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Transcriptional regulation of cell life and death decisions by p73

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Stockholm 2013

ISBN 978-91-7549-081-6

Published by Karolinska institute, Stockholm, Sweden.

Printed by Universitetsservice US-AB.

To all my teachers

Abstract

DNA is the repository of genetic information of an organism and contains the information that regulates its proper morphogenesis. Every day, our DNA is attacked by various DNA damaging agents. Our cells developed several safeguarding mechanisms, which protect DNA from different kinds of damages. Sometimes, these defence mechanisms fail and are unable to repair the DNA damage. This failure can negatively influence various biological mechanisms, *e.g.* cell cycle arrest and/or cell death, and the DNA damaged cells may thus turn into a cancer cells. Cancer cells can later become metastatic and spread to other parts of the body. If their spread is not controlled, they can cause serious illness and even death of an individual. Various cancer treatments aim to inhibit tumor progression by inducing cell cycle arrest and/or cell death, which are significantly mediated by the p53 family of proteins.

p73 is the second member to be identified within the p53 family and shares structure and functions with p53. *P73* generates various isoforms, which include full-length transcriptionally active (TA) isoforms and amino-terminal transactivation domain-deficient (ΔN) isoforms. TA isoforms of p73 are considered to act as tumor suppressors, whereas the ΔN isoforms of p73 act functionally analogous to other oncoproteins by counteracting the tumor suppressive functions of p53 and TAp73. In contrast to the *P53,* which is frequently mutated in a variety of human cancers, *P73* mutations are very rarely found. However, altered expression of p73 or expression of abnormal p73 splicing variants is frequently detected in different type of cancers. In some cancer cell lines overexpression of TAp73α confers resistance to anticancer chemotherapeutic agents.

In our interest to identify transcriptional activities and molecular mechanisms of p73 isoforms that influence drug-induced apoptosis, we found that TAp73α inhibits drug-induced apoptosis by inducing the expression of Hsp72, a cell survival protein, in small cell lung carcinoma cells. TAp73α can also prevent caspase-2-induced apoptosis via inhibiting its enzymatic activity. In contrast, TAp73β induces the expression of $p57^{Kip2}$, which holds tumor suppressor properties. The pro-apoptotic effects of the TAp73β isoform seem to partially depend on the induction of $p57^{Kip2}$. We discovered that different p73 isoforms transactivate cell cycle and apoptosis regulating gene promoters with different capability in a cell type-specific manner. Furthermore, we identified a functional cooperation between p53 family members, in which transcriptional activity of a DBD mutated isoform of TAp73α depends on the p53 status of the cell to transactivate cell cycle regulating *P21* gene promoter.

In conclusion, our findings help to understand the isoform-specific transcriptional activities of p73 that determines its pro- and anti-apoptotic effects, upon drug treatment. These findings are expected to help in the development of new strategies to target cancer efficiently based on the p73 isoform present in the tumour and based on the context of the cell.

LIST OF PUBLICATIONS

I. Nyman U, **Naveen Reddy Muppani**, Zhivotovsky B, Joseph B. Hsp72 mediates TAp73α anti-apoptotic effects in small cell lung carcinoma cells. J Cell Mol Med. 2011 Aug;15(8):1757-68.

II. **Naveen Reddy Muppani**, Nyman U, Joseph B. TAp73alpha protects small cell lung carcinoma cells from caspase-2-induced mitochondrial mediated apoptotic cell death. Oncotarget. 2011 Dec;2(12):1145-54.

III. Ulrika Nyman*, Pinelopi Vlachos*, **Naveen Reddy Muppani**, and Bertrand Joseph. The p73β transcriptional target gene p57Kip2 promotes p73β-mediated mitochondrial apoptotic cell death. Manuscript.

IV. **Naveen Reddy Muppani** *, Ulrika Nyman*, Bertrand Joseph. DNA binding domain independent transcriptional activity of p73 depends on the p53 status of the cell. Manuscript.

* Authors contributed equally to this work

Contents

1. Abbreviations

AIF	Apoptosis inducing factor
Apaf-1	Apoptotic peptidase activating factor-1
asDNA	Anti-sense DNA
ATP	Adenosine 5’ triphosphate
Bax	Bcl2-associated X protein
Bcl-2	B-cell lymphoma/leukemia-2
Bcl-XL	B-cell lymphoma-extra large
BH domain	Bcl-2 homology domain
Bid	BH3 interacting domain death agonist
CAD	Caspase-activated DNase
CBP	cAMP response element-binding protein (CREB)-binding protein
CD95	Cluster of differentiation 95
CDK	Cyclin-dependent kinase
c-FLIP	Cellular FADD like IL-1beta-converting enzyme inhibitory protein
ChIP	Chromatin immunoprecipitation
CKI	CDK inhibitor
Cyt c	Cytochrome C
DBD	DNA binding domain
DD	Death domain
DED	Death effector domain
DISC	Death inducing signaling complex
DNA	Deoxyribonucleic acid
Egr-1	Early growth response-1
Endo G	Endonuclease G
ER	Endoplasmic reticulum
FADD	Fas-associated death domain
FLICE	Fas-associated death domain-like interleukin-1beta-converting enzyme
FLIP	FLICE inhibitory protein
HCC	Hepato cellular carcinoma

Hsp	Heat shock protein
IAP	Inhibitors of apoptosis protein
ICAD	Inhibitor of caspase-activated DNase
JNK	c-Jun N-terminal kinase
LMMP	loss of mitochondrial transmembrane potential
LMP	lysosomal membrane permeabilization
LOH	Loss of heterozygosity
MDM2	Mouse double minute 2
MOMP	Mitochondrial outer membrane permeabilization
mRNA	messenger RNA
NGF	Nerve growth factor
NSCLC	Non-small cell lung carcinoma
NuMa	Nuclear mitotic apparatus protein
OD	Oligomerization domain
p53RE	p53 response element
PARP	Poly (ADP-ribose) polymerase
PIAS-1	Protein inhibitor of activated signal transducer and activator of transcription-1(STAT-1)
Pin1	Protein interacting with never in mitosis A1
pRB	Retinoblastoma protein
PUMA	p53-upregulated modulator of apoptosis
RNA	Ribonucleic acid
SAM	Sterile alpha motif
SCLC	Small cell lung carcinoma
siRNA	Small interfering ribonucleic acid
Smac/Diablo	Second mitochondria-derived activator of caspase/Direct inhibitor of apoptosis-binding protein with low pl
SUMO-1	Small ubiquitin-like modifier-1
TA domain	Transactivation domain
tBid	Truncated BH3 interacting domain death agonist
TF	Transcription factor

TM domain	Transmembrane domain
Tyr	Tyrosine
UV	Ultraviolet
WT	Wild-type
Yap	Yes associated protein
ZEB1	Zinc finger E-box Binding homeobox 1
ΔN isofrom	Amino-terminally truncated isoform

2. Introduction

DNA is the repository of genetic information in every living cell and its integrity and stability are essential during all stages of life. DNA replication and gene expression are regulated differently during various stages of life of an organism. For example, in early Drosophila and Xenopus embryogenesis, the duration of S phase lasts for minutes [27, 133], but in fully differentiated cell types S phase can last longer than ten hours [232]. The developmental signals and molecular mechanisms that control the transcription and replication of DNA during development are poorly understood and they are partly regulated by various families of proteins, like minichromosome maintenance (MCM) family [103], transforming growth factor (TGF)-beta family [340], B-cell lymphoma/leukemia-2 (Bcl-2) family [120], and the p53 family [64]. The p53 family proteins are transcription factors, regulate development, cell cycle, apoptosis and DNA damage response with different capabilities [177].

3. The *P53* gene family

In 1979, six groups of investigators independently reported the discovery of a ~53 kDa protein in human and mouse cells. Five of them reported binding of a ~53 kDa protein to the large T-antigen of simian virus 40 (SV40) in cells infected with this virus and the sixth group found that this protein is expressed in several types of mouse tumor cells [72, 176]. The discovered protein was named p53 and the gene encoding p53 (*i.e., P53*) was initially believed to be an oncogene [87, 138]. However, subsequent studies revealed its potential role as a tumor suppressor protein [13, 176, 228]. Two proteins named p73 and p63 were identified in 1997 and 1998 respectively and these proteins share structural and functional similarities with p53. Later onwards, *P53*, *P63* and *P73* genes are collectively regarded as " The *P53* gene family'', which encodes for the transcription factors (TFs) p53, p63 and p73, respectively.

Members of the p53 family contain an amino-terminal transactivation domain (TAD), a central DNA binding domain (DBD) and a carboxy-terminal oligomerization domain (OD). However, p63 and p73 also contain an additional sterile alpha motif (SAM) domain, which is expected to be involved in protein-protein interactions. p73 contains an additional TA domain and a transcriptional inhibitory domain (TID) within its carboxy-terminal region [268]. The three members of the *P53* family display highest sequence similarity within the TAD, DBD and OD (Fig.1) [331]. At the 5´-terminal region, all members of *P53* gene family contain two alternative promoters (p1 and p2),

which produce full-length transactivating (TA) and 5´-terminally truncated (ΔN) primary mRNA transcripts respectively. The primary transcripts generated from these two promoters undergo alternative splicing at both 5´- and 3´-terminal regions to generate various 5´ and 3´ mRNA isoforms [196, 207, 331].

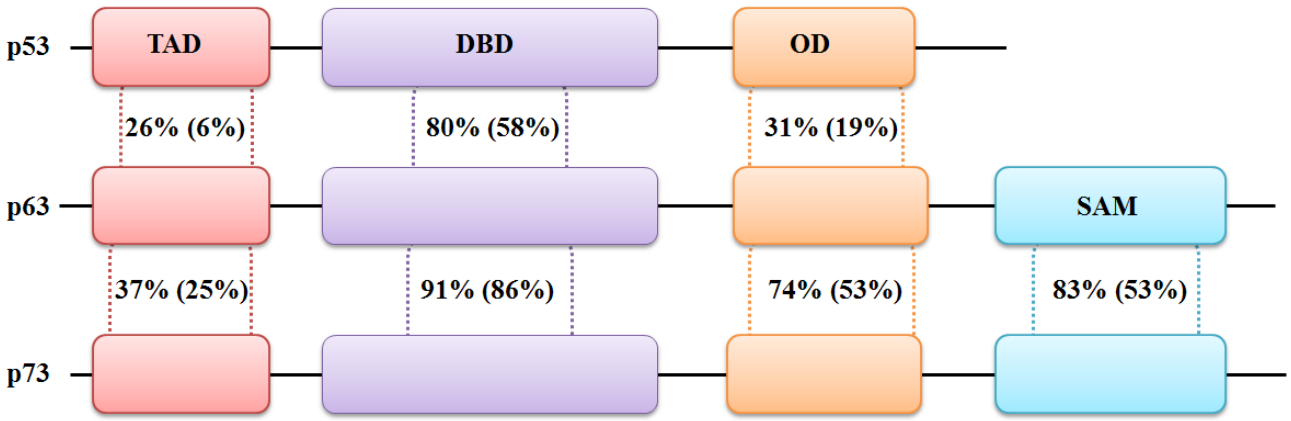


Figure 1. Structure of the human p53 family. Functional domains of p53 family members are depicted. Members of the p53 family contain transactivation domain (TAD), DNA-binding domain (DBD), oligomerization domain (OD) and they are indicated in subtle red, purple and orange colours respectively. However, *P63* and *P73* genes encode an additional sterile α motif (SAM) domain, which is indicated in subtle Aqua colour. Percentage similarity and identity (in brackets) of residues between p53, p63, and p73 is indicated. Values are represented for each individual structural domain [196, 207, 279, 331].

p53 response elements (p53REs) are 20 base pair (bp) DNA segments recognized by the TF p53. p53REs are located within the promoter/enhancer regions across the genome and they are associated with genes that regulate various biological processes, for example, development, differentiation, cell cycle and apoptosis [6, 48, 148, 243]. p53, p63 and p73 proteins are functionally active as tetramers and they recognise p53REs through their DBDs [176, 278]. Though they share structural similarities, their roles in the above mentioned biological processes are dissimilar [176, 254, 331].

3.1. *P53* gene

The *P53* gene encodes the TF p53, which is regarded as ‘‘the guardian of the genome’’ [85]. Identification of p53 created a new era in the cancer research. This protein has attracted the interest of many researchers as it is frequently mutated or deleted in a variety of cancers [329] and p53 knockout mice display spontaneous tumors [83].

P53 contain two distinct upstream promoters (p1′ and p1) and an internal promoter (p2) located in intron four. The use of alternative promoters can lead to the expression of three different 5′-terminal primary mRNA transcripts that give rise to TA, Δ40p53 and Δ133p53 isoforms. Primary transcripts generated from p1 or p1′ promoters undergo alternative splicing at the 5′-terminal region in intron-2 and generate Δ40p53 protein isoforms. Alternative initiation of translation at ATG-40 also generates Δ40p53 protein isoforms. The p2 promoter encodes Δ133p53 protein isoforms. Δ133p53 isoforms are amino-terminal truncated proteins and do not contain the entire transactivation domain and a part of the DNA binding domain [25, 61, 155, 196, 211, 265]. The primary transcripts generated from p1′, p1 and p2 promoters can undergo alternative splicing within the 3′-terminal region and generate *α*, *β*, γ, *δ, ε, ζ* and *ΔE6* isoforms [196, 332].

