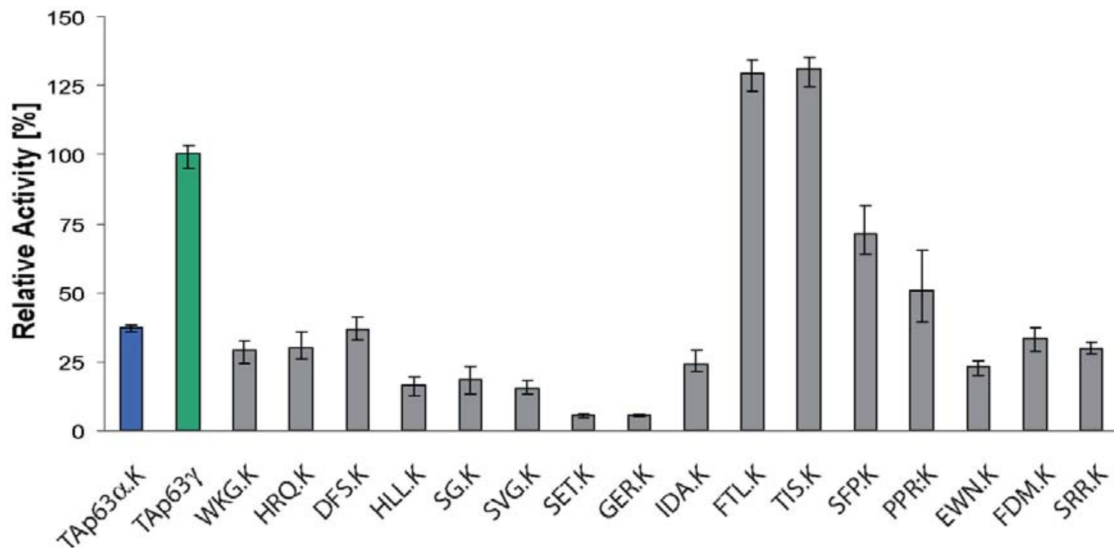

Figure 18: Effects of an affected sumoylation site on the TAp63α constructs. In blue TAp63α with the single mutation K637L, in green TAp63γ.wt [taken from ref. 53].

It can be seen, that those constructs which showed the highest transcriptional activity in the previous experiments increase even more in their relative transcriptional activity with the additional mutation of K637L. The other mutants are as or less transcriptionally active than the construct with the single mutation K637L. When compared to the results of the experiment shown in Fig. 14, the overall increase in transcriptional activity seems to be just an effect of higher protein concentration and not of an altered intrinsic transcriptional activity. To test this hypothesis western blots for all constructs were carried out in the same manner as before. The western blots are shown in Fig. 19.

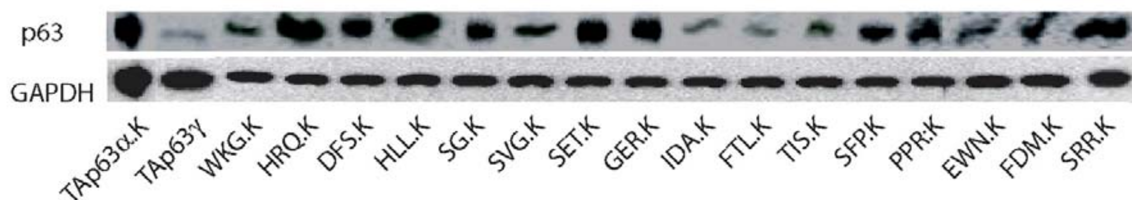

Figure 19: Western Blots of the TAp63α.K constructs and TAp63γ.wt, which have been analyzed in the transactivation assays [taken from ref. 53].

The protein concentration in general is increased when compared to the data in

Fig. 15. Again the most active constructs show relatively low intracellular protein concentrations. The change in protein level for each construct is shown in Fig 20.

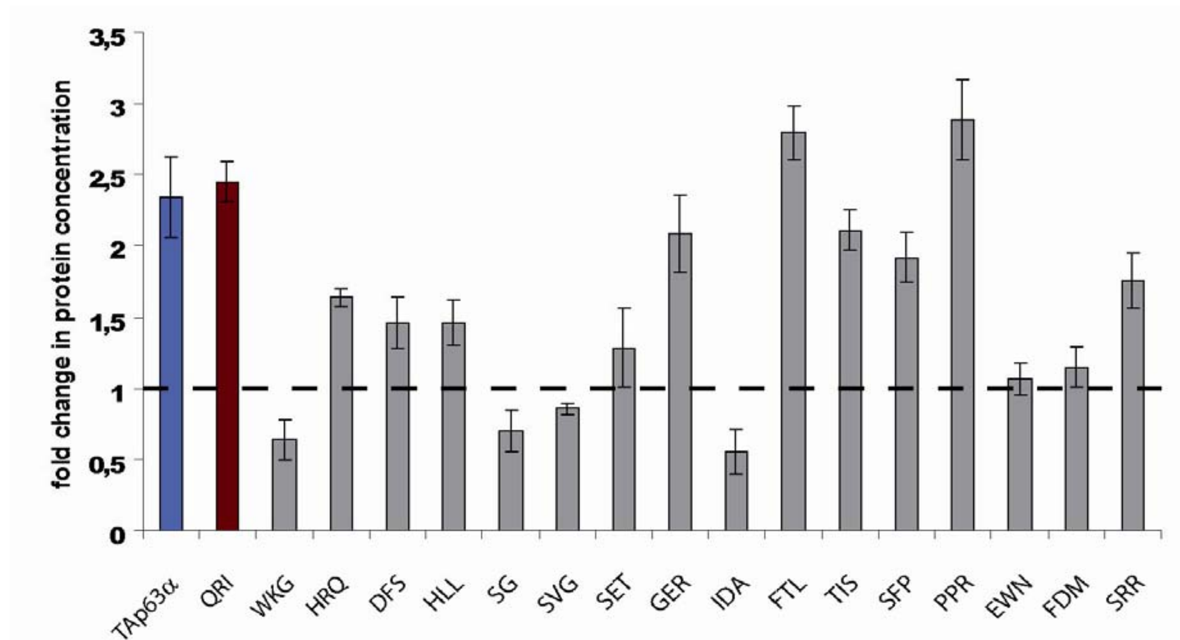

Figure 20: Fold change in protein concentration due to an additionally mutated sumoylation site for each analyzed TI-mutant construct of TAp63α. Constructs, where only the sumoylation site is mutated are indicated in blue (K637L) and red (QRI) [taken from ref. 53].

Most constructs show elevated intracellular protein concentrations after mutation of the acceptor lysine K637 into leucine. The fold change for the construct carrying only this single mutation (blue) is similar to the construct QRI (red), where the receptor lysine remains intact. The normalized transcriptional activities after including the protein concentrations are shown in Fig 21.

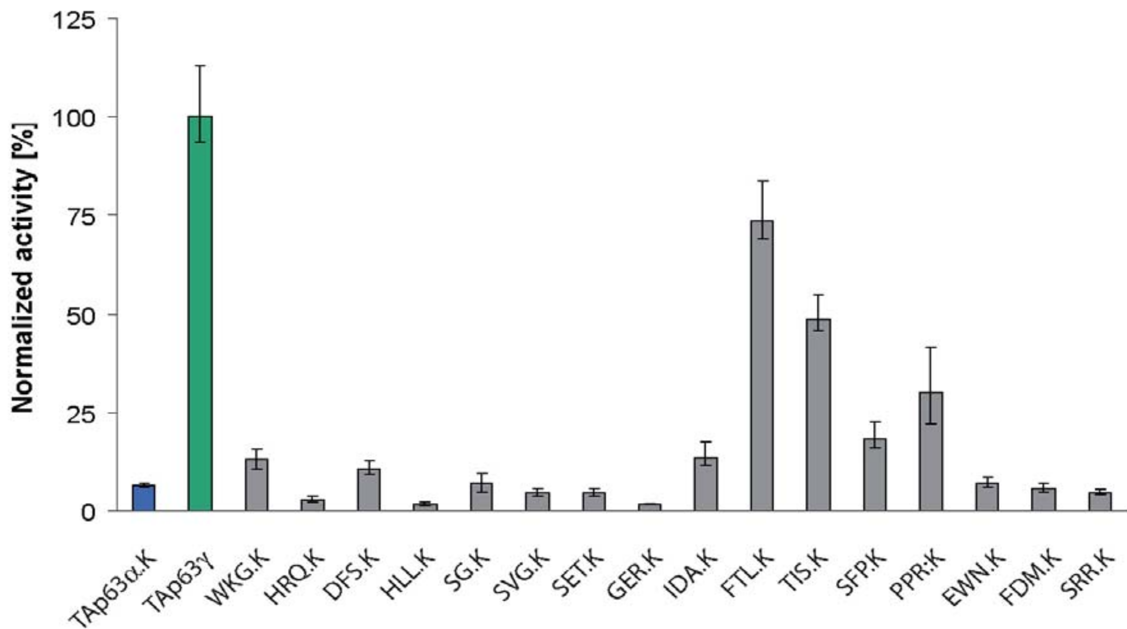

Figure 21: Transcriptional activities of TAp63α constructs with an additional mutation at the sumoylation site (.K). Activities are relative to TAp63γ.wt and normalized to protein concentrations. The single mutation mutant is indicated in blue and TAp63γ in green [taken from ref. 53].

The normalized activities of the mutants show a similar pattern as the normalized activities of the mutants without the additional mutation K637L. Therefore there are two independent regulatory functions located at the C-terminus of TAp63α. One of them is based on sumoylation and influences the intracellular protein concentration. The other one seems to be involved in binding and masking of the TA-domain and the important residues are located in the highly conserved region beginning with F605.