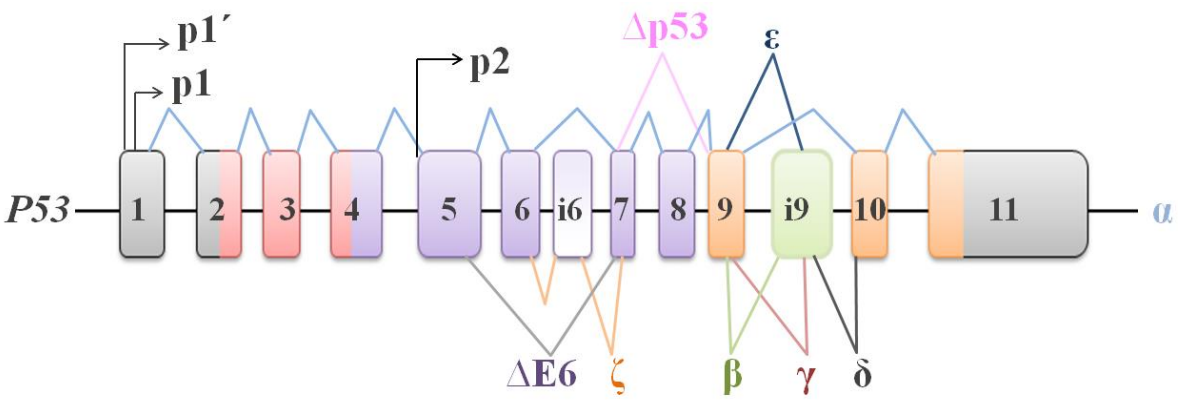


Figure 2. Structure of the human *P53* gene. Alternative promoters (p1, p1′ and p2) and alternative splicing variants of *P53* are depicted. p1 or p1′ promoters of the *P53* gene encodes amino-terminal transactivation (TA) domain containing p53 isoforms. Δ40p53 protein isoforms are encoded from p1 or p1′ promoters and they are amino-terminal truncated proteins due to alternative splicing of intron-2 and/or alternative initiation of translation at ATG-40. Δ40p53 protein isoforms contain only a part of the transactivation domain. Amino-terminal truncated Δ133p53 isoforms, which lack the TA domain and a part of the DBD, are encoded from the p2 promoter of the *P53* gene. Each 5′-terminal mRNA isoform can undergo alternative splicing at the 3′-terminal region and generates *α*, *β*, *γ*, *δ*, *ε*, *ζ* and *ΔE6* carboxy-terminal p53 isoforms. Numbers in the rectangles indicate the exons encoding the p53 protein [194, 196, 219, 332].

p53 is active as a tetramer [54] and it transactivates genes involved in various biological processes like cell cycle and apoptosis [15]. The *P53* gene is commonly mutated in a variety of human cancers [85, 127] and mutations in this gene are reported in more than 50% of the human cancers [329]. Most of the *P53* mutations are located in its DBD region. Some mutations in DBD of p53 do not completely abolish its transcriptional activities, but in many cases these mutations alter transcriptional activities of p53. Donehower *et al.*, in 1992 first reported that *P53*$^{-/-}$ mice are viable, but highly susceptible to spontaneous tumorigenesis [83]. p53 knockout mice revealed that p53 plays an important role in tumor suppression [83, 343]. p53 regulates cell cycle related genes, and thereby controls G1 and G2/M cycle checkpoints [2, 37, 125, 203, 264]. p53 induces expression of pro-apoptotic proteins and induces apoptosis [34, 239, 252, 255, 281, 328].

Both the amino- and carboxy-terminal domains of the p53 protein undergo post-translational modifications like phosphorylation and acetylation, which regulate the functions of p53 [63, 205]. Mutant p53 alters the transcriptional activities of the wild-type (WT) p53 [357] and in some cases it contributes to chemoresistance [85]. A subset of tumor derived mutant forms of p53 interacts with p73 and alters its transcriptional activities [106].

p53 is also associated with the regulation of normal embryonic development. *P53*$^{-/-}$ mice exhibit abnormalities in the central nervous system [7]. p53-deficient mice embryos display abnormal brain and head morphology, and exhibit exencephaly [270]. Decreased pregnancy rate and litter size were observed in matings with *P53*$^{-/-}$ female mice, but not with *P53*$^{-/-}$ male mice [130].

3.2. *P63* gene

The *P63* gene was identified in 1998 and is located at chromosome 3q27–29 [345]. *P63* encodes the TF p63, which is renowned as ‘‘the guardian of the female germ line’’ [21]. Due to use of alternative promoters (p1 and p2) it can produce both TA and ΔN 5′-terminal mRNA isoforms. Moreover, alternative splicing within the 3′-terminal region of these two primary transcripts generates various carboxy-terminal p63 isoforms named α, β, γ, δ and ε [332]. ΔNp63 isoforms counteract the transcriptional activities of TAp63 isoforms and p53 [221]. Similar to other TFs of the p53 family, p63 is active as a tetramer. The p63 isoforms can form complexes among themselves or with mutant p53 and p73. These multiple interactions increase the complexity in understanding the biological roles of the p63 [174, 185].

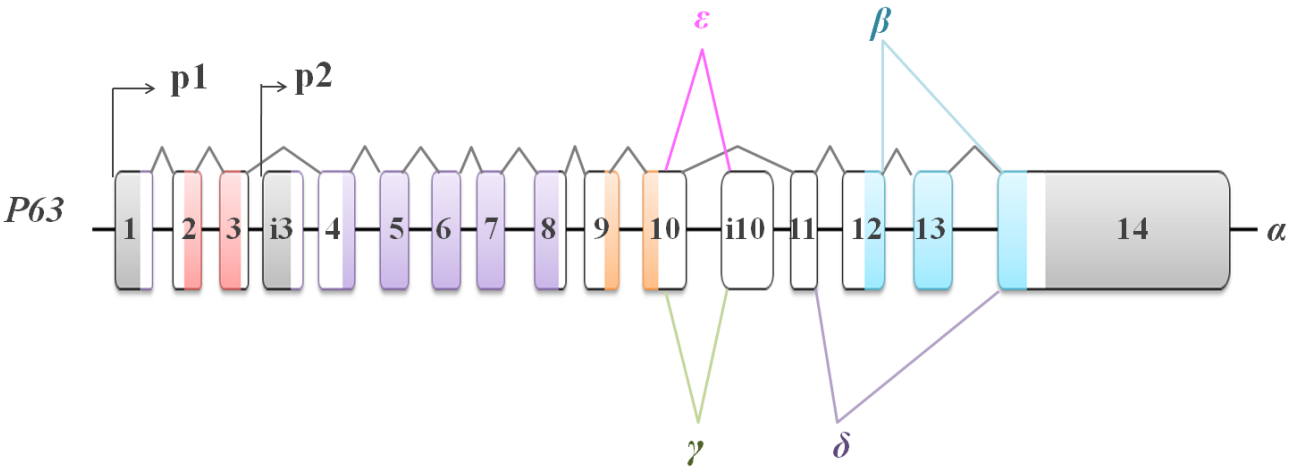


Figure 3. Structure of the human *P63* gene. The *P63* gene contains two alternative promoters (p1 and p2). Full-length transactivation domain containing TAp63 isoforms are encoded from the p1 promoter of the *P63* gene. ΔNp63 isoforms, which lack the transactivation domain, are encoded from the p2 promoter of the *P63* gene. Numbers in the rectangles indicate the exons encoding the p63 protein [35, 36, 219].

p63 plays key roles during embryonic development and regulates ectodermal structures such as hair, skin, teeth and nails [210, 260]. It also regulates the development of limbs, facial features and the urinary system. Mutations in the *P63* gene are associated with various disorders like Rapp-Hodgkin syndrome (RHS), ectrodactyly-ectodermal dysplasia clefting (EEC) syndrome, limb mammary syndrome (LMS) ankyloblepharon-ectodermal defects-cleft lip/palate (AEC) syndrome and split hand/foot malformation type 4 (SHFM4) [260]. *P63*$^{-/-}$ mice revealed that p63 plays an important role in regulating the normal development of epithelia, wound healing, and in preventing the premature ageing as well as tumorigenesis [19, 100, 298].

p63 regulates various cellular activities like cell growth, proliferation, differentiation, cell adhesion, cell cycle and apoptosis [30, 45, 98, 341]. Altered expression levels of the p63 protein are detected in various cancers such as bladder, prostate, lung, breast and cervical cancer [334].

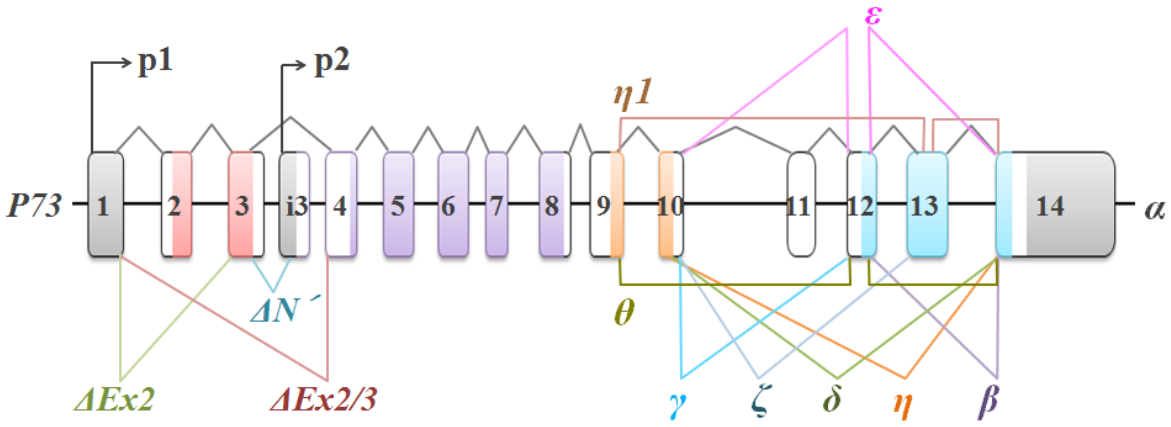
3.3. *P73* gene

P73 was identified in 1997 and maps to the chromosome region 1p36.33, a locus identified to be deleted or hypermethylated in various human cancers [29, 44, 140, 149, 154, 292]. Since its discovery it has been extensively studied, mainly due to its remarkable similarity with *P53*. The *P73* gene encodes the TF p73. Depending on the cell type, p73 is expressed as several structural variants, which may differ either at the amino- and/or carboxy-terminal region. Two different 5´-terminal primary mRNA transcripts (TA and ΔN) are generated as a result of transcription of the alternative promoters (p1 and p2) of the *P73* gene. These primary mRNA transcripts undergo alternative splicing at the 5´-terminal region and generate ΔEx2, ΔEx2/3 and ΔN´ isoforms [290, 291].

Subsequently, 5´-terminal primary mRNA transcripts undergo alternative splicing at the 3´-terminal region and generate nine different putative isoforms named, *α, β, γ, δ, ε, θ, ζ, η* and *η1* [332]. Among all p73 isoforms, TAp73α is the longest and contains a proline rich SAM domain. TAp73β is shorter than TAp73α and lacks the carboxy-terminal SAM domain found in p73α. Differential expression of TA and ΔN isoforms of p73 is expected to be influenced by the regulatory elements present in each promoter and by tissue-specific splicing of its primary mRNA transcripts.

All p73 isoforms contain a DBD and an OD. The carboxy-terminal α and ζ isoforms contain the entire SAM domain. The OD is responsible for homooligomerization and for formation of heterodimers with p63 [65]. ΔNp73 isoforms lack the transactivation domain and are frequently overexpressed in various human cancers, but not in the corresponding normal tissues [11, 353].

(A)



(B)

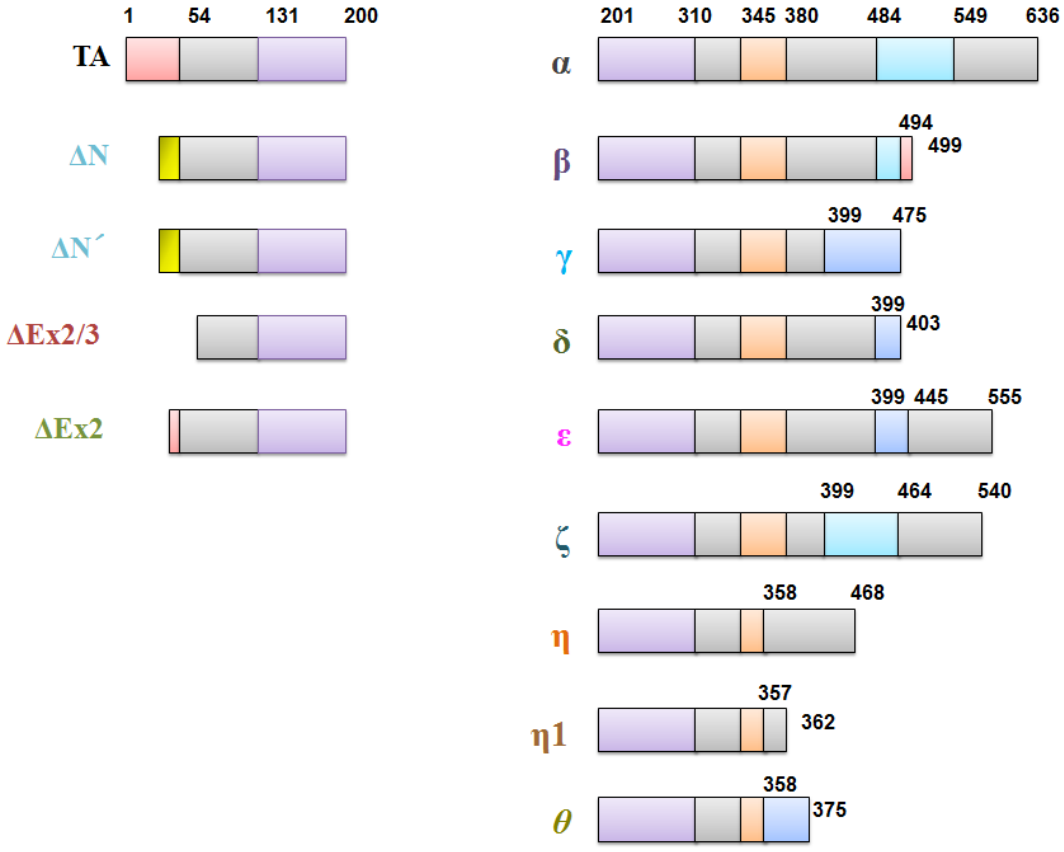


Figure 4. The structure of the human *P73* gene. (A) *P73* gene contains two alternative promoters (p1 and p2). Alternative splicing of the primary transcripts, which are generated from these two promoters undergo alternative splicing both at 5´- and 3´-terminal regions and generates several mRNA variants. (B) Amino-terminal (left) and carboxy-terminal (right) isoforms of p73 are depicted. Theoretically 45 different isoforms can be generated from the *P73* gene as a result of alternative splicing [48, 194, 206, 248, 268, 288]. Initial positions of the amino acids that located at different domains are indicated.