6.1.4 High transcriptional activity is connected to low intracellular protein levels

It is obvious that the TAp63α-constructs which carry mutations between F605 and R616 show high transcriptional activity while they have a low intracellular concentration. This is similar to TAp63γ. To elucidate if this is a consequence of the increased transcriptional activity or other reasons i.e. degradation of the protein further experiments were carried out. An additional mutation in the DNA-

binding domain (DBD, R279H) was included to TAp63α.FTL (FTL) and TAp63α.FTL.K (FTL.K), which leads to complete transcriptional inactivity of these constructs. The intracellular protein concentration of the transcriptionally active and the transcriptionally inactive constructs was analyzed by western blots. The results are shown in Fig. 22.

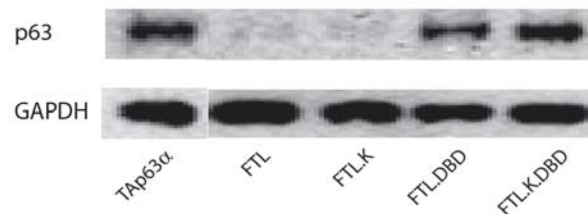

Figure 22: Western Blots of the transcriptionally active TAp63α-constructs and the respective DNA-binding domain mutants and TAp63α wt [taken from ref. 53].

Figure 22 clearly shows that the transcriptionally inactive constructs have a higher intracellular concentration than the active ones. Therefore there seems to be an additional regulation step for transcriptionally active TAp63α-forms which acts at the protein level. Such a control mechanism might be essential for keeping the cellular environment stable.
The concentration of FTL.DBD.K is increased in comparison to FTL.DBD also in this case with the additional mutation K637L. The impacts caused by mutation of the sumoylation site (FTL.K.DBD) are similar to the wt even though the amino acids FTL are mutated to alanine.

6.1.5 Transcriptional activity of the TID-mutants on the *bax* promoter

So far the data for transcriptional activity have been obtained on the *p21* promoter. To test if there is any promoter specific effect the transcriptional activity for the analyzed constructs containing mutations in TI and / or sumoylation site were carried out in the same way as on the *p21* promoter on the *bax* promoter. The corresponding data is shown in Fig. 23.

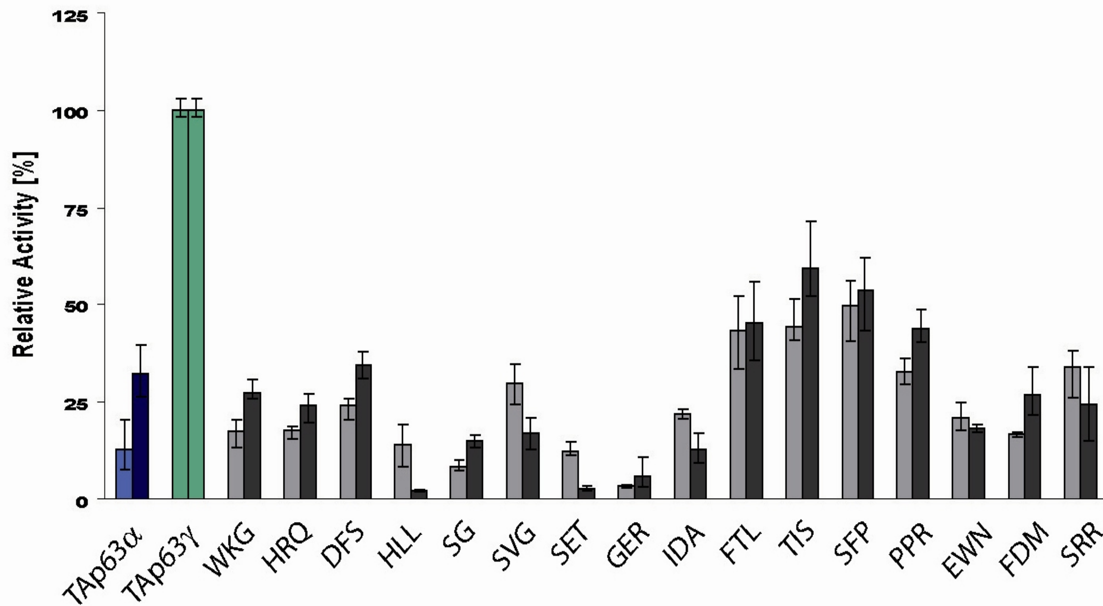

Figure 23. Relative transcriptional activity for the TAp63α constructs on the *bax* promoter. Grey bars show the constructs with the mutations in the TI domain. Black bars show the constructs with the additional mutation K637L. The wt of TAp63α is indicated in light blue, the corresponding sumoylation mutant in dark blue. TAp63γ is indicated in green [taken from ref. 53].

As on the *p21* promoter the constructs with mutations in the stretch between F605 and R616 are the most active ones, suggesting that the same amino acids are crucial for the inhibitory function. Surprisingly the additional K637L mutation leads to no additional increase in the transcriptional activity in most of the constructs while in the wild type this single mutations leads to an increase in transcriptional activity (as on the *p21* promoter).

To test if the effect of sumoylation is promoter-dependent for active TAp63α forms western blots were performed to obtain the intracellular concentration levels. This was carried out for TAp63α.wt, TAp63γ.wt, TAp63α.FTL and the corresponding constructs of TAp63α with the additional K637L mutation. The transcriptional activities and the corresponding western blots are shown in Fig. 24.

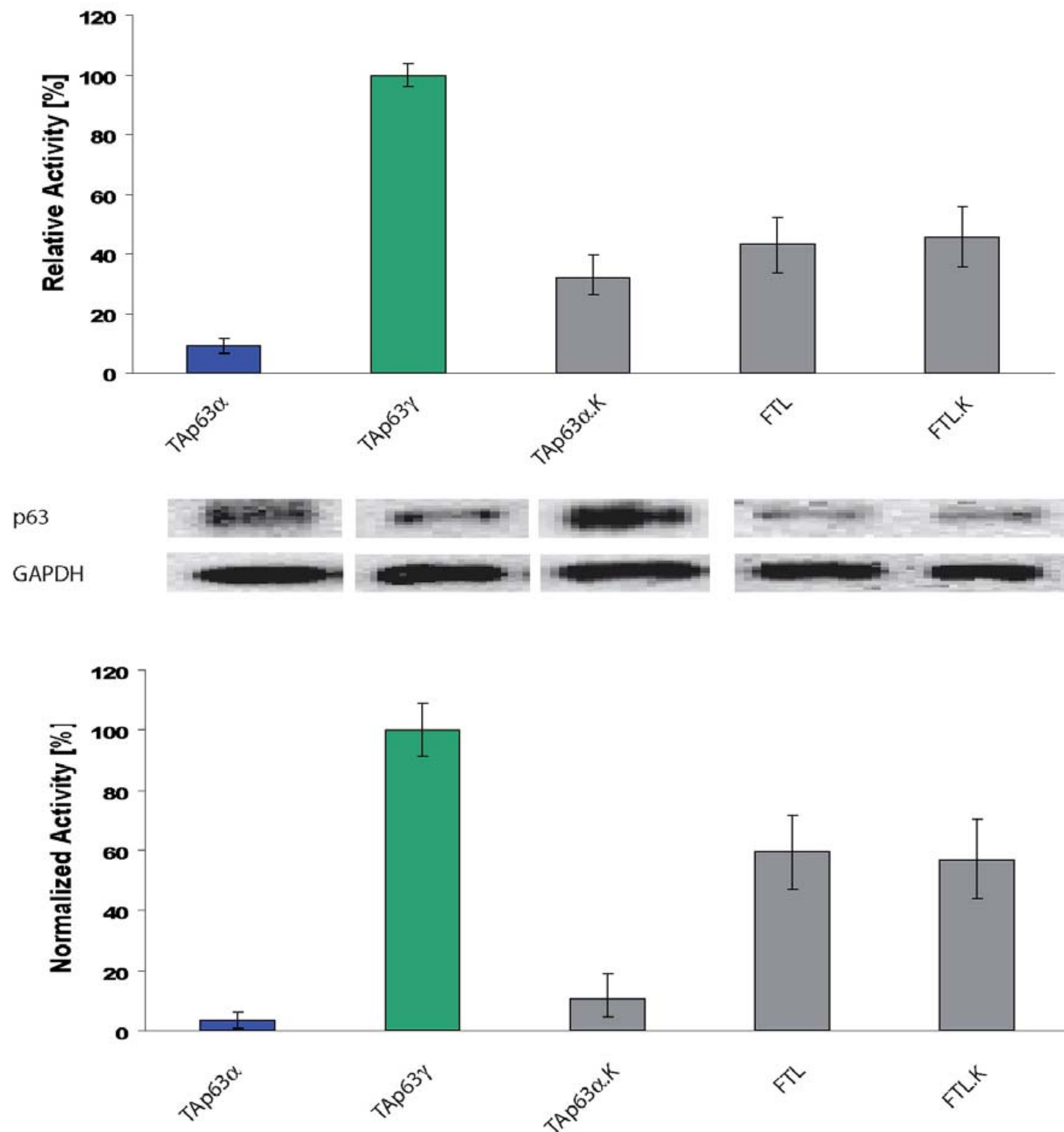

Figure 24: Relative and normalized transcriptional activities and protein levels for the TAp63α constructs and TAp63γ on the *bax* promoter [taken from ref. 53].

The result for TAp63α and TAp63α.K is similar to that obtained on the *p21* promoter. In contrast to that TAp63α.FTL and TAp63α.FTL.K show the same relative transcriptional activity and intracellular concentration on the *bax* promoter. The resulting normalized activity per protein on the *bax* promoter is the same in case of FTL and FTL.K as on the *p21* promoter. The identified stretch of amino acids seems to be important for the intrinsic regulation of the protein in both cases and therefore seems to be promoter independent, while sumoylation seems to depend on the promoter to which the protein binds.