3.4. Interplay between p53 family isoforms

Protein-protein interactions regulate protein stability, activity, and its biological function. These interactions may cause reversible conformational changes in proteins that influence their functions in various signaling networks. These interactions are reversible and influenced by various factors. Due to use of alternative promoters and alternative splicing, members of the *P53* gene family generate a vast number of protein isoforms. These individual protein isoforms have different capabilities to regulate various biological processes like development, differentiation, apoptosis and DNA damage response [177]. Moreover, the interactions between these family members have significant impact on each other's function.

In head and neck squamous cell carcinomas and in breast tumors ΔNp63α inhibits pro-apoptotic effects of TAp73 [174]. In non-small cell lung carcinoma (NSCLC) H1299 cells, transcriptional activities of p73α, β, γ and δ isoforms over the *P21*gene promoter are inhibited by a mutant p53 [295, 337]. A transcriptionally inactive mutant of p73α containing an amino acid substitution (R292H) within its DBD, stabilizes and activates p53 to induce *P21* expression [213]. This suggests that mutations in p73 might not cause a loss of its tumor suppressive activities as they still have the ability to stabilize and activate p53 [213].

ΔNp73 can form complexes with WT p53 in an ovarian carcinoma and endometrial carcinoma. Surprisingly, these complex formations are identified only in the tumors from patients, but not in the corresponding normal tissues [353], suggesting that these complex formations might have significant roles in cancer. ΔNp73 protein isoforms act in a dominant negative fashion over functions of TAp73 and p53 proteins and inhibit their transcriptional activities. ΔNp73 protein isoforms also inhibit both TAp73- and p53-mediated apoptosis [11, 353].

$p63^{+/-}$ $p73^{+/-}$ double heterozygous mice have been shown to have a shorter life span than $p63^{+/-}$ or $p73^{+/-}$ mice [100, 226]. $p63^{+/-}$ $p73^{+/-}$ double heterozygous mice develop spontaneous tumors and malignant tumors at a higher frequency as compared to $p63^{+/-}$ or $p73^{+/-}$ mice. Interestingly, more aggressive tumor phenotypes (higher tumor burden and metastases) and shorter life spans are noticed in $p53^{+/-}$ $p73^{+/-}$ and $p53^{+/-}$ $p63^{+/-}$ mice than in $p53^{+/-}$ mice [100, 226].

4. p73

Transcription factors regulate transcription of gene promoters and they also participate in cellular signaling networks. Among these transcription factors one of the well-studied is p53. p73 is a structural and functional homologue of p53, and transcriptionally regulates various biological processes.

4.1. Transcriptional activities of p73

Transcription factors are proteins and they recognize specific DNA sequences within promoter/enhancer regions of their target genes with their DBD. p73 transcriptionally regulates various genes involved in development, differentiation, cell cycle and apoptosis. p73 is active as a homotetramer. However, it can form heterotetramers with p63 [141]. It recognizes p53REs within the promoter/enhancer regions of various genes [91]. TAD of p73 is responsible for transactivation of its target genes. Our group identified a second TAD at the carboxy-terminal region of p73 that is located within amino acid residues 381-399 [236, 304].

p73 isoforms can bind to p53 REs and transcriptionally activate a subset of p53-target genes, which induce cell cycle arrest, apoptosis, and senescence in a p53-like manner [145, 146, 149]. In 2005, Zhu *et al.*, described that the same gene can be differentially transactivated by different isoforms of p73 [362]. Description of some genes that are differentially regulated by p73α and p73β are presented in Table 1.

Genes regulated by p73 isoforms	Fold increase in relative mRNA expression levels with respective p73 isoform	
	p73α	p73β
P21	17,5	3,2
MDM2	14,8	1,4
GADD45	4,3	1,5
BAX	3,6	0,8
14-3-3σ	3	17

Table 1. Differential induction of cellular targets by p73α and p73β [362].

Our group identified that TAp73α, but not TAp73β induces the expression of anti-apoptotic Hsp72 protein in SCLC cells. We also reported that in several cell lines $p57^{Kip2}$ protein expression is induced by TAp73β, but not by TAp73α. Furthermore, our group found that co-expression of p53 with DBDmut p73α (a R268Q, R300C mutant of p73) promotes nuclear localization of DBDmut p73α protein. Increased nuclear localization of DBDmut p73α by p53 seems to promote the recruitment of DBDmut p73α at the *P21* gene promoter and thereby inducing its transactivation. Association of p73 with other proteins and its posttranslational modifications appears to influences its transcriptional activities [59, 197, 215, 297, 355].

4.2. Role of p73 in development

Gene expression is carefully regulated during embryonic development. Temporal and spatial control of gene expression is monitored by several mechanisms such as lionization (X-chromosome inactivation), epigenetic modifications, TF-dependent transcription and mRNA splicing. Thousands of proteins carefully coordinate with each other during all stages development of a living organism. The interactions between these proteins and their temporal expression are important for its proper development. p73 is one of the proteins that plays important roles in development. p73 isoforms seem to play a role in the terminal differentiation of human skin keratinocytes [215]. The loricrin and involucrin genes are induced during the keratinocyte differentiation and they are recognized as molecular markers of epidermal differentiation [253]. p73γ and p73δ transactivates loricrin and involucrin gene promoters [68], suggesting a role of p73 in keratinocyte development.

Downregulation of the N-Myc proto-oncogene [310] and upregulation of the Rb tumor suppressor [67, 256] are reported during differentiation of neuroblastoma cells *in vitro*. In N1E-115 murine neuroblastoma cells, decrease of N-Myc levels and increase of the pRB levels are noticed upon overexpression of p73β [67], suggesting that p73β might be involved in neuroblastoma differentiation. p73 is accumulated during the murine neuroblastoma cell differentiation induced by retinoic acid treatment [67], further supporting that it might play an important role in differentiation.

Withdrawal of NGF induces apoptosis in sympathetic neurons of mice and it has been shown that ectopic expression of either ΔNp73α or ΔNp73β protects sympathetic neurons from NGF depletion induced apoptosis [250]. Moreover, ΔNp73β interacts with p53 and inhibits p53-induced neuronal apoptosis during development of the mouse nervous system [215, 250], collectively suggesting that ΔNp73β is important for the survival of neurons. ΔN isoforms are predominantly expressed during development of the brain [336] suggesting that p73 expression might be important for normal

neuronal development. In support of this idea, $\Delta Np73^{-/-}$ mice were shown to have a reduced neuronal density in the brain [336]. p73 null mice show central nervous system defects and pheromone abnormalities, hippocampal dysgenesis with aberrant lower blade of the DG (dentate gyrus), loss of CR (Cajal-Retzius) cells, hydrocephalus and abnormalities in the limbic telencephalon. Moreover, *P73*$^{-/-}$ mice are infertile due to defects in early embryonic development [312], and a majority of p73 knockout mice die at an early stage due to chronic infections [346].

4.3. Regulation of p73 expression and its activity

Activity and function of a protein is influenced by factors such as its level of expression, post-transcriptional modifications and interactions with other proteins. p73 is expressed differently in different cells depending on the stage of life. Deregulation of p73 can alter its transcriptional activities on genes involved in biological processes and also its participation in cellular signaling networks. p73 expression, activity and function are regulated at four different levels.

4.3.1. Transcription of *P73*

Transcriptional regulation of the *P73* gene is important to maintain its proper cellular levels. In several cancers, altered levels of p73 expression were identified and low or high levels of p73 can significantly alter the biological activities regulated. Stimuli, such as DNA damage [49], viral factors [39] and transcriptional regulators [102, 172] can regulate *P73* gene expression at the transcriptional level. A regulatory 1 kb stretch of the intron 1 region upstream of the initiating ATG codon in exon 2 of *P73*, contains six binding sites for the transcriptional repressor ZEB1 (δEF1). Inhibition of ZEB1 (δEF1) increases p73 expression [102]. p1 and p2 promoters of *P73* are located within exon 1 and intron 3, respectively. Upstream of and within the p1 promoter lies putative binding sites for transcriptional regulators like Sp1, E2F, AP-2 and Egr-1,-2,-3 [81]. The p1 promoter of *P73* contains three E2F, a transcription factor family, binding sites and *in vitro* binding of E2F1 to two of these binding sites mediates an increased expression of TAp73 transcripts [268, 282]. Early growth response factor-1(Egr-1) is one of the rapidly induced early response genes to various environmental signals [344]. Egr-1transactivates the p1 promoter, but not the p2 promoter of the *P73* gene [350]. The adenovirus early region 1A (E1A) also transactivates its p1 promoter, but not the p2 promoter [99]. E2F1, c-Myc, and E1A induces expression of full-length p73α and p73β [352].

Much less is known about transcriptional regulation of the p2 promoter. This promoter contains a p53 RE [114] and is transcriptionally regulated by both p53 [153, 326] and TAp73 [223]. ΔNp73 physically interacts with TAp73 and p53, and regulates their functions by blocking their transcriptional activity thus mediating a dominant negative feedback loop [223].

Loss of p73 mRNA expression is noticed due to hypermethylation (a histone mark for gene silencing) of *P73* in hematological malignancies like acute lymphoblastic leukemia (ALL) and Burkitt's lymphomas [58]. *P73* was also found hypermethylated in 94 % of natural killer (NK) cell lymphomas [29], suggesting an epigenetic regulation of p73 expression.

4.3.2. Post-transcriptional modifications of p73 mRNA

As mentioned earlier, as a result of alternative splicing, the p73 primary transcripts generated form the alternative promoters can be expressed as various carboxy- and amino-terminal variants (refer to **Figure 4A.)**. The p1 and p2 promoters of the *P73* gene encode amino-terminal TAp73 and ΔNp73 isoforms, respectively. p2 promoter generates, two mRNAs (ΔNp73 and ΔN′p73) differing in their 3′-untranslated region by two-way usage of exon 3′. However, they encode an identical ΔNp73 protein, which lacks the amino-terminal transactivation domain found in TAp73 proteins [136]. p73Δex2 and p73Δex2/3 5′-terminal spliced transcripts are also detected in several human tumor cells [289]. Expression of p73Δex2, a 5′-terminal spliced variant of p73 transcript with the deletion of exon 2 was detected in the ovarian tumor cell lines, but not in the human normal ovarian surface epithelial (HOSE) primary cultures [227]. Deletion of exon 2 would delete the first 48 amino acid residues at amino-terminal region as compared to the full-length TAp73. In hepatocellular carcinomas, aberrant spliced p73 variants *i.e.*, p73Δex2, p73Δex2/3 that do not contain exon 2, and exon 2 and 3, respectively have been identified [291].

Among the p73 variants, TAp73α is the longest isoform and contains all 14 exons. Carboxy-terminal β ,γ, δ, ζ, and ε isoforms do not contain the exon 13, exon 11, exons 11–13, exons 11 and 12, and exons 11 and 13, respectively [136]. Ishimoto O *et al.*, reported that among 33 tested human tissue, the ΔN′p73 transcript was only detected in the pancreas and the ΔNp73 transcript was only detected in fetal lung and corpus callosum among eight sub regions of the brain. p73α expression was identified in a variety of human tissue like, brain, pancreas, testis, prostate and cerebellum, whereas p73β expression was identified in lymph nodes, spinal cord and cerebellum. p73γ expression was detected in liver, small intestine, fetal lung, and cerebellum (for further details, refer to [136]).

Expression of p73η, which lacks the exons 10-13, was detected in neuroblastoma and in the MCF-7 breast carcinoma cell line [279]. Its expression was not detected in B-lymphocytes from seven healthy donors. The p73η´ is generated from splicing of two nucleotides at 3´end of exon 9 and complete splicing of exons 10-12, whereas the last 18 nucleotides of exon 13 are retained. In p73θ, exons 10, 11 and 13 are completely spliced out, but the exon 12 is retained. In contrast to the p73η, p73η´ and p7θ isoforms have been also identified in non-tumor lymphocytes.

Although p73 can be expressed as 45 different putative isoforms (refer to **Figure 4B.)**, to my knowledge, no human tissue or cell line is yet identified that expresses all of these isoforms. Expression of p73 spliced variants seems to be regulated by the tissue-specific mRNA splicing mechanisms.

4.3.3. Post-translational modifications of p73

Post-translational modifications are the chemical modification of one or several amino acids of a protein. Functions of a protein can be greatly influenced by the modification of functional groups, such as acetate, phosphate, lipids and carbohydrates, of its amino acids. These modifications can be reversible and influence the structure, function, stability, localization and activity of a protein.

c-abl, a non-receptor tyrosine kinase, phosphorylates TAp73α/β and ΔNp73α/β and stabilizes them. c-abl phosphorylates TAp73α on tyrosine (Tyr)-99, and to a lesser extent on Tyr-121 and Tyr-240 [1, 315, 351], and its kinase activity is required for pro-apoptotic activity of p73 [1]. c-Jun N-terminal kinase (JNK) physically interacts with and phosphorylates p73. JNK-mediated phosphorylation promotes p73 transcriptional activity on growth arrest and DNA damage 45 *(GADD45)* and *BAX* gene promoters [143]. p300, a histone acetyltransferase (HAT), acetylates p73α after DNA damage, and inhibition of p300 by Lys-CoA decreases apoptosis induced by p73α [59].