6.1.6 Subcellular localization of inactive and active TAp63α constructs

To exclude the possibility that an altered subcellular localization might be the reason for changes of transcriptional activity, immunofluorescence data was carried out. The results are shown in Fig. 25.

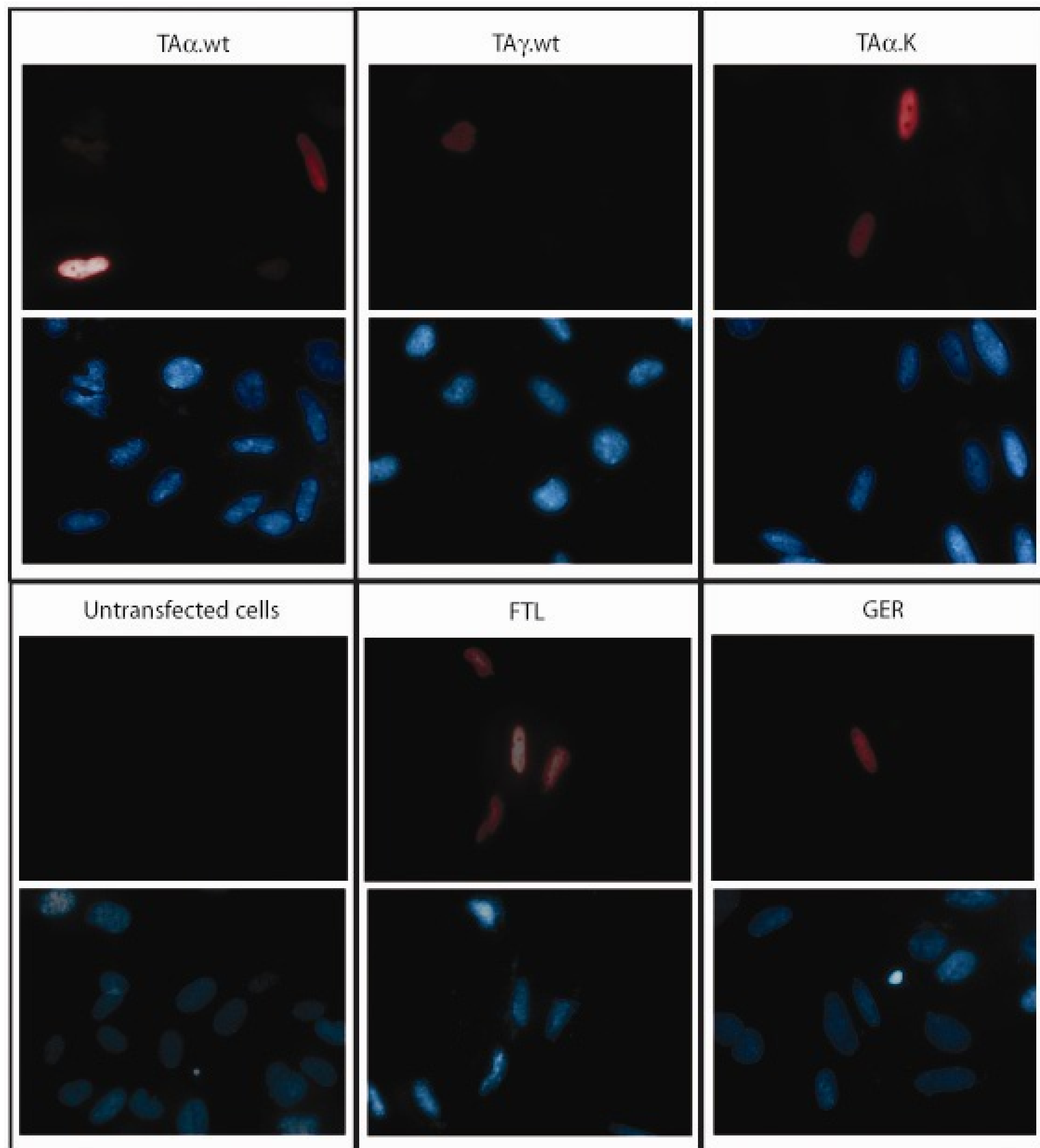

Figure 25: Immunofluorescence data for constructs of TAp63α and TAp63γ.wt transfected in SAOS2 cells. In red the signal of the Cy3-coupled anti-myc antibody is shown, in blue the DAPI stain of the nucleus [taken from ref. 53].

The data displayed in Fig. 25 show exclusive nuclear localization of the analyzed TAp63 constructs. Therefore the differences in transcriptional activity are not caused by an altered subcellular localization of TAp63.

6.1.7 Effects of a deleted QP domain

All presented results of TAp63α were obtained for the full-length protein. To investigate if the QP domain between the OD and SAM has an influence either on the identified region (FTL) or the sumoylation the transcriptional activity of several constructs without the QP domain was measured [Fig. 26].

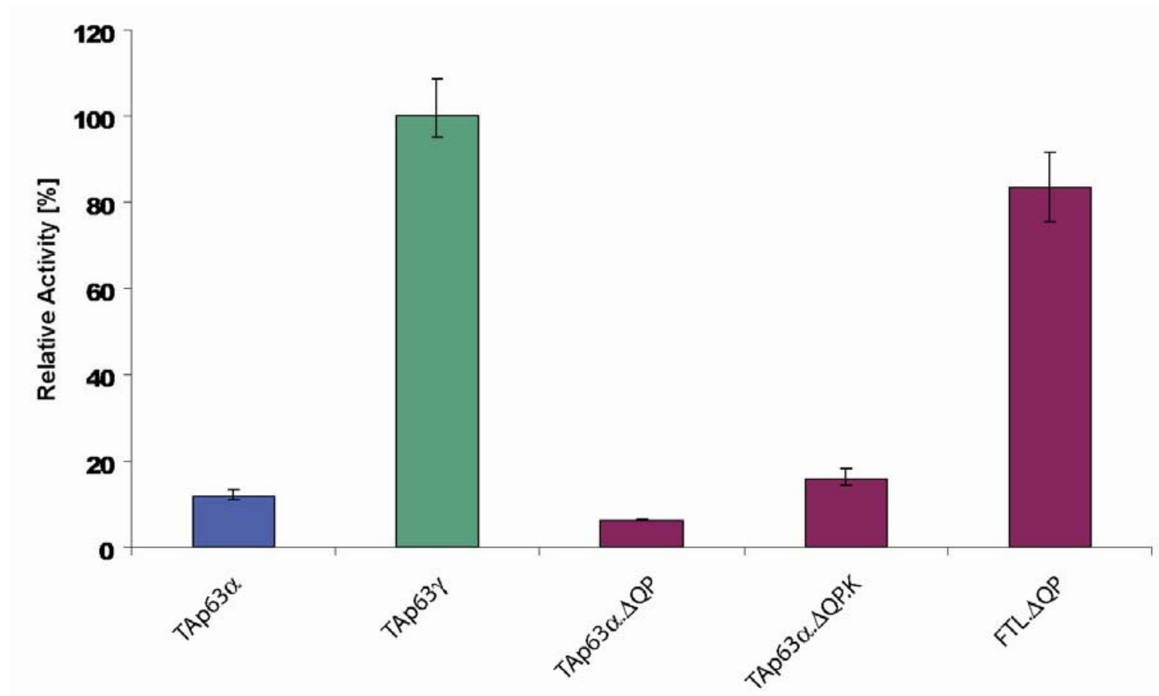

Figure 26: Transcriptional activity of several TAp63α constructs lacking the QP domain relative to TAp63γ in SAOS-2 cells. In blue TAp63α wt, in green TAp63γ wt [taken from ref. 53].

Figure 26 shows that the previously observed effects on transcriptional activity are similar in constructs without the QP domain. Therefore the QP domain seems to have no influence on the previously described effects (Sumoylation, FTL).

6.2. Implications on ΔNp63α

6.2.1 Impacts of mutations in the TID on the inhibitory potential of ΔNp63α

The experiments presented in 6.1 have shown effects of AA F605 to R616 on the transcriptional activity of TAp63α. To test if these results also influence ΔNp63α, which also has a TI-domain, additional experiments were carried out. The TAp63α construct FTL showed the highest transcriptional activity of all TAp63α constructs. Therefore an analogue mutation was carried out in ΔNp63α. Other constructs were created with additional mutations in the DBD for both ΔNp63α.FTL and ΔNp63α.wt. The ΔNp63α constructs were tested for transcriptional activity on the *p21* promoter. The results are shown in Fig. 27.