Protein inhibitor of activated STAT-1(PIAS-1) binds and sumoylates p73α and stabilizes it. This sumoylation leads to the inability of p73α to transactivate *BAX* and *P21* gene promoters. PIAS-1 expression starts at the end of the G_1 phase, peaks during the S phase, and completely disappears in the G_2 phase of the cell cycle. This PIAS-1-induced p73-mediated inhibition of p21 expression seems to facilitate G_1/S transition [217].

4.3.4. Interactions of p73 with various proteins and its degradation

As mentioned earlier, protein-protein interactions regulate activity and stability of a protein. Various proteins interact with p73 and post-transcriptionally modify it, thereby influencing its stability and biological functions.

ΔNp73 physically interacts with p73 and p53 and inhibits their pro-apoptotic functions [224]. The p53 inhibitor Mdm2 interacts with p73α. However, in contrast to Mdm2-p53 interaction, Mdm2 interaction with p73α does not promote its degradation, but inhibits its transcriptional activity [42, 354]. Interestingly, p73 transactivates *MDM2* promoter [171, 327, 362], suggesting that a feedback regulatory loop exists in the p73-Mdm2 signaling network. In this network, Mdm2 blocks the transcriptional activity of p73, but does not affect its levels. p300 and cAMP response element-binding protein-binding protein (CBP) binds to p73α and enhances its transcriptional activity, thus act as transcriptional co-activators. Moreover, p300, a transcriptional co-activator enhances the apoptotic function of p73α [355]. Both Mdm2 and p300/CBP bind to the amino-terminal region of p73. The interaction of Mdm2 with p73 blocks the p73-p300/CBP interactions. This might be a reason for the transcriptional inactivation of p73 by Mdm2 [354]. c-Myc, a proto-oncogene protein, physically interacts with p73α and inhibits p73α-mediated transcriptional activation of *P21* and *BAX* gene promoters [330]**.** Yes associated protein (Yap) interacts with p73 and enhances its transcriptional activity on the *BAX* promoter [296]. Human Papillomavirus (HPV)-E6 protein associates with p73 and inhibits its ability to transactivate the *P21* gene promoter [245]. Protein interacting with ‘‘never in mitosis A (NIMA) 1’’ (Pin1), a prolyl isomerase, stabilizes p73 by physically interacting with it. Pin1 is required for p73 to transactivate genes involved in apoptosis [197]. c-Abl interacts with p73 via SH3 (Scr-homology-3) domain and phosphorylates it. Phosphorylation of p73 by c-abl stabilizes p73 protein and enhances its apoptotic function [110].

Proteolysis is the process of breakdown of proteins into smaller polypeptides or amino acids. Some enzymes, *i.e.* proteases, hydrolyse peptide bonds to degrade proteins. p73 degradation is regulated, in part, by the ubiquitin proteasome system, which is a sophisticated cellular proteolytic machinery in many eukaryotic cells [42, 109, 156, 165]. Promyelocytic leukemia (PML) protein physically interacts with p73, and promotes p300-mediated acetylation as well as stabilization of p73. PML reduces ubiquitinylation levels of p73, thereby protecting it from the ubiquitin proteasome system-mediated degradation [20]. Pirh2, a p53 regulated RING finger E3 ubiquitin ligase, physically interacts with p73. Pirh2 polyubiquitinates p73, thereby promoting its ubiquitin proteasome mediated degradation [147].

The degradation of p73 seems to be isoform-dependent, for example, upon DNA damage, ΔNp73 is degraded more rapidly than TAp73 [195, 267, 324]. TAp73α induces the expression of PIR2, a ring finger domain ubiquitin ligase p73-induced RING 2 protein that differently regulates the stability of TAp73 and ΔNp73 isoforms. Co-expression of PIR2 and TAp73α results in an increase in TAp73α protein levels, whereas co-expression of PIR2 and ΔNp73α results in a decrease in ΔNp73α levels. This suggests that PIR2 differently regulates TAp73 and ΔNp73 protein levels [277]. p73α and p73β undergo differential degradation by the proteasome, where p73α is sensitive and p73β is not sensitive to this degradation [212]. p73α, but not p73β, is covalently sumoylated by the small ubiquitin-like modifier-1 (SUMO-1) on lysine residue 627. Although SUMO-1-mediated modification is not absolutely required for degradation of p73, sumoylated p73 is more rapidly degraded by the proteasome than unmodified p73 [212].

5. Apoptosis

Cell death mechanisms like anoikis, necrosis, autophagy and apoptosis play important roles in maintaining the tissue homeostasis. These cell death mechanisms are important for regulating development, cell growth, immune response and clearing of redundant or unnecessary cells [93]. Apoptosis is a well-studied cell death mechanism in which cell blebbing, shrinkage, and fragmentation of chromosomal DNA and nuclei takes place. Billions of cells die by apoptosis in the human body every day, just to keep balance with the numbers of new cells generated from the body's stem cell populations [121, 259].

5.1. Morphological and Biochemical features of an apoptotic cell

During the process of apoptosis a lot of biochemical and morphological changes occur in the apoptotic cell. Caspases, a family of cysteine and aspartic proteases, are activated. They play a major role in execution of apoptosis by cleaving and/or activating several substrates, for example, endonucleases, nuclear lamins, PARP (poly-ADP ribose polymerase), PAK2 (p21-activated kinase 2). Subsequently, the apoptotic cell loses microvilli and desmosomes, and then loses contact with the surface or adjacent cells. Activated endonucleases cleave nuclear DNA into internucleosomal fragments. Thus, DNA ladder pattern formation upon regular agarose gel electrophoresis can be identified. The plasma membrane extends and seals the detached solid cellular material into vesicles

named ‘‘apoptotic bodies’’. These apoptotic bodies are packed with densely crowded cellular organelles and fragments of the nucleus.

Ladder pattern of DNA fragmentation, caspases activation and apoptotic body formations are key features of an apoptotic cell death. In the immune system well developed organisms, such as humans and mice, phagocytes accumulate at the site where apoptotic cells are located. They recognize the apoptotic cells through receptors and engulf them. Engulfed apoptotic cells are then processed within the phagocytes [89], thus no signs of inflammation are noticed.

5.2. Stimuli that initiate apoptosis

The process of apoptosis can be initiated by stimuli like DNA damage (*e.g.* caused by ionizing radiation and alkylating agents), cell membrane damage (*e.g.* caused by UV light, oxidizing agents and heat) and stimuli that activate death receptors (*e.g.* Tumor necrosis factor-α and Fas ligand)

5.3. Proteins and Organelles involved in regulation of apoptosis

The apoptotic process is regulated by a variety of proteins and organelles. However, their roles in the regulation of apoptosis are still not fully understood.

5.3.1. Caspases

Caspases play important roles in initiation and execution of apoptosis and inflammation. They are expressed as pro-enzymes (zymogens) in the cell. Upon proper stimuli, caspases are processed and activated. To date, 14 caspases have been identified and they are involved in inflammatory responses (*e.g.* caspase-1, -4, -5 and -12) or apoptosis. The caspases that are involved in apoptosis are classified as initiator caspases (caspase-2, -8, -9 and -10) or effector caspases (caspase-3, -6 and -7) [5].

Processing and activation of caspases are regulated by a number of molecules, such as inhibitor of apoptosis protein (IAP), fas-associated death domain (FADD), apoptotic protease activating factor-1 (Apaf-1), Bcl-2 family members and FADD-like interleukin-1beta-converting enzyme (FLICE) inhibitory protein (FLIP) [86]. Overexpression of pro-apoptotic caspases induces apoptosis in various cell lines. For example, overexpression of caspase-2 induces apoptosis in small cell lung carcinoma (SCLC) cells [218]. Active caspase-2 cleaves full-length Bid to active Bid (also

known as truncated Bid or tBid). tBid activates Bax, thereby promoting the release of pro-apoptotic proteins from mitochondria. Overexpression of caspase-2 promotes translocation of Bax to the mitochondria, thereby inducing mitochondria-mediated apoptotic cascade [28, 118, 170, 193].

5.3.2. Heat shock proteins

Heat shock proteins (Hsps) were discovered in the larvae of *Drosophila melanogaster* after exposing them to heat shock [166]. Their levels are also elevated under other types of stress stimuli. Hsps are encoded by the *HSP70* and *HSP90* gene families. Molecular weights of Hsps range from ˜15 to 110 kDa. Based on their function and molecular weights they are divided into several groups (for more details, refer to [126, 333]). Hsps are localized in different compartments of the cell, like cytosol, mitochondria, endoplasmic reticulum (ER) and nucleus. They are involved in the maintenance of protein structures, refolding of misfolded proteins, prevention of protein aggregation and the degradation of unstable proteins [40, 51, 76, 214, 242].

Hsps also play key roles in the regulation of apoptosis. Hsp27, -70, and -90 proteins function as anti-apoptotic, whereas Hsp60 functions as pro-apoptotic proteins. They act at multiple levels of the apoptotic signalling pathway [80], for example, HSPA1A/Hsp72/Hsp70–1 (referred to later as Hsp72) inhibits lysosomal membrane permeabilization, thereby inhibiting the release of cathepsins and other proteases, which are involved in the apoptotic signaling pathway [23, 119]. In addition, Hsp72 inhibits the apoptotic cascade by inhibiting JNK, preventing the translocation of Bax to the mitochondria [183], by interacting with apoptosis-inducing factor (AIF) and Apaf-1 [271], and by inhibiting the processing of caspase-9 and -3 [16].

5.3.3. The Bcl-2 family of proteins

Bcl-2 family proteins are prime regulators of the intrinsic apoptotic pathway and govern mitochondrial outer membrane permeabilization (MOMP). Bcl-2 homology (BH) domains are characteristic features of the Bcl-2 family proteins, which share one or more of the four BH domains (*i.e.* BH1-4). Bcl-2 family members are categorized as anti- or pro-apoptotic proteins. The anti-apoptotic proteins (*e.g.* Bcl-2, Bcl-XL, Mcl- 1, A1 and Bcl-W) contain all four BH (BH1,-2,-3 and -4) domains and a carboxy-terminal transmembrane (TM) domain. The pro-apoptotic family members (*e.g.* Bax, Bak and Bok) contain the TM domain and BH1,-2 and -3, but not BH4. Other

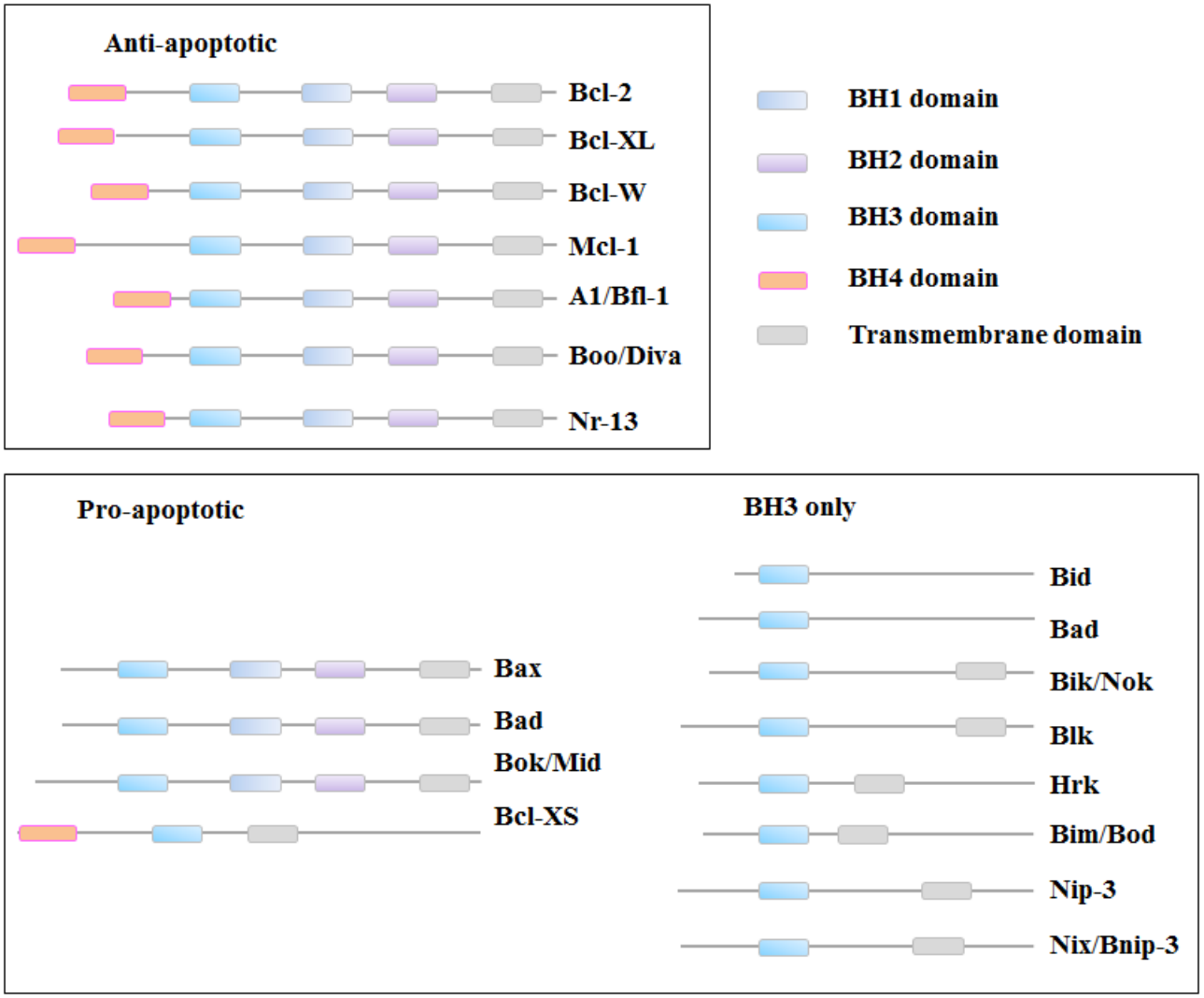


Figure 5. Structure of the Bcl-2 family of proteins. The Bcl-2 family proteins are key regulators of the intrinsic apoptotic pathway and govern mitochondrial transmembrane potential and subsequent release of the cytochrome c (Cyt c). Members of the Bcl-2 family contain characteristic Bcl-2 homology (BH) domains. The anti-apoptotic Bcl-2 family members contain all four BH (*i.e.* BH1-4) domains and their overexpression inhibits apoptosis. They prevent BH3-only proteins induced oligomerization of the pro-apoptotic Bcl-2 family members, for example, Bax/Bak in the mitochondrial outer membrane. Most of anti-apoptotic Bcl-2 family members contain transmembrane (TM) domains thus they are typically associated with membranes. The pro-apoptotic Bcl-2 family members promote apoptosis by forming pores in the outer mitochondrial membrane. The BH3-only subfamily of pro-apoptotic Bcl-2 family members contains only BH-3 domain and/or TM. The mammalian BH3-only protein family contains eight members (Bid, Bad, Bim, Bik, Bmf, Noxa, Puma and Hrk) and upon overexpression they promote apoptosis. Bid is activated via proteolysis mediated by caspase-8 and active Bid (*i.e.* tBid) activates Bax/Bak and induces releases of Cyt c [60, 75, 124, 157, 308].

pro-apoptotic proteins of the Bcl-2 family contain the BH3 domain and TM (*e.g.* Bik and Bnip) or only the BH3 domain (*e.g.* Bad, Bid, Bim and Egl-1).