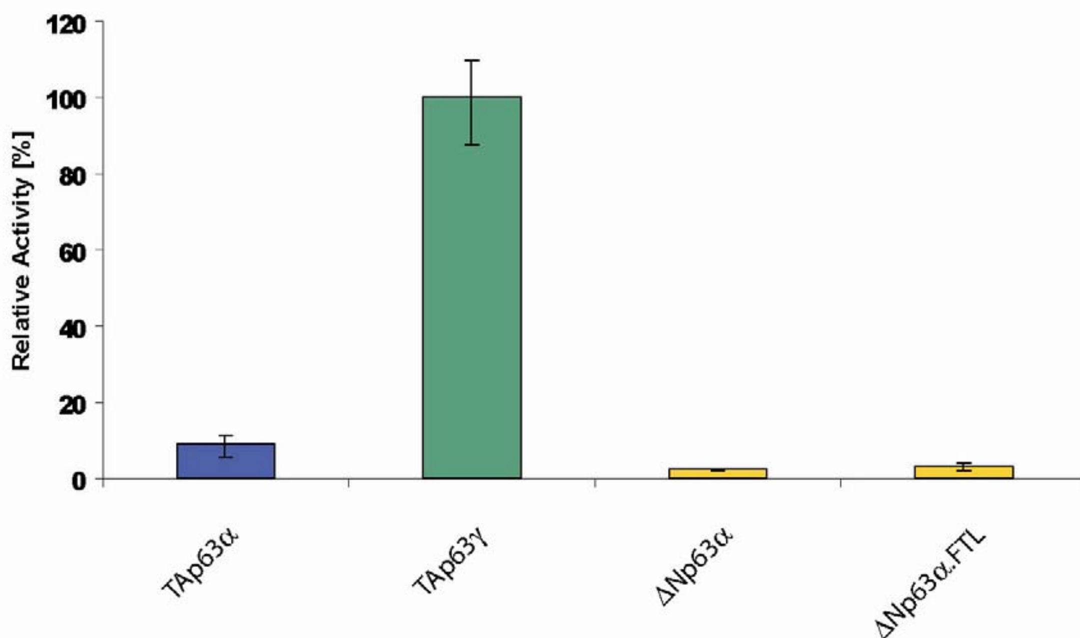

Figure 27: Transcriptional activity of TAp63α, TAp63γ and ΔNp63α constructs on the *p21* promoter relative to TAp63γ in SAOS-2 cells. In blue TAp63α wt, in green TAp63γ wt [taken from ref. 53].

As expected the ΔNp63α constructs both show low transcriptional activity on the *p21* promoter. Therefore this mutation has no effect on the transcriptional activity of ΔNp63α on the *p21* promoter. To test, if the mutation has any effect on the

inhibitory potential of ΔNp63α an inhibition assay was performed in the following experiment. Constant amounts of plasmid-DNA encoding TAp63γ wt and increasing amounts of plasmid-DNA encoding various ΔNp63α constructs had been co-transfected. The results on the *p21* promoter are shown in Fig. 28.

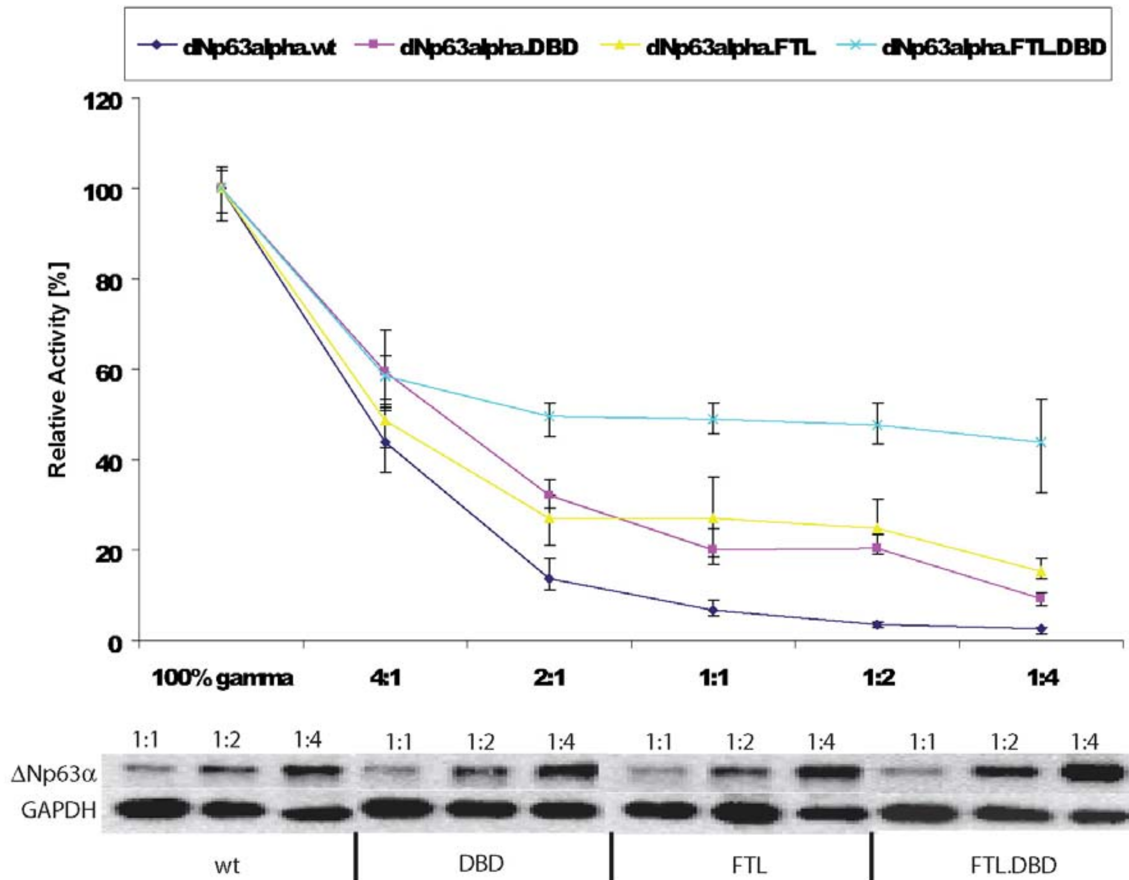

Figure 28: Relative transcriptional activity of TAp63γ transfected with increasing amounts of different ΔNp63α constructs and respective western blots [taken from ref. 53]. Ratios between transfected TAp63γ and ΔNp63α plasmid DNA are shown on the x-axis and above the protein levels.

The shown western blots of the three highest plasmid concentrations of the ΔNp63α constructs show increasing protein concentrations with each titration step. For nomalization of the transcriptional activities only the data points for the three highest plasmid concentrations of ΔNp63α were used. This data is shown in Fig. 29.

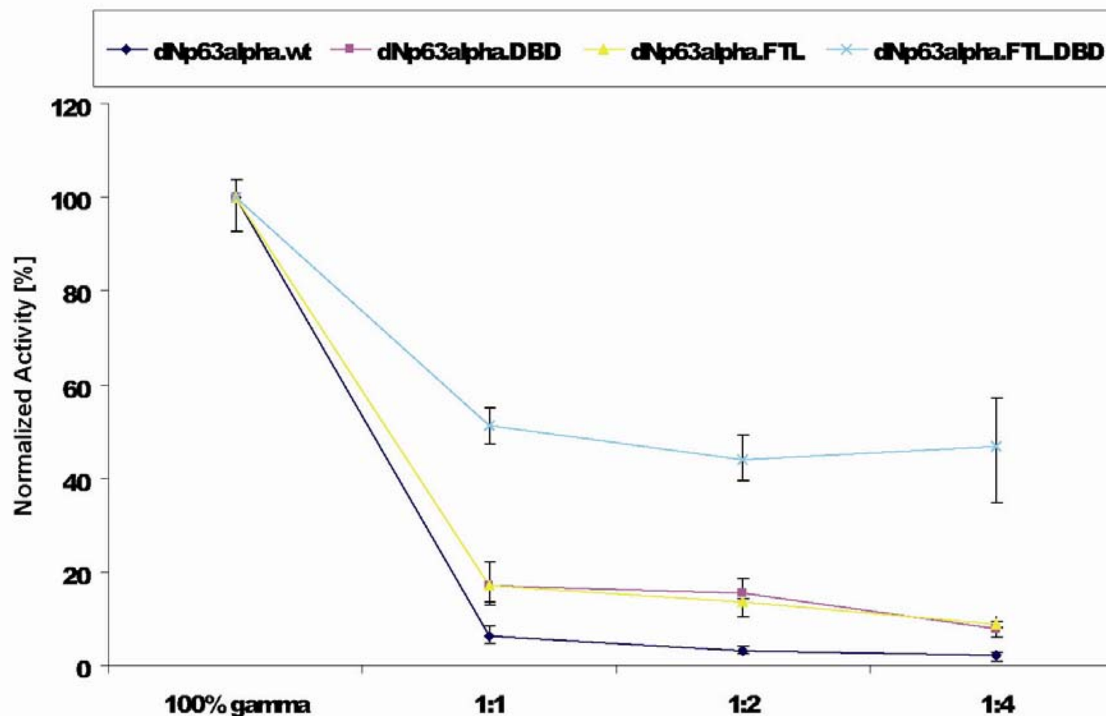

Figure 29: Normalized transcriptional activity of TAp63γ cotransfected with increasing amounts of different ΔNp63α [taken from ref. 53].

ΔNp63α behaves strongly dominant negative towards TAp63γ. The net transcriptional activity decreases rapidly with increasing amounts of ΔNp63α. ΔNp63α.FTL and ΔNp63α.DBD are less but still inhibiting while ΔNp63α.FTL.DBD is a weak inhibitor. The relatively high inhibitory potential of ΔNp63α.FTL seems to be due to the higher binding affinity to the promoter. This might be due to an indirect inhibition based on promoter squelching and independent of the C-terminal inhibition via the TA-TI-interaction. The two opposing effects compensate each other and the overall inhibitory potential of ΔNp63α.FTL seems to be similar to that of the wild type. But the inhibition caused by the C-terminus is significantly reduced which can be seen by comparison of ΔNp63α.DBD and ΔNp63α.FTL.DBD. The weak inhibitory potential of ΔNp63α.FTL.DBD shows that it only inhibits by forming tetrameres via the oligomerization domain. The overall activity in the case of ΔNp63α.FTL.DBD is 50%, assuming that at high protein levels of the ΔN forms of p63 most of the tetramers should consist of mixed tetrameres in which the cotranslationally formed dimers are the basic unit. This point will be elucidated in 6.3.

6.2.2. ΔNp63α is able to prevent the transcription of certain genes

A higher affinity to a promoter is a mechanism how ΔNp63α might be capable of shutting down genes by blocking the binding site for other transcription factors. Experiments with several p63-isoforms were carried out by Reverse Transcriptase-PCR in SAOS2 cells to elucidate if there is any difference in the mRNA level of the investigated genes, which are important for osteogenesis [Fig. 30]. Those cells were derived from an osteosarcoma cell line and should therefore be suitable for these differentiation experiments.

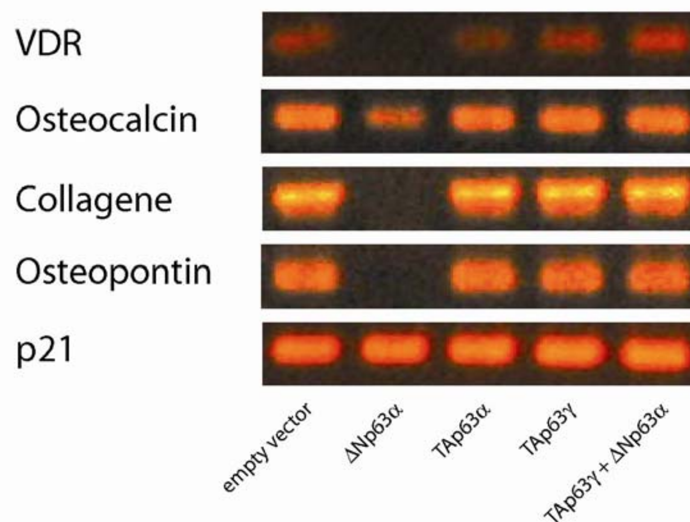

Figure 30: DNA-levels of several genes (*VDR*: Vitamin D receptor) obtained by RT-PCR corresponding to the levels of mRNA of these genes inside transfected SAOS2 cells. *P21* as a target of p63 is the control.

There is a difference in the mRNA level between those cells transfected with ΔNp63α and the others. The Vitamin D receptor (VDR) plays a crucial role for transcription of the other involved genes, therefore ΔNp63α seems to block the promoter of *VDR* and maybe the other genes important for osteogenesis. But the observed result could also be a sequential effect and the VDR normally induces these genes which show decreased mRNA level. The mRNA level of Osteocalcin is also decreased while the mRNA level of p21 seems to be the same. Therefore the DNA-binding affinity of ΔNp63α seems to be not sufficient to block the promoter of this gene efficiently. For all genes involved in Osteogenesis the level

of the PCR-product is decreased (Osteocalcin) or there is even no signal. This data supports the idea of ΔNp63α inhibiting other factors important for transcription like VDR, maybe by preventing their transcription.

6.3. Analysis of TAp63γ

6.3.1 Impact of oligomerization state on transcriptional activity

The data shown before suggests that a mixed tetramere of ΔNp63α.FTL.DBD and TAp63γ might act like a dimer of TAp63γ. Further validation of this hypothesis is delivered by transcriptional activity data of the TAp63γ-constructs shown in Fig. 31.

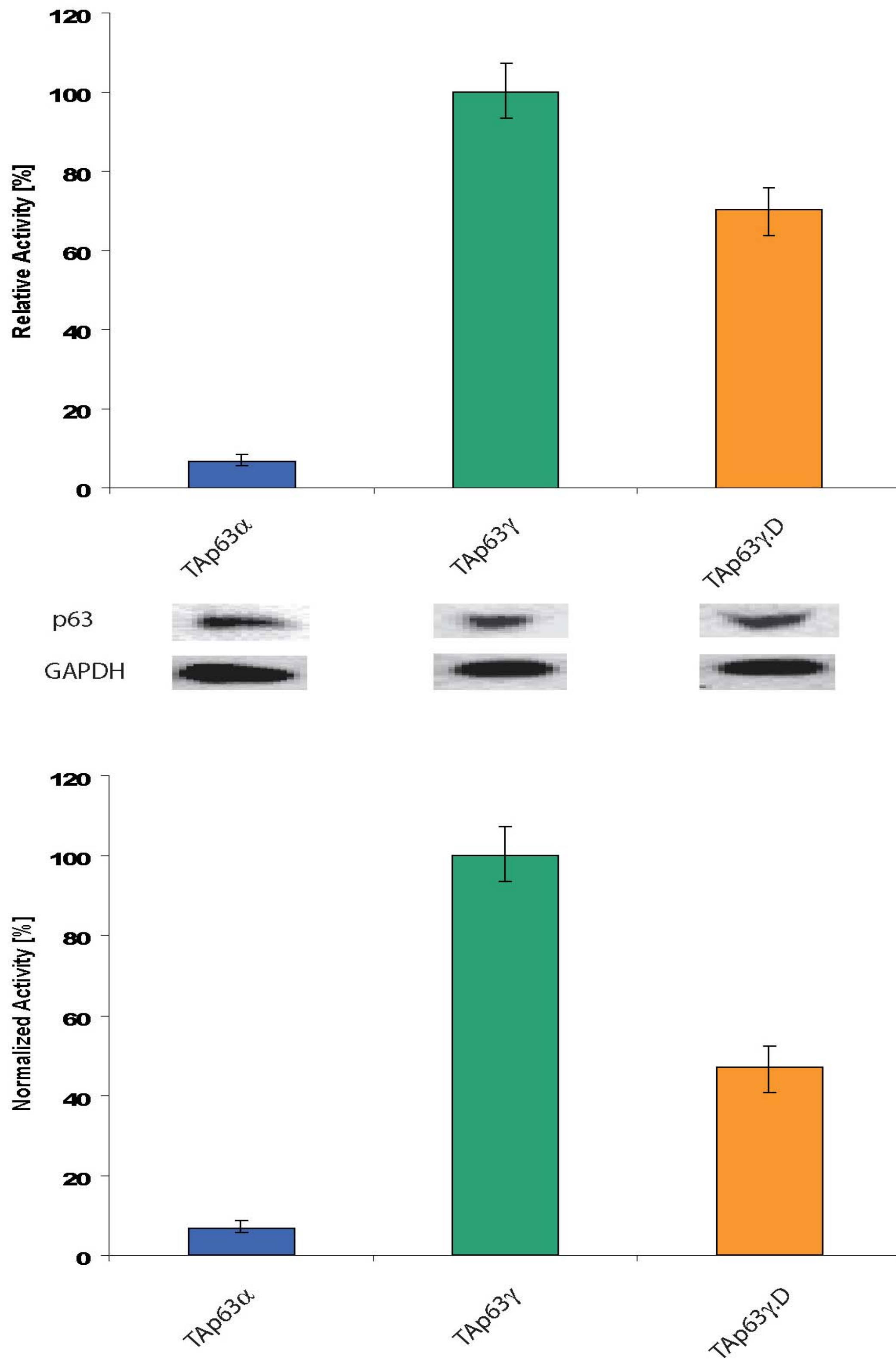

Figure 31: Relative and normalized transcriptional activity of TAp63α.wt (blue) and TAp63γ.D (M374Q, I378R) to TAp63γ.wt (green) in SAOS-2 cells [taken from ref. 53].

To test if the oligomeric state of the protein is a dimer instead of a tetramere two

p63 constructs containing of the oligomerization domain and the SAM-domain (ODΔQPSAM) were expressed recombinantly in *E.coli* and analyzed by gelfiltration, one of them with the mutations M374Q and I378R in the tetramerization interface. The gelfiltration data is shown in Fig. 32.

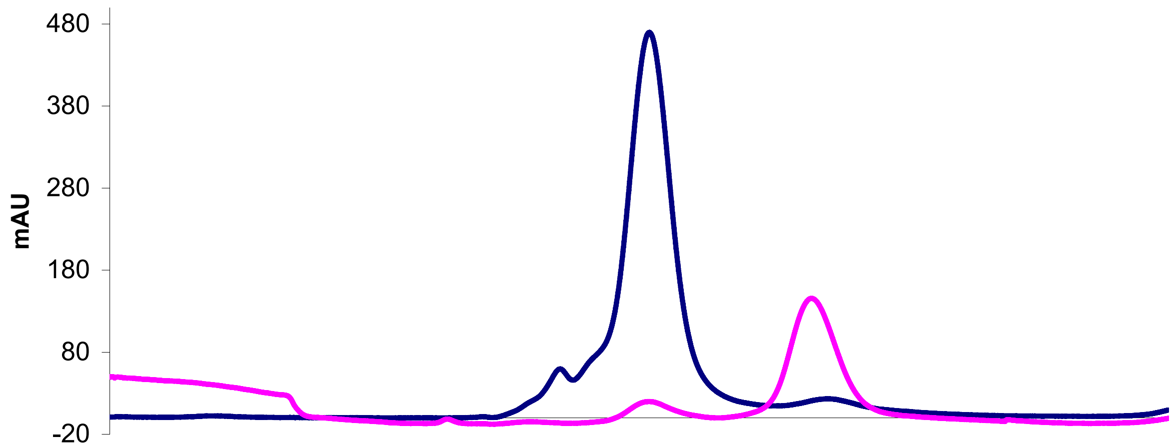

Figure 32. Elution profile of ODΔQPSAM wild type (blue, 56 ml)) and the construct with the mutations in the tetramerization interface (purple, 73 ml) (M374Q, I378R) on a Superdex™ 200 Highload 16/60 column.

The observed volumes correspond to the molecular weight of either tetrameres or dimers. An equilibrium between tetrameres and dimers can be observed in both cases, but the equilibrium is shifted significantly with introduction of the dimer mutations (M374Q, I378R) towards the dimeric state of the protein. Nonetheless only a qualitative measurement was carried out and no quantitative assay. The mutations in the tetramerization interface therefore have effects on the oligomerization state in the truncated p63-construct, assuming similar effects in the full length protein.