Bcl-2 family members can form homo- or heterodimers among themselves [41]. Anti-apoptotic Bcl-2 proteins are mostly found in membranes of the ER, mitochondria and the nucleus. Most pro-apoptotic Bcl-2 family proteins are localized in the cytosol. Upon initiation of the death cascade, pro-apoptotic Bcl-2 family proteins undergo conformational changes and integrate into the outer mitochondrial membrane, and promote loss of mitochondrial transmembrane potential [90, 115, 167, 339].

Bax is a globular protein that consists of nine α helices [300]. It is a soluble protein located in the cytosol. The amino-terminal part of the Bax protein (*i.e.* amino acid residues 20-37) contains a mitochondrial targeting sequence, and deletion of this sequence results in the termination of its mitochondrial localization ability [46]. During the process of apoptosis Bax undergoes conformational changes and translocates to the mitochondria [339], subsequently mitochondria lose their transmembrane potential [168]. Cleavage of Bid by caspase-8 leads to its activation [107, 182]. Cleaved active carboxy-terminal fragment of Bid (*i.e.* tBid), translocates to the mitochondria. tBid activates Bax/Bak proteins, thereby promoting MOMP [75, 157].

Overexpression of anti-apoptotic Bcl-2 protein decreases the pro-apoptotic responses of irradiation and chemotherapy [152], whereas overexpression of pro-apoptotic proteins, *e.g.* Noxa, Puma and Bax, promotes drug-induced apoptosis [184, 360]. Del Poeta *et al*., reported that the ratio between Bax and Bcl-2 (pro- and anti-apoptotic proteins, respectively) influences both a longer overall survival (OS) and disease-free survival (DFS) in acute myeloid leukemia (AML) [71].

Two models exist, which explain the roles of BH-3 only proteins in promoting apoptosis. The first model is called the ‘‘direct activation model’’, in which BH-3 only proteins bind to Bax or Bak, and induce their conformational change, which leads to their activation. The second model is called the ‘‘neutralization model’’, in which pro-apoptotic BH-3 only proteins bind and inactivate the anti-apoptotic Bcl-2 family of proteins like Bcl-XL and Bcl-2 [50].

According to ‘‘The rheostat model’’ proposed by Korsmeyer *et al.*, formation of heterodimers and the balance between the pro- and anti-apoptotic proteins of the Bcl-2 family creates a life or death switch for a cell. This model suggests that pro- and anti-apoptotic proteins directly counterbalance

each other functions, where more abundant protein regulate whether the cell dies or survives [163]. According to a model proposed by Edlich *et al.*, Bax is constantly translocated to the mitochondria in healthy cells, where anti-apoptotic Bcl-2 family members, *e.g.* Bcl-XL, bind to Bax and retranslocate it into the cytoplasm. Blocking of anti-apoptotic Bcl-2 proteins by BH3-only proteins, *e.g.* Bim, results in Bax accumulation on the mitochondria [84].

5.3.4. Mitochondria

Mitochondria are membrane bound organelles that produce ATP (adenosine 5'-triphosphate). They are found in eukaryotes and have been described as ''the energy power house of the cell'' [202]. Apart from their role in generation of energy and well established role in the induction of apoptosis [113, 139], they are also involved in cellular processes such as differentiation [73], cell cycle [97, 240], cell growth [94] and cell signaling [302].

Pro-apoptotic Bcl-2 family proteins regulate the release of pro-apoptotic proteins from the mitochondria. During the process of apoptosis, one or several of the BH3-only proteins (Bad, Bid, Bik, Bim, Bmf, Hrk, Nix, Noxa, Bnip3 and Puma) undergo activation and directly or indirectly activate Bax and/or Bak. For example, tBid, Bim and Puma directly bind and activate Bax and Bak. Active Bax and Bak induce the loss of mitochondrial transmembrane potential (LMMP). As a result pro-apoptotic mitochondrial proteins like Cyt c, second mitochondrial activator of caspases/direct inhibitor of apoptosis proteins (IAP) binding protein with low pl (Smac/Diablo), AIF and endonuclease G (Endo G) are released into the cytosol.

5.3.5. The Cip/Kip family of proteins

Cyclin-dependent kinase (CDK) inhibitors (CKI's) bind and inhibit the activity Cyclin/CDK complexes, which promote cell proliferation [57] to prevent abnormal cell proliferation [283, 284]. To date, seven CKI's have been identified in mammals and they are classified into two families according to their structural and functional similarities [225]. The Cip/Kip (CDK interacting protein/kinase inhibitor protein) family comprises $p21^{Cip1}$ (also called Waf1, Sdi1, or CAP20 and later it is referred to as p21), $p27^{Kip1}$ and $p57^{Kip2}$ [225]. These three members have a conserved cyclin-dependent kinase binding/inhibitory domain at its amino-terminal region. The other family, designated Ink4, comprises p16(INK4a), p15(INK4b), p18(INK4c) and p19(INK4d) [62]. These family members have four ankyrin motif tandem repeats in common. The Ink4 family of proteins

inhibits the activity of CDK4 or CDK6 specifically, whereas the Cip/Kip family of proteins shows a broad spectrum of inhibitory effects on cyclin/CDK complexes [74].

p21 is induced by various stress stimuli, and its induction may cause cell cycle arrest. Overexpression of p21 results in cell cycle arrest [108]. p21 plays an important role in preventing tumor development by suppressing tumor growth *in vitro* and *in vivo* [108]**.** Mice lacking p21 are more susceptible to chemically induced skin carcinoma [246, 314]. p21 holds pro-apoptotic functions and its overexpression promotes the drug-induced apoptotic response in a variety of cell lines [162, 187]. p21 overexpression induces apoptosis in xenografts of human cervical cancer cells [316].

$p57^{Kip2}$ expression promotes drug-induced apoptosis in several cell lines [273, 325]. p73β-induced apoptosis was reduced after inhibition of $p57^{Kip2}$ by siRNA, suggesting that $p57^{Kip2}$ mediates the apoptosis [112]. These observations indicate roles of the $p57^{Kip2}$ during apoptosis. However, the precise mechanisms of $p57^{Kip2}$ in apoptosis are still unclear. On the other hand, some studies have also shown anti-apoptotic roles for p21 [9, 131] and $p57^{Kip2}$ [47].

5.3.6. Lysosomes

Lysosomes are acidic, single-membrane bound organelles present in eukaryotic cells. They are also called the suicide bags of the cells. They contain several acid hydrolases, including phosphatases, nucleases, glycosidases and proteases [12]. Of the lysosomal proteases, the cathepsin family is well characterized. According to the amino acids of their active sites that confer catalytic activity, the cathepsins are subdivided into cysteine (cathepsin B, C, F, H, K, L, O, S, V, W, and X), serine (cathepsin A and G) and aspartic (cathepsin D and E) cathepsins [356].

A wide range of apoptotic stimuli, such as death receptor activation, oxidative stress, DNA damage and growth factor starvation [38, 95, 160, 293], can induce lysosomal destabilization, which will later be referred to as lysosomal membrane permeabilization (LMP). For many years, lysosomes were thought to be mainly involved in necrotic and autophagic cell death. Accumulating evidences are indicating that partial lysosomal membrane permeabilization (LMP) and consequent release of lysosomal proteases into the cytosol may initiate or execute the apoptotic cell death [117, 173, 293, 318, 319]. Partial or selective lysosomal permeabilization can lead to release of other hydrolases, H^+ (causing acidification of the cytosol) and Ca^{2+} into the cytosol during onset of apoptosis [43]. The lysosome-mediated apoptotic pathway can be activated by death receptors, lipid mediators and photodamage [117]. In some circumstances LMP is an early event and essential for apoptosis

signaling to proceed, while under other circumstances LMP occurs at a late stage of the cell death process that amplifies the death signals [142].

Microinjection of cathepsins into the cytosol initiates apoptotic cell death [24, 262, 280]. Disruption of lysosomal membrane results in the release of cathepsins (e.g. cathepsin D, cathepsin B and L) into the cytosol. Once in the cytosol, they activate Bid, degrade the anti-apoptotic Bcl-2 protein and X-linked inhibitor of apoptosis (XIAP), thereby initiating the apoptotic cascade [38, 53, 160, 320]. This results in Cyt c release from mitochondria and activation of caspases. In activated human T lymphocytes, upon staurosporine treatment, cathepsin D, which is released from the lysosomes, induces Bax activation independent of Bid cleavage. This results in mild outer mitochondrial membrane permeabilization, a result limited to AIF release [22].

5.4. Apoptotic pathways

Apoptosis can be triggered by various stimuli, which can initiate either of the two following apoptotic signalling pathways.

5.4.1. Extrinsic pathway

Members of the tumor necrosis factor (TNF) receptor superfamily are popularly known as ‘‘death receptors’’. These death receptors contain cysteine rich extracellular domains and a cytoplasmic domain, the “death domain (DD)’’ [10]. These death receptors are involved in transmitting the extracellular death signals to the intracellular apoptotic signalling cascade. Transmembrane death receptor and death signal interactions initiate the extrinsic apoptotic pathway. Stimulation of these death receptors by corresponding ligands results in the cytosolic recruitment of an adapter molecule named FADD through the interaction between DDs. Thereafter, FADD recruits DED (death effector domain)-containing procaspase-8 through DED/DED interactions, thus forming the death inducing signalling complex (DISC) [52, 204]. Formation of DISC facilitates the autocatalysis of procaspase-8 and thereby its activation [359].

Cellular-FLIP (c-FLIP), an anti-apoptotic regulator, binds to FADD and/or caspase-8 and negatively regulates their activities. c-FLIP prevents the DISC formation, thereby inhibiting the extrinsic apoptotic cascade [269].

5.4.2. Intrinsic pathway

Diverse non-receptor-mediated stimuli such as radiation, hypoxia, hyperthermia, toxins, viral infections, and free radicals can induce LMMP [88]. LMMP results in the release of pro-apoptotic factors (*e.g.* Cyt c, AIF, Endo G and Smac/Diablo) into the cytosol from the mitochondria. Released Cyt c takes part in the formation of the apoptosome, which is composed of Cyt c, Apaf-1 and procaspase-9. Apoptosome formation facilitates activation of caspase-9, which is involved in the further processing and activation of other caspases, like caspase-3 and -7 [201].

IAPs are a family of anti-apoptotic proteins. IAPs can bind and inhibit the activity of caspases, thereby inhibiting the apoptotic cascade. When Smac/Diablo is released from the mitochondria, they interact with IAPs and antagonizes them, thereby facilitating the activation of caspases [201].

5.4.3. Execution phase of extrinsic and intrinsic apoptotic pathways

The extrinsic and intrinsic apoptotic pathways leads to the execution phase of the apoptotic pathway, in which "effector/executioner" caspases (caspase-3, -6, and -7) play key roles. Caspase-3 can process and activate many substrate proteins including endonucleases, *e.g.* inhibitor caspase-activated DNase (ICAD). Cleavage of ICAD results in the release of caspase-activated DNase (CAD). Released CAD translocates into the nucleus where it cleaves DNA into internucleosomal fragments [306]. Active caspase-3 and -7 cleave and/or activate several substrates such as cytosolic endonucleases and proteases, *e.g.* PARP [33], cytokeratins, cytoskeletal protein fodrin [198], and nuclear protein NuMA (nuclear mitotic apparatus protein) [301]. These activated cellular components are involved in the degradation of the nucleic acids and cytoskeletal proteins.

AIF and Endo G are released from the mitochondria after LMMP and they have nuclease activity. They can directly translocate into the nucleus and participate in nuclear fragmentation. These series of events lead to the morphological and biochemical changes seen in the apoptotic cells [88, 286].

LMMP can occur due to various stimuli and LMMP results in the release of Cyt c from the mitochondria. Cyt c couples with Apaf-1 and procaspase-9 to form the apoptosome, which facilitates activation of procaspase-9. IAPs prevent the activation of caspases. Smac/Diablo released from the mitochondria blocks the activity of IAPs, thereby promoting activation of caspases.