6.3.2 Deletion study of the C-terminus of TAp63γ

The experiments and results described in the analysis of the TI-domain [Fig. 16, 17] show that there are regulatory elements at the C-terminus of the p63α

isoforms, which have important biological functions. Even though there is no assigned role of biological relevance for TAp63γ described so far, the question arises if this most active isoform of p63 is controlled in a similar manner like the α-isoforms or if it is controlled similar to p53 to which it shares high structural similarity. Because of the lack of previous data on important regions, in contrast to TAp63α, a deletion and no mutational analysis was carried out in the beginning on TAp63γ. For this purpose several constructs were created and tested for transcriptional activity in SAOS2 cells. The corresponding data is shown in Fig. 33.

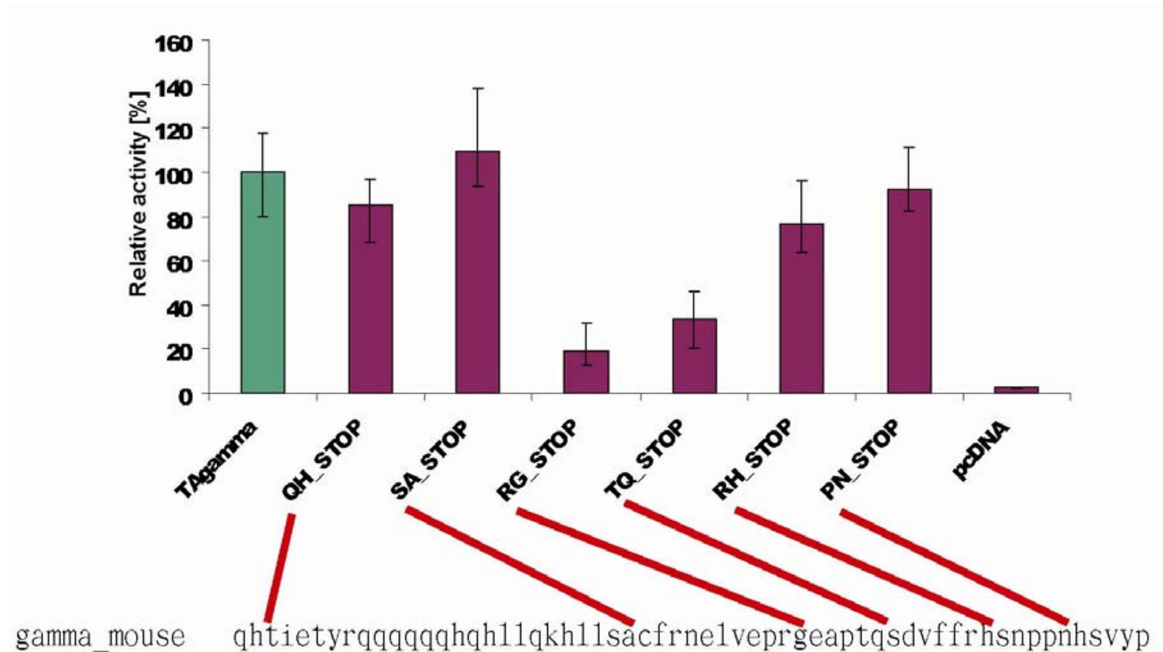

Figure 33. Relative transcriptional activities of TAp63γ deletion constructs to the wt (green) in SAOS-2 cells. The murine gene sequence is indicated below the activity data. The red lines indicate at which amino acid the STOP-codon was introduced in the construct.

The results show that the transcriptional activity of the protein does not change dramatically until the deleted number of amino acids is about twenty (TQ_STOP). The deletion of more amino acids (RG_STOP) has no additional negative effect, only after additional deletion of twelve more amino acids (SA_STOP) the transcriptional activity returns to wild type levels. In the region is one serine (S428) and threonine (T426). In an additional experiment those residues were mutated to alanines (PAQA) and the transcriptional activity was measured. This

data is shown in Fig. 34.

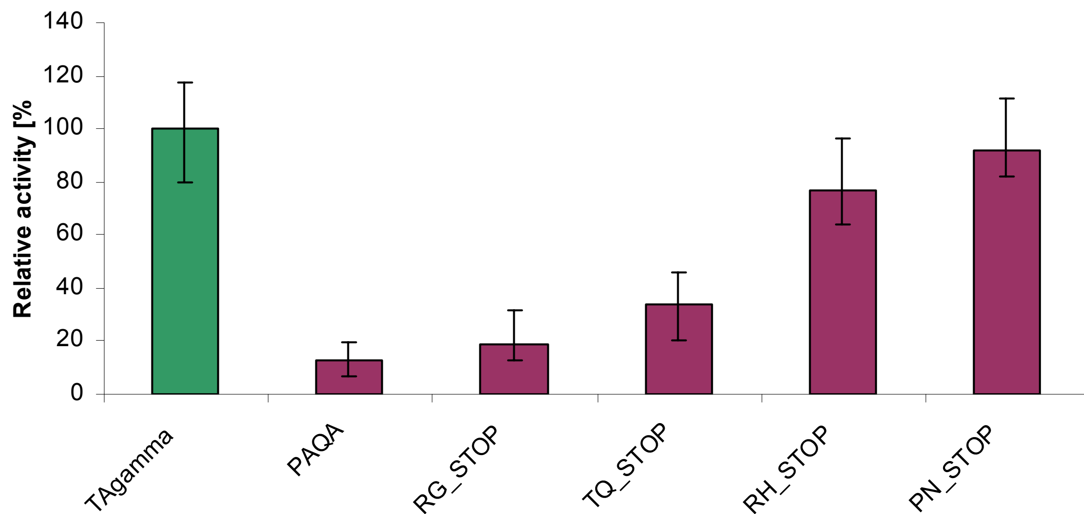

Figure 34. Transcriptional activities of an alanine mutant of TAp63γ (PAQA) and TAp63γ deletion constructs relative to the wt (green) in SAOS-2 cells.

It can be seen that the transcriptional activity of the double alanine mutant is nearly at the same level as of the construct RG_STOP which lacks both amino acids. Therefore these amino acids might be important for transcriptional activity of the protein. Since both amino acids might get modified post-translational the probability for phosphorylation was elucidated.

6.3.3 Phosphorylation predication

To investigate the possibility of the identified amino acids of getting phosphorylated a phosphorylation predication was carried out [Fig. 35].

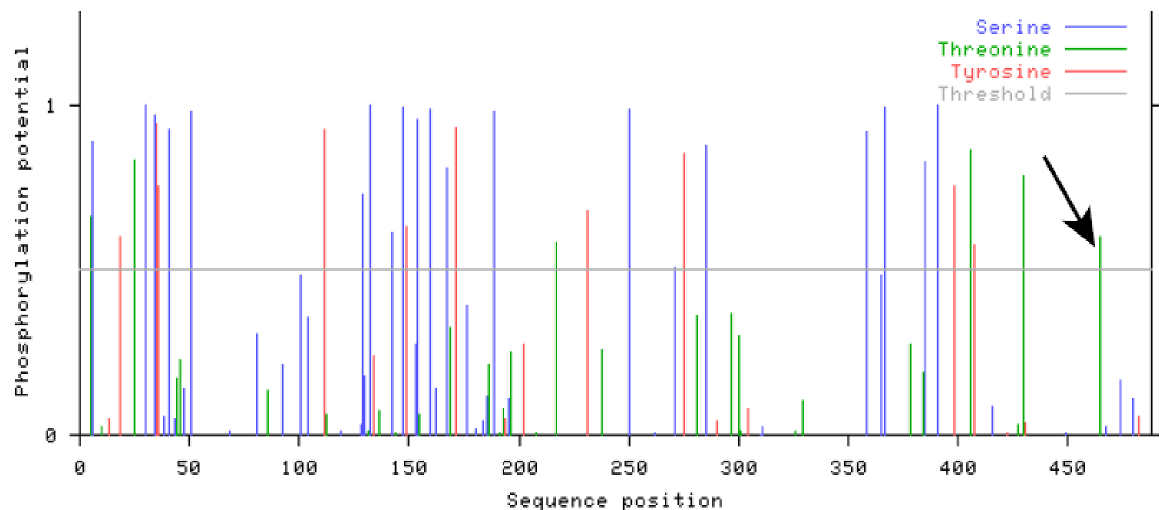

Figure 35. Phosphorylation prediction of murine TAp63γ. The threonine mutated in the alanine mutant shown before is indicated by an arrow. The height of the bars stands for the phosphorylation potential of the possible phosphorylation sites in the whole protein [taken from ref. 88].

The phosphorylation prediction shows that one of the identified amino acids might get phosphorylated. The phosphorylation potential for the T426 is above 0.5, what might mean that phosphorylation occurs at this site.

7. Discussion

7.1 Functional analysis of the C-terminus of TAp63α

Due to the biological relevance of the transcription factor p63, which is involved in key decision processes during ectodermal development, maintenance of mature skin and in genotoxic stressed oocytes, its regulation is of outstanding biological significance.

In contrast to p53, which is mainly regulated by the interaction with MDM2 the regulation of p63 is more complex due to the expression of at least six different isoforms. Two of these isoforms have an extended (full-length) C-terminus comprising two independent regulatory elements, which contribute to the auto-inhibition of TAp63α and the inhibitory effects of ΔNp63α.

Mutational analysis was carried out on the C-terminus of TAp63α to identify amino acids which modulates the transcriptional activity if mutated into alanine. The pattern of the transactivation activity [Fig. 14] obtained by luciferase assays identifies a region with increased transcriptional activity when mutated within the TID beginning with F605. Increasing transcriptional activity can also be observed if the sumoylation site is affected. This effect of a mutated sumoylation site will be pointed out later in this discussion.

The transcriptional activities of all measured constructs were normalized by measuring the intracellular concentration of the protein which was determined by western blot [Fig. 15]. This was done to exclude the possibility that the higher transcriptional activity is just because of concentration effects and not because of intrinsic effects. Earlier investigations had revealed that the most transcriptionally active isoform TAp63γ shows a significantly reduced intracellular concentration compared to transcriptionally impaired mutants [89]. In western blots the more active constructs of TAp63α are less concentrated than those with lower transcriptional activity or TAp63α.wt, leading to an even more increased normalized activity of these constructs relative to the others and the wild type [Fig. 16].

The presented results show that transcriptional activity of TAp63α seems to be inhibited via a stretch of amino acids in the Transactivation inhibitory domain. The identified region seems to be important for regulation of TAp63α by binding to and masking the TA-domain. Pulldown experiments showed, that this region is crucial for binding the TA-domain *in vitro* [53]. This might occur by a conformational change.

A point mutation in the identified amino acid stretch has not been reported to date, but patients with AEC syndrome carry mutations in the TI domain just N-terminal to this stretch (R598L, D601V) [90]. Also the biological impact for these findings is provided by the recent discovery that TAp63α plays a major role in protecting the female germ line [32]. The carried out biochemical experiments during this thesis and the finding that ionizing radiation leads to activation of TAp63α via phosphorylation and the phosphorylation of TAp63α is coupled to cell death of oocytes correlate with each other. Therefore it is most likely that some post-translational modification is taking place in this region (FTL) if the inhibition

has to be abolished. It is noticeable that there are two highly conserved threonines (within FTL) and one highly conserved serine (within TIS) within this important stretch, therefore phosphorylation could be a possible mechanism for activation of the protein. This leads to the assumption that TAp63α is kept in a transcriptionally inhibited conformation with the help of the TI domain. Maybe also the oligomerization domain is involved and the oligomeric state is affected by the TA-TI interaction, but this has to be elucidated. The identified short stretch of amino acids might be essential for keeping the protein in a closed conformation and therefore inhibiting its transcriptional activity. This might be due to direct binding to one or more domains of p63. Experiments carried out before had shown direct interaction between the bacterially expressed TA and the TI domains of p63 [15]. Together with the finding that phosphorylation is essential for activation of TAp63α in Oocytes this might implicate that several functions of the protein are blocked by forming a closed conformation with several other domain-domain interactions. Unfortunately a mutation of T606 to glutamate as a phosphorylation mimic does not lead to a significant increase in transcriptional activity of TAp63α in SAOS2 cells. This could either mean that this mutation is not a good mimetic of phosphorylation in terms of breaking all important contacts, or the activation mechanism is more complex and additional modifications are needed.

In the case of p53 several phosphorylations occur during activation of the protein [43,44,49,50]. Unfortunately, a kinase which phosphorylates amino acids in this stretch of the TID (FTL) of p63 has not been identified to this point.

The surprising absence of missense mutations in this region of the TID could be explained in several ways. Primary a missense mutation could have a lethal effect. Due to the gene structure of p63 all α-isoforms in nearly every cell will be affected if such a mutation would occur in an early stage of the organism, leading most likely to death. Especially possible implications on the ΔNp63α isoform might have consequences for the development. Hypothetically a mutation in Oocytes leading to an active form of TAp63α would lead to infertility, so this mutation is eliminated from the gene pool. Another possibility for the absence of observed mutations in the core of the TID is that a syndrome would only arise in case of dominant negative or gain of functions effects. If only one allele is

affected the consequences on development seems to be manageable due to the lack of a phenotype in heterozygous knock-out mice for p63 [27]. Haploid insufficiency only has consequences in rare cases of heterozygous deletions of the p63 gene in human patients [34]. Therefore expression from the second, non-mutated allele seems to be sufficient to compensate possible effects of the mutated allele during development and maintenance of epithelial tissue.

An increased transcriptional activity for TAp63α can also be observed if the sumoylation site is affected. This seems to be another way of regulating TAp63α via its C-terminus. The mutation of I636 or the acceptor lysine K637 in TAp63α leads to an increase in transcriptional activity. Nearly all constructs where the acceptor lysine K637 is mutated into leucine show increased transcriptional activity [Fig. 18]. Analysis by Western blot reveals, that this is the consequence of an increased protein level [Fig. 19].

Alanine mutations in the active region again show a low intracellular concentration relative to the others in this experiments even though the protein concentration increased due to the mutation of the sumoylation site. Overall, for most constructs the additional mutation at the sumoylation site leads to an increased protein concentration [Fig. 20]. This corresponds to the overall increased transcriptional activity. So the increased transcriptional activity of the constructs containing the K637L mutation is just due to concentration effects what can be seen after normalization [Fig. 21]. The normalized activities show a very similar pattern to those experiments carried out with the constructs containing an intact sumoylation site [Fig. 16]. Sumoylation therefore seems not to alter the intrinsic activity of p63 itself, but changes its intracellular concentration leading to a shifted transcription rate of the target genes.

Accordingly sumoylation might constitute another control mechanism independent from the other proposed control mechanism. Nevertheless the biological relevance of sumoylation still has to be confirmed, possibly by investigating the sumoylation state of p63 in arrested oocytes.

Concerning the importance of sumoylation, there have also been reports about nonsense mutations in patients with SHFM4 syndrome, where protein forms exist that are only truncated by a few amino acids [91]. These mutations seem to affect only the sumoylation site leaving the rest of the TID and its interactions intact.

The observed syndromes might be caused by the ΔNp63α isoforms, due to the phenotype of these mutations. The removal or destruction of the sumoylation site is enough to lead to the SHFM4 syndrome [34,55]. In other cases of the SHFM4 syndrome the mutations occur in the transactivation and the DNA binding domain [92]. Two of the reported mutations in the DNA binding domain affect lysines involved in regulation of the intracellular protein level by ubiquitination of these residues by Itch [93]. Accordingly, for the SHFM4 syndrome there might be a link to impaired degradation of ΔNp63α.

ΔNp63α becomes sumoylated in several different cell lines (HaCaT human keratinocyte, FaDu human hypopharynx squamous carcinoma, Dok human dysplastic oral keratinocyte) or after transfection in SAOS2 cells [54]. Also a direct interaction of TAp63α and ΔNp63α with Ubc9, which is essential for sumoylation, has been demonstrated in transiently transfected human embryonic retina 911 cells [55]. It is possible to completely abolish the association among p63 and SUMO-1 by a K637E mutation, which also leads to a dramatically increase of the transcriptional activity of TAp63α on the *RGC* promoter. Furthermore, sumoylation is important for regulation of another p53-family member and regulates the intracellular concentration of p73 [56].

The results in this thesis together with previous findings show an interesting correlation between high transcriptional activity and low intracellular concentration [89]. There seem to be additional mechanisms regulating the intracellular concentration of transcriptionally active forms without affecting transcriptionally repressed forms of the protein. This hypothesis is supported by the finding that the intracellular concentration of TAp63α.FTL (barely detectable) is highly increased by an inactivation of this construct via an additional mutation in the DBD [Fig. 22]. This shows that even if the conformation is changed due to the mutation in the TID the protein seems to be stable if not transcriptionally active. It might be that the degradation of p63 only takes place if a transcriptionally active complex is formed on the DNA, which is regulated via ubiquitination by Itch, MDM2 or other E3-ligases that interact with p63 [93-99]. A difference between transcriptionally active and inactive forms has been observed i.e. for the estrogen receptor hERα as well [100].

All shown results were obtained using a reporter vector containing the *p21*

promoter region p63 binds to. To test if these results are restricted to this promoter or if the functionality is more general all transactivation experiment were carried out again using a promoter construct containing the *bax* promoter instead of the *p21* promoter [Fig. 23]. The results for the constructs with mutations in the TID with an intact sumoylation site show the same pattern as in the case for the *p21* promoter, suggesting that these identified amino acids are essential for keeping the protein in its inhibited form not only on the *p21* promoter.

For the wild type of TAp63α a mutation of the sumoylation site has the same effect on the *bax* promoter as on the *p21* promoter. But for most of the other constructs an additional mutation which affects the sumoylation site seems to have no additional effect in terms of an additionally increased transcriptional activity. This could mean that some regulation mechanisms depend on the controlled promoter. While a mutation of the important amino acids (FTL) again leads to an activation of the protein and a single mutation of the sumoylation site has an effect on intracellular protein concentration the two effects are not able to become additive in a double mutation.

To investigate the intracellular concentrations for the experiments on the *bax* promoter western blots of TAp63α.wt, TAp63α.K, TAp63α.FTL, TAp63α.FTL.K and TAp63γ.wt were carried out [Fig. 24]. The data shows that in case of the wild type and a single mutation in the sumoylation site the normalized transcriptional activity and therefore the result on the *bax* promoter is comparable to the result on the *p21* promoter in this case. The concentrations of TAp63α.FTL with and without an additional mutation of the sumoylation are similar to each other and consequently also the normalized activities are similar. As a consequence the normalized transcriptional activity of these constructs is comparable to the data on the *p21* promoter.

The obvious difference on the *bax* promoter and the *p21* promoter is, that there is no accumulation effect on the transcriptional activity due to the additional mutation in the sumoylation site for most constructs. Maybe sumoylation is dependent on the conformation of the protein and this can be different on various promoters.

The transcriptional activity of a protein can also be influenced by its localization within the cell. For p73 localization signals seem to be important for its regulation

[101]. At least the decreased activity of constructs like TAp63α.GER could be the result of a different subcellular localization, therefore immunofluorescence has been carried out [Fig. 25]. The results clearly show all analyzed construct show exclusive nuclear localization. Accordingly the altered activities are not the result of differences in cellular localisation.