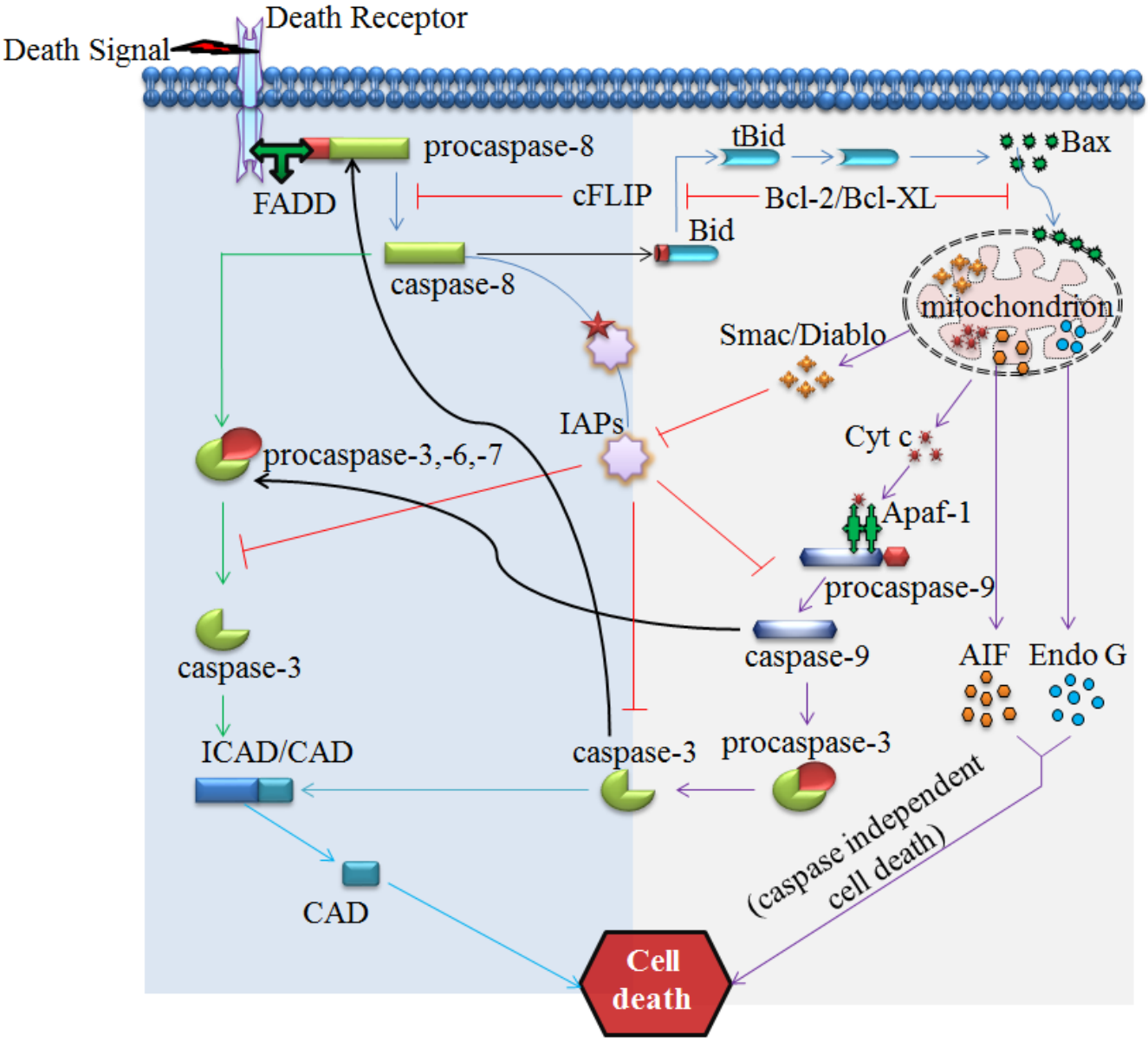


Figure 6. An overview of the extrinsic and intrinsic pathways of apoptosis. Initiation of apoptosis can lead to the activation of any of the two apoptotic pathways, extrinsic or intrinsic pathway. The death receptor activated extrinsic pathway is mediated by caspase-8, whereas the intrinsic pathway is mediated by caspase-9. In the extrinsic pathway, activation of the death receptor leads to the recruitment of an adaptor molecule named FADD to its cytosolic DD. Then FADD couples cytosolic DD of death receptors to procaspase-8. This promotes the autocatalysis and activation of caspases-8. cFLIP, an inhibitory protein, prevents caspase-8 activation.

Crosstalk between the two apoptotic pathways occurs via caspase-8-mediated cleavage and activation of Bid. Activated Bid further activates Bax, thereby engaging the mitochondria-mediated intrinsic pathway. Similarly, caspase-3, which is activated in the intrinsic pathway, cleaves and

activates procaspase-8, thereby initiating the activation of components of the extrinsic pathway [17, 128, 129].

5.5. Role of apoptosis in diseases

Abnormalities in the apoptotic process have a significant role in the progression or regression of diseases. Increased apoptosis can cause neurodegenerative diseases, *e.g.* Alzheimer's disease, Parkinson's disease, Huntington disease [275], as well as myelodysplastic syndromes [238] and acute myocardial infarction [238]. Decreased apoptosis is noticed in autoimmune disorders, in viral infections like, Herpes viruses and Adenoviruses, and in various cancers [150].

6. Role of p73 in cell life and death decisions

Transcriptional dependent and independent activities of p73 play important roles in regulation of cell life and death decisions. Regulation of cell life and death decisions by p73 appears to have significant impact on development and cancer treatments.

6.1. Role of p73 in cell life

Billions of cells are generated and simultaneously died during various stages of human development. Cell death is important for sculpturing various parts of the body, for example, in formation of joints and separation of fingers. Cell death has to be carefully regulated for the proper morphogenesis of an organism. An increase in the cell death than required level can cause abnormal organ formation and various diseases.

p73 protects the cells from cell death in a variety of situations. In cultured cortical neurons of mice, a decrease in the number of wild-type *P73* alleles caused an increase in JNK activity, which promotes apoptotic signalling. Release of lactate dehydrogenase (LDH) into the medium is a biochemical indicator of necrosis. In these cells, a decrease in the *P73* copy number increased the release of LDH into the medium [32, 77, 335].

ΔNp73 inhibits TAp73α-, TAp73β- and TAp73γ-induced transactivation of pro-apoptotic Cluster of differentiation 95 *(CD95)* [220] and *BAX* [220, 224] gene promoters, and thereby confers chemoresistance [220, 266]. ΔNp73 interacts with p73α and it inhibits p73α-induced neuronal cell death in a dose-dependent manner [223]. ΔNp73β physically interacts with p53 and increased expression of ΔNp73β rescues sympathetic neurons from p53-induced cell death [250]. ΔNp73β overexpression is noticed in malignant NIH3T3 cells, and injection of these cells into mice generates tumors [290]. In mice, overexpression of ΔNp73α or ΔNp73β isoform protects neurons from apoptosis induced upon withdrawal of the nerve growth factor (NGF). ΔNp73 isoforms are predominantly expressed in developing brain of mice [250], suggesting that ΔNp73 isoforms play important roles in brain development by counteracting p73- and p53-induced cell death.

Full-length p73 is overexpressed in a variety of cancers. p73 expressing colorectal adenocarcinoma showed greater vascularity than p73-negative tumors [116]. In a human ovarian cancer cell line, overexpression of TAp73α causes an upregulation of DNA damage repair proteins and provides resistance towards DNA damaging agent treatments [323]. TAp73α represses drug-induced apoptosis in SCLC cells by preventing caspase-3 activation and PARP cleavage [235]. We identified that TAp73α induces the expression of Hsp72, prevents the loss of mitochondrial transmembrane potential and loss of lysosomal membrane permeability, and thereby inhibits drug-induced apoptosis in SCLC [237]. We also reported that TAp73α represses the enzymatic activity of caspase-2 and inhibits caspase-2-induced Bax activation, as well as loss of mitochondrial transmembrane potential, thereby protecting SCLC cells from caspases-2-induced apoptosis [218].

6.2. Role of p73 in cell death

Cell division, proliferation and differentiation are important for the formation of organs and for the proper function of the immune system. Cell division is a biological process that generates new cells. As a result of cell division, huge numbers of cells are produced every day in the human body. Therefore, cell division has to be carefully regulated during all stages of the life of an individual to maintain tissue homeostasis. Different types of cell death mechanisms counterbalance the excess cells and control cell number. Cell death is also a defensive mechanism that removes infected, mutated or damaged cells. Abnormal cell growth can occur due to defects in cell death mechanisms. Transcriptional dependent and independent activities of p73 are involved in regulation of cell death.

p73 transactivates genes, which are involved in apoptosis. TAp73α, β and γ transactivates the *CD95* gene promoter [220]. p73 transactivates the *BAX* gene promoter [208, 220] and induces Bax

translocation from cytosol to the mitochondria [208]. Puma induces apoptosis in a Bax/Bak-dependent manner [208]. p73 induces the transcription of *PUMA* [208] and Puma promotes translocation of Bax to the mitochondria [358], thereby promoting Cyt c release from them. *NOXA*, a pro-apoptotic BH3 only member of the Bcl-2 family, is transactivated by p73 [200]. Scotin, a pro-apoptotic protein located in the ER and nuclear membrane [34], promotes caspase-dependent apoptosis upon UV irradiation. p73α transactivates the *SCOTIN* gene promoter [309]. Our group found that, TAp73β induces the expression of $p57^{Kip2}$, which contributes to mitochondria-mediated apoptosis.

Overexpression of p73 induces apoptosis in various cancer cell lines [158, 241]. In NCI-H82 cells overexpression of TAp73β promotes apoptosis upon drug treatment. In DU-145 prostate cancer cells, caspase-1 overexpression enhances apoptosis after ionizing radiation [338]. Lung endothelial cells of caspase-$1^{-/-}$ mice showed a reduction in the serum withdrawal-induced apoptosis [159], suggesting that caspase-1 plays an important role in apoptosis. TAp73α and TAp73β can transactivate the *CASPASE-1* gene promoter [137]. Loricrin, a major component of the cornified cell envelope, can induce programmed cell death [348] and *LORICRIN* gene promoter is transactivated by TAp73γ [68]. Transcriptional regulation of various pro-apoptotic gene promoters like *NOXA*, *BAX*, *SCOTIN* and *CD95* can explain the isoform-specific involvement of p73 in apoptosis. Apart from regulating cell proliferation, the transcription factor E2F1 is also involved in the induction of apoptosis. E2F1 directly transactivates TAp73. Functional inactivation of p73 reduces E2F1-induced apoptosis [135], suggesting that p73 plays an important role in E2F1-induced apoptosis.

Many chemotherapeutic drugs induce the expression of p73, which in turn enhances drug-induced apoptosis [158]. Downregulation of p73 results in the inhibition of cisplatin-induced apoptosis [4]. Upon drug treatments, p73 elicits apoptosis in a caspase-dependent manner. In chemosensitive head and neck squamous cell carcinoma (HNSCC) Ca9-22 cells, p73 is responsible for etoposide-induced caspase-8 activation and apoptosis [189]. Knockdown of p73 using small interfering ribonucleic acid (siRNA) blocks caspase-3 and -7 activities in etoposide-treated Ca9-22 cells, suggesting its role in activation of caspase-3 and -7 [189]. Reduced cytotoxicity of chemotherapeutic drugs upon inhibition of p73 expression, suggests the importance of p73 in sensitizing cells to cancer treatments. p73 is cleaved by caspases both *in vitro* and *in vivo* during apoptosis induced by DNA damaging drugs and its cleaved fragments are localized to mitochondria and augment tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis [276]. In support, purified recombinant TAp73α induces Cyt c release from isolated mitochondria

[276], suggesting that it has a transcription-independent activity in induction of the intrinsic apoptotic pathway.

6.3. Factors influencing the cell life and death decisions by p73

p73 is a multifunctional protein that regulates both cell life and cell death. Various cellular and environmental factors direct the regulation of cell life and death decision by p73. These factors influence the choice of genes to be transactivated by p73. TAp73 is able to transactivate its own intrinsic promoter (p2), thereby regulating the expression of anti-apoptotic ΔNp73 isoforms. As mentioned earlier, ΔNp73 isoforms are predominantly expressed in developing brain [250] and seem to protect neurons by counteracting p53- and p73-mediated apoptosis. A subset of tumor derived p53 mutants, but not WT p53, reduces the transcriptional activity of p73α on *P21* and *BAX* gene promoters, hence reduce apoptosis induced by p73 [78, 295].

The impact of various isoforms of p73 in the transactivation of genes, which regulate cell cycle or apoptosis, differ [237, 362]. Our group reported that, in SCLC cells, TAp73α represses drug-induced apoptosis, whereas TAp73β promotes it. Increasing levels of TAp73α inhibits the pro-apoptotic effect of TAp73β in a dose-dependent manner, upon drug treatment and vice versa. Counteractions of TAp73α and TAp73β on each other's function suggest that the more abundant isoform determines whether cell die or survive.

p73 is acetylated by p300 after DNA damage. This acetylation activates the pro-apoptotic functions of p73 and directs it to transactivate pro-apoptotic gene promoters [59, 355]. A non-acetylatable mutant of p73 transactivates the cell cycle related *P21* gene promoter, but not the pro-apoptotic *P53AIP1* gene promoter [59]. Pin1 physically associates with p73, stabilizes it and promotes its transcriptional activity on genes involved in apoptosis [188, 191, 197]. Upon DNA damage, a cleaved fragment of protein kinase Cδ (PKCδ) phosphorylates serine residue 289 of p73β and enhances p73β-mediated apoptosis [258]. p73α mediated apoptosis is enhanced by Chk-1-mediated phosphorylation of p73α at serine residue 47 [111]. Upon DNA damage the transcriptional co-activator Yap is activated by c-abl-dependent phosphorylation. Active Yap displays increased affinity to p73 and promotes selective recruitment of p73 on pro-apoptotic *BAX* and *P53AIP1* gene promoters [179, 297]. In contrast, Akt-mediated phosphorylation of Yap suppresses Yap's ability to promote p73-mediated transactivation of pro-apoptotic gene promoters [14].