The data about the identified active region (FTL) leads to the hypothesis that the increased activity of some constructs is due to a break up of important domain-domain interactions. There is a region present in p63, called the QP-domain, that is of unclear relevance for structure and function of the protein. To test the hypothesis that this domain is not involved in the domain-domain interactions suggested between the identified amino acids (FTL) and other domains analogue experiments to those before have been carried out. In these experiments the used constructs lack the QP-domain and transcriptional activity was measured [Fig. 26]. The resulting data is very similar to the data of the experiments with the full length TAp63α and supports the idea that the QP-domain is not essential for this aspect of regulation of p63.

To summarize, the transcriptional activity of TAp63α seems to be regulated in two different ways, one of them based an intrinsic mechanism (FTL) and one based on sumoylation. There is a stretch of amino acids present in the TID, that seems to be important for this intrinsic activation mechanism. Transcriptionally inactive contracts show a higher intracellular concentration than active ones. The intrinsic mechanism seems to be similar on the investigated two promoters while sumoylation seems to be promoter dependent. For these two distinct control mechanisms the QP-domain seems not to be essential. To test if the identified amino acids, which have an impact on transcriptional activity, also have implications for the other α-isoform of p63, experiments with analogue mutations were carried out on ΔNp63α.

7.2 Implications on ΔNp63α

The dominant negative function of ΔNp63α towards TAp63α and therefore the function of the C-terminus in ΔNp63α is unclear. To test if the analogue

mutations, which lead to increased transcriptional activity in TAp63α have any effect on inhibitory abilities of ΔNp63α or have other effects the most significant mutation (FTL) was applied to ΔNp63α. First of all the consequence on the transcriptional activity of ΔNp63α on the *p21* promoter was tested by a transactivation assay [Fig. 27]. ΔNp63α does not get transcriptionally active on the *p21* promoter due to this mutation. The protein has the same transcriptional activity as the wild type in this experiment. Due to the fact that ΔNp63α binds to enhancer elements of certain genes and promotes transcription in this way [102] it might be possible that the enhancing ability is altered by the mutation of FTL, but this has to be elucidated in further experiments.

To investigate the implications on the inhibitory potential towards TAp63γ cotransfections with increasing amounts of DNA encoding ΔNp63α were carried out and overall transcriptional activity was measured [Fig. 28]. The difference between the inhibitory potential of ΔNp63α.wt and ΔNp63α.FTL is not that significant. Both proteins nearly inhibit all transcriptional activity with increasing amounts of transfected DNA and, according to the western blots, also increasing protein concentration of ΔNp63α. But it is important to remind that ΔNp63α can inhibit transcriptional activity in two different ways, either by directly interacting via the TID or indirectly by blocking the promoter. To distinguish between these effects an additional mutation in the DNA binding domain was added to ΔNp63α.FTL and net transcriptional activity was compared to ΔNp63α.DBD in titration experiments with TAp63γ. The two constructs carrying a mutation in the DBD are not capable of inhibiting TAp63γ indirectly by promoter binding.

Like in the case of ΔNp63α and ΔNp63α.DBD the main part of the inhibition does not take place due to promoter binding, but seems to be because of direct inhibition mechanisms. A comparison between ΔNp63α.DBD and ΔNp63α.FTL.DBD shows that the inhibitory potential of ΔNp63α.FTL.DBD and most obviously as well as that of ΔNp63α.FTL by direct inhibition is greatly affected by the mutation in the TID. To rule out concentration effects the protein concentrations have been determined and activities have been normalized [Fig. 29].

The western blots show that the double amount of transfected DNA of all ΔNp63α

constructs lead to doubled intracellular protein concentrations. The curves for the normalized net transcriptional activities are similar to the curves for the relative net transcriptional activities. For ΔNp63α.FTL there seem to be two opposed effects. The indirect inhibition by DNA binding seems to be increased in this construct. With respective to the data obtained for TAp63α this result is not surprising. Maybe the mutation of FTL leads to an increased DNA-affinity in both isoforms. On the other hand direct inhibition via the TID seems to be abolished in the case of ΔNp63α.FTL, also according to the data for TAp63α. This clearly shows the significance of the identified amino acids for both α-isoforms.

At the C-terminus of both isoforms the identified amino acids influence the function of the proteins. The mutation of these amino acids maybe interrupts interactions within the protein and leads to an opening of the closed structure in both isoforms. The role of this regulation mechanism for ΔNp63α is not that obvious as in the case of TAp63α. But the consequence of ΔNp63α overexpression on certain genes important for osteoblastic differentiation [103] is demonstrated by RT-PCR [Fig. 30]. At least one promoter, most likely of the *VDR*, seems to be controlled by ΔNp63α. The decreased mRNA-level of the other genes might be a sequential effect due to the decreased intracellular level of VDR. It is unclear if the shown result might also be a result of a direct interaction of ΔNp63α with another protein. But it is important to mention that in the introduced case there is a massive overexpression of ΔNp63α. In the natural case there might be a mechanism to achieve the same goal without the need of such a massive overexpression by increasing the DNA binding affinity of ΔNp63α. For a complete silencing (or enhancing) of the target genes with lower protein concentrations this would be essential. In this case the identified possible control mechanism would be of biological relevance, as an "active" form of ΔNp63α would have a higher affinity for DNA. This would be a way to regulate ΔNp63α without the need of any additional isoforms.

As mentioned before ΔNp63α plays a crucial role in epithelial development. Maybe ΔNp63α is kept active in these tissues by the same kinase which activates TAp63α in Oocytes. Further experiments could proof this hypothesis i.e. if ΔNp63α also gets phosphorylated at the identified amino acids. The suggested

way of regulating ΔNp63α in a similar manner than TAp63α but with different effects could be a way how epithelial development in terms of stem cell survival or differentiation is regulated.

7.3 Analysis of TAp63γ

The previous experiments about the inhibitory potential of ΔNp63α.FTL.DBD also showed that the maximal inhibition is around 50 % of the starting value. This could mean that at these concentration levels of ΔNp63α.FTL.DBD and TAp63γ.wt mainly homo- tetrameres of ΔNp63α.FTL.DBD with no transcriptional activity or hetero- tetrameres of the two different isoforms exist. A hetero-tetramere of TAp63γ and ΔNp63α.FTL.DBD theoretically would behave like a dimer of TAp63γ due to only two functional DBD and TA domains. It is important to mention that the ΔNp63-construct is only able to inhibit via oligomerization with TAp63γ and neither by TA-TI interaction or promoter blocking. To investigate how a dimer of TAp63γ behaves in a transactivation assay an experiment was done with such a construct [Fig. 31]. After normalization the Dimer construct of TAp63γ (TAp63γ.D) has around 50 % transcriptional activity of the wild type. The same value as achieved in the titration experiment with TAp63γ.wt and ΔNp63α.FTL.DBD. It seems like a tetramere is required for high transcriptional activity of TAp63γ, a point that might also be important for TAp63α as already speculated before. To proof that the mutations in the tetrameric interface (M374Q, I378R) of the oligomerization domain really affect tetramerization, a shortened construct, which can be bacterially expressed, containing the oligomerization and the SAM domain with a deleted QP domain was used and analyzed via gelfiltration [Fig. 32]. In contrast to the wild type the construct containing the two mutations in the OD elutes at a higher volume and the elution volume corresponds to the molecular mass of a dimer. It is therefore assumed that the same mutation in TAp63γ leads to an inability in forming functional tetrameres. The cell culture data on TAp63γ.D shows a clear impact on the transcriptional activity, which seems to be due to the oligomeric state of the protein.

To examine if there are amino acids within the C-terminus of TAp63γ that have an impact on transcriptional activity of the protein (similar to TAp63α) a deletion analysis was carried out and transcriptional activity was measured for these constructs [Fig. 33]. It is markable that the transcriptional activity does not change until a certain number of amino acids of the C-terminus are deleted. After this point the transcriptional activity is affected dramatically, but recovers after deletion of a number of additional amino acids. Those are the only deletions which show a significant effect on the relative transcriptional activity.

To further elucidate this, two amino acids within the region which led to a drop in transcriptional activity after deletion were mutated to alanine, T426 and S428. The mutations had the same effect on the transcriptional activity like the deletion of this region [Fig. 34]. This might mean that post-translational changes are taking place at these amino acids which are necessary for the function of the protein. Maybe these residues are interacting with other nearby residues in this region if they are post-translationally modified, which also means that they can be regulated i.e. by phosphorylation. A deletion of additional amino acids might result in an activation because the interaction partners which have acted inhibitional and are bound by the phosphorylated threonine and serine in the active conformation of TAp63γ are lost. But this is also speculative and further data has to be obtained on that point. The phosphorylation prediction shows that at least one of the two identified amino acids gets phosphorylated [Fig. 35]. Nevertheless is it necessary to proof this like in the case of the other isoforms by additional experiments.

8. References

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Curriculum Vitae

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Publications

Straub* WE., Weber* TA., Schäfer B., Candi E., Durst F., Ou HD., Melino G., Dötsch V. The C-terminus of p63 Contains Multiple Regulatory Elements with Different Functions. (2009). Submitted.

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Coutandin D., Löhr F., Niesen FH., Ikeya T., Weber TA., Schäfer B., Bullock AN., Yang A., Güntert P., Knapp S., McKeon F., Ou HD., Dötsch V. Conformational stability and activity of p73 require a second helix in the tetramerization domain. (2009). Submitted.

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School and military service

11/1998 – 07/1999	Military service
06/1998	University-entrance diploma
08/1989 – 07/1998	Secondary school (Albert-Einstein-Schule in Maintal)
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Computing skills	Microsoft operating systems, basic knowledge of Linux
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Driving licence	car up to 7,5 t

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- Marathon
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- Volleyball
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Eidesstattliche Erklärung:

Die vorliegende Doktorarbeit habe ich, Tobias Weber, selbstständig verfasst und keine anderen als die angegebenen Quellen verwendet.

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Tobias Weber
Frankfurt am Main, 17. Juni 2009