The apoptosis stimulating protein of p53 (ASPP) family consists of ASPP1, ASPP2 and iASPP (inhibitor of ASPP) [299]. DBD of p73 is responsible for the sequence-specific DNA binding and is

an interaction site for ASPP1 and ASPP2 [215]. This interaction specifically activates transcriptional activity of p73 on pro-apoptotic gene promoters like *BAX*, *PUMA*, and *PIG3*, but not cell cycle regulating *P21* and *MDM2* gene promoters [18]. p53 inactivation is upregulating the p73 expression via E2F1-mediated transcription [313], suggesting that the presence of p53 can influence the relative levels of p73, thereby influencing the p73-mediated cell life or death decisions.

In conclusion, regulation of cell life and death decisions by p73 is influenced by the presence of other p53 family members, type of p73 isoform (TA or ΔN) generated, balance between its pro- and anti-apoptotic isoforms, its association with various interacting partners and its post-transcriptional modifications.

7. *P73* in cancer

Uncontrolled cell growth and spread of abnormal cells in various parts of the body are some features of cancer. If the spread is not controlled, it can cause the death of an individual. Various factors contribute to the generation of cancer. For example, UV radiation from sun causes skin cancer, smoke from tobacco causes lung cancer. Viral infections, such as hepatitis B virus (HBV), hepatitis C virus, and human papilloma virus (HPV) can cause cancer. In addition, metabolism within the cells produces free radicals as byproducts. These free radicals have the ability to cause DNA damage. It is estimated that an individual cell can suffer up to a million DNA changes in a day [56]. Our cells have developed exquisite DNA damage-repair mechanisms that protect us from DNA damage-related unfavorable outcomes [56]**.** During cell division DNA is replicated and segregated into daughter cells. A proper segregation of DNA into the daughter cells is very important for proper cell division and development of an organism. The cells have developed safeguarding mechanisms known as checkpoints, which ensure that everything is in order before allowing the next event to proceed during cell cycle progression.

Various DNA repair mechanisms detect and repair the damaged DNA, and maintain its integrity . In the case of irreversible damage, cell death mechanisms are activated to eliminate unwanted or damaged cells. A failure to repair the damaged DNA can produce mutations. If these mutations are not repaired, *e.g.* due to failure of protection and repair mechanisms, they are passed on to the daughter cells. As a result mutated proteins with altered functions could be produced within the cell and may cause disturbances in the signaling mechanisms in which they are involved. Sometimes

these mutations negatively regulate signaling pathways associated with cell cycle, DNA damage repair, and cell proliferation, and thus enhance the risk of malignant transformation.

Tumors are classified into various groups based on their primary site of origin, histological type, or type of body tissue from which they originate. Different types of tumors exhibit different clinicopathological features [169, 249, 287, 303, 342]. However, cancer cells share some general features, which are described as ‘‘the hallmarks of cancer’’. These common features include, self-sufficiency in growth signals, evading programmed cell death, sustained angiogenesis, unlimited replicative or proliferative capability, tissue invasion and metastasis, and evading the growth suppress signals (for review, refer to [123]).

The discovery of p53 was a major breakthrough in cancer biology research. Tumor suppressive functions of the p53 protein are linked to its ability to transactivate a variety of target genes that regulate DNA damage repair, cell cycle progression and apoptosis, and participate in a number of different signaling cascades. Mutations in the *P53* gene can produce transcriptionally inactive p53 proteins that have lost their tumor suppressive function [216, 261]. Compared to *P53*, mutations in *P73* are very rare. However, amino acid substitutions or deletions in p73 were identified in a variety of cancer derived cells, *e.g.* the G264W mutation in NSCLC NCI-H1155 cells, Del418 mutation in SCLC DMS92 cells, and Del603 mutation in lung carcinoma A427 cells [132, 349]. Deb *et al.*, described a mutant of TAp73α in MCF-7 cells having two amino acid substitutions (R268Q, R300C) within the DBD region [69]. Two mutations were identified in neuroblastomas (NBLs), one somatic (P405R) and one germline (P425L), which are amino acid substitutions at the carboxy-terminal region of p73 [134]. In a primary tumor of a breast cancer patient, one somatic missense mutation of glutamine from arginine at codon 269 within exon 7 was reported [122].

Although *P73* is rarely mutated, altered expression levels of p73 and its abnormal splicing variants were identified in several human cancers [178]. Decreased levels of p73 were identified in pancreatic adenocarcinoma [190], breast cancer [3], thyroid cancer [96] and osteosarcoma [244]. In some hepatocellular carcinoma (HCC), increased levels of p73 expression were identified and this was correlated with a lower mean survival time for the patients [307]. Increased expression of TAp73α was identified in cancers such as cervical cancer [331], medulloblastoma [363], B-cell chronic lymphocytic leukaemia [234], ovarian carcinoma [231], gastric adenocarcinoma [151], bladder cancer [347] and thyroid cancer [104]. ΔNp73 isoforms are frequently upregulated in some tumors compared with their normal tissue of origin [353]. ΔNp73 isoforms express pro-oncogenic properties and act as dominant negative inhibitors over pro-apoptotic TAp73 and p53 by physically

interacting with them [223, 224, 353]. Thus, ΔNp73 isoforms functions as oncoproteins and can contribute to the cancer progression. Interestingly, TAp73α, β and γ isoforms are able to induce transcription of *ΔNp73* promoter [331]. Increased expression of TAp73α in a variety of human cancers and its ability to transactivate oncogenic *ΔNp73* suggests it could be a pro-oncogene. In support, we identified that under certain experimental conditions, TAp73α exhibits anti-apoptotic effects, for example, it protects SCLC NCI-H82 cells from caspase-2, as well as from drug-induced apoptosis. However, these oncogenic effects of TAp73α seem to be restricted to certain cell types.

In Saos-2 (Sarcoma osteogenic) cells, mutated p53 causes functional inactivation of p73 by physically interacting with it, thereby promoting Saos-2 colony formation [199]. Physical interaction between mutant p53 and p73 also inhibits the transcriptional activity of p73 on *P21*, a cell cycle regulator [106]. Hence, functional inactivation of p73 seems to be one reason for cancer in p53 mutated cells. Increased incidences of spontaneous tumors are noticed in TAp73 null mice [312]. As mentioned earlier, a more aggressive tumor phenotype (higher tumor burden and metastases) and shorter life span was noticed in $P53^{+/-}$ $P73^{+/-}$ mice than $P53^{+/-}$ mice [100, 226], suggesting p73 might be compensating for the loss of p53 tumor suppressive functions in $P53^{+/-}$ mice. Loss of heterozygosity (LOH) of the *P73* was observed in esophageal cancer [229], hepatocellular cancer [209], gastric cancer [178], neuroblastoma and breast cancers [149, 164, 266, 285]. Moreover, in primary neuroblastoma tumors, low levels of p73 expression were identified [233]. Furthermore, p73 inhibits vascular endothelial growth factor (VEGF) gene expression [272], strongly supporting its tumor suppressive functions.

p53 family members are involved in regulation of cell cycle and cell death. In a variety of cancer treatments, the used drugs target cell cycle or cell death pathways. Our data indicate that TAp73α and TAp73β elicits opposite effects (anti- and pro-apoptotic effects, respectively) on drug-induced apoptosis. Thus, consideration of the status of p73 isoform expressions seems to be necessary during chemotherapy, otherwise the selected drugs might promote cancer progression rather than curing it.

8. Aim of the thesis

- To determine isoform-specific transcriptional activities of p73 that influence drug-induced apoptosis.
- To uncover the molecular mechanisms of p73 isoforms by which they exhibit pro- and anti-apoptotic effects.
- To elucidate the structural and functional requirements for p73 to regulate cell life and death decisions.
- To discover the transcriptional activities of various isoforms of p73 on cell cycle and apoptosis related gene promoters based on the context of the cell.

9. Results

9.1. Paper I

Hsp72 mediates TAp73α anti-apoptotic effects in small cell lung carcinoma cells.

Our group previously showed that TAp73α inhibits etoposide-induced apoptosis in SCLC, whereas TAp73β promotes it. Increasing levels of TAp73α inhibited apoptosis promoted by TAp73β upon etoposide treatment. Different isoforms of p73 differently transactivate genes, which are involved in various biological processes [362]. We speculated that opposite effects TAp73α and TAp73β on drug-induced apoptosis might be due to isoform-specific transcriptional activities.

Hsp72 is an anti-apoptotic protein and acts at multiple levels on the apoptotic signalling pathway [80]. It inhibits lysosomal membrane permeabilization [23, 119], JNK and translocation of Bax to the mitochondria [183]. It interacts with AIF [257] and Apaf-1 [271], inhibits the processing of caspase-9 and -3 [16, 180], thereby inhibiting progression of apoptosis.

We speculated that the anti-apoptotic effects of TAp73α in SCLC might be associated with its transcriptional activity and that it might transactivate the anti-apoptotic *HSP72*. We found that TAp73α, but not TAp73β transactivates *HSP72* promoter, induces Hsp72 mRNA levels and Hsp72 protein expression.

In NCI-H82 cells, antisense knockdown of Hsp72 resulted in an abolishment of the anti-apoptotic effect of TAp73α upon drug (etoposide) treatment. Moreover, Hsp72 knock down resulted in activation of Bax, loss of mitochondrial membrane potential and lysosomal membrane permeabilization in these cells even in the presence of TAp73α.

Interestingly, overexpression of Hsp72 abolished the pro-apoptotic effects of TAp73β upon drug treatment. Furthermore, our results suggested that TAp73β counteracts the anti-apoptotic effects of TAp73α by preventing its transcriptional activities on *HSP72* promoter.

Inhibitory effects of TAp73α on drug-induced apoptosis are associated with its ability to inhibit caspase-3 and PARP cleavage. Interestingly, under similar conditions TAp73β promoted both caspase-3 and PARP cleavage [235]. In addition to the previous reports on pro- and anti-apoptotic effects of p73 isoforms, our study on transcriptional activities of carboxy-terminal TAp73 isoforms provides evidence that they govern the cell life and death decisions at the transcriptional level. Moreover, our studies explain a molecular mechanism on how certain tumors with TAp73α overexpression are resistant to drug treatments.

9.2. Paper II

TAp73α protects small cell lung carcinoma cells from caspase-2-induced mitochondrial mediated apoptotic cell death.

In paper I, we demonstrated that anti-apoptotic effects of TAp73α are mediated by Hsp72 in SCLC NCI-H82 cells. Apart from transactivating anti-apoptotic genes, TAp73α is also able to inhibit the activation of pro-apoptotic proteins, for example, it inhibits caspase-3 activation and PARP cleavage, thereby negatively regulating apoptosis [235].

Caspase-2 is the second caspase to be cloned and its overexpression induces apoptosis in various cell lines. During the progression of apoptosis, it cleaves full-length Bid to activated tBid and engages the mitochondria-dependent cell death pathway [28]. We speculated that TAp73α might exert anti-apoptotic effects by inhibiting caspase-2-induced apoptosis. In fact, our results demonstrate that TAp73α inhibits apoptosis induced by caspase 2. In contrast, under our experimental conditions TAp73β promoted caspase-2-induced apoptosis.

Interestingly, TAp73α co-expression significantly reduced caspase-2 enzymatic activity in NCI-H82 cells. Overexpression of caspase-2 promotes Bax translocation to the mitochondria and induces the mitochondrial mediated apoptotic cascade [28, 118, 170]. Co-expression of TAp73α prevented caspase-2-induced mitochondrial dysfunction and Bax activation. These results suggest that TAp73α operates at the initiation phase of apoptosis, upstream of mitochondria and at level of the Bax activation.

To determine the structural domains that are required for the anti-apoptotic functions of TAp73α on caspase-2-induced apoptosis, we took advantage of plasmids encoding mutations in different functional domains of TAp73α. Our mutational studies revealed that TAp73α requires the functional DBD and SAM domain for its anti-apoptotic effect, on caspase-2-induced apoptosis.

In addition to the previously described anti-apoptotic effects of TAp73α, our findings provide biochemical and molecular mechanisms on how TAp73α negatively influences the caspase-2-induced apoptosis in SCLC cells.

9.3. Paper III

TAp73β transcriptional target gene p57^{Kip2} promotes p73β-mediated mitochondrial apoptotic cell death.

In papers I and II, we showed the molecular mechanisms on how TAp73α exerts anti-apoptotic functions upon drug-induced apoptosis. In these papers, we also reported that drug (etoposide) and caspases-2-induced apoptosis were enhanced by TAp73β. TAp73β has been shown to be a potent inducer of apoptosis as compared to the TAp73α [161, 288]. In paper III, we aimed to identify the TAp73β regulated molecular mechanisms that execute its pro-apoptotic effects.

p57^{Kip2} is a CKI and holds tumor suppressive functions. p57^{Kip2} expression levels are downregulated in a variety of human cancers [31] and loss of its expression was correlated with poor survival rate of cancer patients with laryngeal squamous cell carcinoma [92]. Overexpression p73β, but not p73α or p73γ, induces the expression of p57^{Kip2} [26]. It has been shown that p57^{Kip2} potentiates drug-induced apoptosis [273]. We hypothesised that the pro-apoptotic effects of TAp73β might be depend on its ability to induce expression of the p57^{Kip2} protein.

In this paper, we demonstrate that in various cell lines TAp73β, but not TAp73α, induces expression of p57^{Kip2}. Gonzalez *et al.*, reported that p57^{Kip2} mediates pro-apoptotic activity of p73β [112]. However, the precise mechanism by which TAp73β regulates the p57^{Kip2} expression and its roles in TAp73β-induced apoptosis was unclear. In this study, we reveal that TAp73β, but not TAp73α, binds to a putative p53RE located at 1753 base pairs upstream of *P57^{Kip2}* transcription initiation site. Furthermore, transcriptional activity of TAp73β on the *P57^{Kip2}* promoter is counteracted by TAp73α. To detect whether pro-apoptotic effects of TAp73β upon drug treatment are mediated by p57^{Kip2}, we took advantage of siRNA-mediated silencing of p57^{Kip2} expression. Overexpression of TAp73β and p57^{Kip2} promoted staurosporine (STS)-induced loss of mitochondrial membrane potential and apoptosis in HeLa cells. siRNA-mediated silencing of p57^{Kip2} repressed TAp73β-induced mitochondrial dysfuntion, Bax activation and apoptosis. These results suggest that upon drug treatment, p57^{Kip2} sensitizes these cells to TAp73β-induced apoptosis via engaging the mitochondria-mediated apoptotic pathway. The present data reveals a possible mechanism to often observed pro-apoptotic activities of TAp73β.

Our data provide an additional evidence for the target-specific transcriptional activities of carboxy-terminal TAp73α and TAp73β isoforms that mediate their anti- and pro-apoptotic effects, upon drug treatments.

9.4. Paper IV

DNA binding domain independent transcriptional activity of p73 depends on the p53 status of the cell.

The p73 isoforms have been shown to have different transcriptional activity [362] and they have distinct roles during various stages of development [215, 219]. Interplay between the p53 family members has been shown to have a significant effect on development, proliferation, tumorigenesis and response to anti-cancer treatments [101, 144, 175, 192]. In papers I and III, we reported that TAp73α and TAp73β exhibit opposite effects on drug-induced apoptosis and their anti- and pro-apoptotic effects partly depend on their target-specific transcriptional activities.

P53 is commonly mutated or deleted in a variety of human cancers. In contrast to the *P53*, *P73* mutations are more seldom reported in human cancers and cancer derived cells [70, 122, 132, 134, 222, 247, 349]. Mice lacking the TAp73 isoforms exhibit increased incidence of spontaneous tumors [311]. On the other hand, altered expression levels of various isoforms of p73 and expression of its abnormal splicing variants are noticed in several cancers [3, 82, 96, 104, 151, 230, 234, 244, 317, 332, 347, 363]. Certain tumor-derived mutants of p53 interact with TAp73 isoforms and inhibit their transcriptional activity [79, 106, 192, 294, 337]. It is thus important to take into account that the mutations in TAp73 may influence the biological functions of a subset of p53 mutants and/or p53.

In this paper, we aimed to identify cell context-specific functions of p73 isoforms. We hypothesized that TAp73α, TAp73β and ΔNp73α isoforms and DBDmut p73α, which contains two amino acid substitutions (R268Q and R300C) within its DBD region [70], exhibit cell type-specific transcriptional activities. Indeed, our gene reporter assays (GRAs) revealed that they exhibit different transcriptional activities in different cell lines on cell cycle and apoptosis related *BAX* and *P21* gene promoters. Interestingly, we identified that DBDmut p73α transactivates the *P21* gene promoter in HEK-293 cells, but fails to transactivate the same promoter in NCI-H82 cells. Our subsequent GRAs using wild-type (WT) p53 and p53 null cells and siRNA-mediated silencing of p53 in HEK-293 cells revealed that the ability of of DBDmut p73α to transactivate *P21* gene promoter depends on the p53 status of the cell. We further identified that upon co-expression DBDmut p73α and p53 physically interact with each other and stabilize their respective protein expression level in cells. Furthermore, we identified that co-expression of p53 promotes nuclear localisation of the DBDmut p73α. To identify whether p53 promoted nuclear localization of

DBDmut p73α promotes its recruitment on *P21* promoter, we performed ChIP experiments in HEK-293T cells. Our ChIP experiment revealed that p53 was required for the recruitment of DBDmut p73α to the *P21* promoter.

Our findings suggest that in different cells transcriptional activities of isoforms of p73 are different and their transcriptional activities depend on the context of the cell. Furthermore, our findings reveal a functional cooperation network between p53 and p73, where DBDmut p73α seems to act as a transcriptional co-factor for p53.

10. General discussion and Future perspectives

p53 was identified in 1979 and it attracted the interest of numerous researchers because the *P53* gene was found to be frequently mutated or deleted in a variety of human cancers. Initially *P53* was thought to be an oncogene, however, its tumor suppressor functions were well established in 1989 [13]. p73, a structural homologue of p53 was identified in 1997. The structural similarity initially suggested that it might share functional similarities with p53 [178]. Indeed, p73 shares functional properties with p53 and it recognizes p53REs with its DBD, and transactivates them with its TAD. p73 transcriptionally regulates various biological processes such as differentiation, development, cell cycle and apoptosis. Though p73 shares structural and functional similarities with p53, it also has its own biological functions.

The p73 isoforms have distinct transcriptional activities, for example, in yeast, mouse and human cells, they exhibit different capability to transactivate p53 regulated promoters [78, 186, 208, 251, 289] and specific target genes [26, 361]. Full-length TAp73α and TAp73β differently regulate cell cycle progression [321], apoptosis [66, 112, 235, 362] and differentiation [181].

Full-length transcriptionally active TA isoforms of p73 are considered to act as tumor suppressors. Transactivation domain-deficient ΔN isoforms of p73 act functionally analogous to other oncoproteins by inactivating the transcriptional activity and apoptosis induced by p53 and TAp73. Thus ΔNp73 isoforms are regarded as oncoproteins. Overexpression of ΔNp73 isoforms was identified in a variety of human cancers further supporting their oncogenic properties. Interestingly, overexpression of the pro-apoptotic TAp73α isoform was identified in several human cancers such as gastric adenocarcinoma [151], thyroid cancer [104], ovarian carcinoma [231], prostate cancer [8] and bladder cancer [347]. Moreover, in an ovarian cancer cell line, overexpression of TAp73α results in resistance to chemotherapeutic agents [322]. Nyman *et al*., reported that TAp73α inhibits drug-induced apoptosis in a cell type-dependent manner [218]. It was shown that overexpression of TAp73α cause chemoresistance, but the precise mechanism to how it does so is not yet fully understood. As p73 can transcriptionally regulate various biological processes, we wanted to identify whether the apoptosis inhibitory effects of TAp73α depend on its transcriptional activity. The cell survival protein Hsp72, a member of the heat shock family of proteins, inhibits apoptosis at multiple levels. In several cancers overexpression of Hsp72 was reported [55, 105, 274] and a variety of chemotherapeutic drugs induce expression of Hsp72 that results in decreased chemosensitivity of cancer cells. Moreover, ΔNp73α induces expression of Hsp72 [305]. In paper I, we report that TAp73α induces the expression of Hsp72 and that its anti-apoptotic effects partially depend on the

induction of Hsp72. Interestingly, TAp73β did not induce Hsp72 expression. TAp73α transactivation of *HSP72* can to some extent explain the anti-apoptotic properties of TAp73α and its involvement in the resistance to chemotherapeutic agents in certain cell types.

In SCLC cells, TAp73α represses drug-induced apoptosis, upstream of the mitochondria at the level of Bax activation. Caspases cleave several substrates in the cell and promote the progression of apoptosis. Caspase-2 acts upstream of mitochondria and promotes Cyt c release during drug-induced apoptosis [263]. Active caspase-2 engages mitochondria in apoptosis by directly cleaving full-length Bid to activated tBid and promotes Bax translocation to the mitochondria.

In an attempt to elucidate the molecular mechanisms by which TAp73α mediates its anti-apoptotic effects, we tested whether it can inhibit caspases-2-mediated apoptosis. In paper II, we report that TAp73α inhibits caspase-2-induced apoptosis through inhibiting caspase-2 enzymatic activity, Bax activation, and loss of mitochondrial transmembrane potential and resulting apoptosis. In contrast, TAp73β promoted caspase-2-induced apoptosis. In an attempt to identify the domains required for TAp73α to elicit anti-apoptotic effects, we found that the DBD and SAM domains of TAp73α are required for its inhibitory effect on caspase-2-induced apoptosis in SCLC cells. Requirement of the SAM domain suggests that binding of specific co-factors influences its anti-apoptotic effects. This hypothesis is further supported by SAM deleted TAp73α failing to inhibit caspase-2-induced apoptosis, and rather significantly promoted it. Certainly, TAp73β, which does not have the SAM domain, seems to be not able to associate with inhibitory molecules, thus seems to promote caspases-2-induced apoptosis. Previous studies have focused on transcriptional activities of p73 to explain its pro- and anti-apoptotic activities. Identifying its transcription-independent activities in regulation of cell life and death decisions is also an interesting area of p73 research.

$p57^{Kip2}$, a cyclin-dependent kinase inhibitor, regulates cell proliferation and acts as a tumor suppressor protein. Recent studies revealed that $p57^{Kip2}$ mediates drug-induced mitochondria-mediated cell death [325]. In paper III, we reveal that TAp73β, but not TAp73α, induces the expression of $p57^{Kip2}$. Furthermore, knockdown of $p57^{Kip2}$ showed that TAp73β-induced $p57^{Kip2}$ expression contributes to mitochondria-mediated apoptotic events.

The observation based on papers I, II and III, suggests that carboxy-terminal TAp73α and TAp73β isoforms exhibit opposite effects on cell life and death decisions. In these papers, we also showed that TAp73α and TAp73β counteract each other's pro- and anti-apoptotic functions in a dose-dependent manner. It seems that the balance between amino- and carboxy-terminal isoforms play important roles in overall tumor development and chemoresistance. Therefore, consideration of the

presence of specific isoform of p73 and their relative level of expression in cancer cells seem to be necessary during cancer therapy to achieve an effective treatment.

These opposite effect on cell life and death decision and differential transcriptional activity of p73 isoforms might be influenced by structural differences at their carboxy-terminal region. TAp73α, but not TAp73β, possess a carboxy-terminal SAM domain, which is expected to be involved in protein-protein interactions. It has been shown that several transcriptional co-activators physically interact with p73 at the carboxy-terminal region and regulates its transcriptional activity. Differential binding of co-factors to their carboxy-terminal region might be one of the reasons for their differential transcriptional activity.

Functional differences between p73 isoforms were previously described. TAp73α was shown to repress drug-induced apoptosis in SCLC cells, but to promote it in NSCLC cells [235]. In yeast, mouse and human cells, isoforms of p73 show different ability to transactivate p53 regulated promoters [78, 186, 208, 251, 289]. Functional cooperation between p53 family members was previously described. For example, a more aggressive tumor phenotype (higher tumor burden and metastases) and shorter life span were noticed in $P53^{+/-}$ $P73^{+/-}$ mice, compared to $P53^{+/-}$ mice [100, 226], indicating that p73 functionally compensates the loss of p53 in the $P53^{+/-}$ mice. Some mutants of p53 physically interact with p73 and inhibit its transcriptional activity [106, 297], whereas some mutants of p53 bind to p73, but do not alter its transcriptional activity [106]. In paper IV, we report that in different cell lines, isoforms of p73, *e.g.* TAp73α, TAp73β and a DBD mutant of TAp73α transactivate *P21* or *BAX* promoters with different capabilities. In this paper, we also report a functional cooperation between DBDmut TAp73α and p53. In p53 null cells, DBDmut p73α is transcriptionally inactive and fails to transactivate either cell cycle or apoptosis regulating gene promoters (*P21* and *BAX* promoters, respectively). Interestingly, DBDmut p73α was able to transactivate the *P21* promoter only in p53 expressing cells. In this paper, we report that p53 promotes nuclear localization of DBDmut p73α and promotes its recruitment at the *P21* promoter. Our observations suggest that the context of the cell influences the transcriptional activity of DBDmutp73α.

Various isoforms of p73 have their specific transcriptional target genes and an individual isoform can differentially transactivate the same gene based on the cell type. The transactivation ability of a specific isoform seems to be dependent on the context of the cell, its association with various interaction partners, and the presence of certain transcriptional co-factors. Its association with ASPP1 and/or ASPP2 induces the transcriptional activity of p73 to transactivate pro-apoptotic gene promoters, *e.g. BAX* and *PUMA*, but not cell cycle regulating *P21* and oncogene *MDM2* gene

promoters [18]. Different post-translational modifications of p73 were shown to direct it towards the selective induction of either cell cycle arrest or apoptosis [59, 65]. Not only the transcriptional activities of p73, but also its cellular signaling activities seem to influence the fate of the cell [276].

As various isoforms of p73 seem to exhibit different roles in biological processes depending on the cell context, it is very interesting to identify their effect on the fate of the cell at different stages of development based on the cell context. It is important to identify the functions of specific isoforms of p73 as they are frequently deregulated in various types of human cancers. A better understanding about individual isoform functions based on the cell context might help us to develop new strategies that in the future can more effectively target the tumor.

11. Concluding remarks

This thesis reveals transcriptional and molecular mechanisms about how different isoforms of p73 regulate cell life and death decisions of the cell.

In paper I, we identified that TAp73α inhibits drug-induced apoptosis, whereas TAp73β promotes it. TAp73α, but not TAp73β, induces the expression of the cell survival protein Hsp72, by transactivating its promoter. Antisense knockdown of Hsp72 results in an abolishment of the anti-apoptotic effect of TAp73α in SCLC cells upon etoposide treatment, suggesting that Hsp72 expression mediates its anti-apoptotic effects. Moreover, Hsp72 co-expression prevents the pro-apoptotic effects of TAp73β. Furthermore, TAp73β counteracts the anti-apoptotic effect of TAp73α by inhibiting TAp73α-induced Hsp72 expression in a dose-dependent manner.

In paper II, we revealed that TAp73α represses caspase-2-induced apoptosis via inhibition of its enzymatic activity in NCI-H82 cells. Co-expression of TAp73α inhibits caspase-2-induced Bax activation, LMMP and resulting apoptosis. Furthermore, we identified that TAp73α requires the functional DNA binding domain and SAM domain for its anti-apoptotic effect, on caspase-2-induced apoptosis.

In paper III, we identified that *P57^{Kip2}* is a direct target gene for TAp73β, but not for TAp73α. We identified a potential p53 response element within the *P57^{Kip2}* promoter region for the recruitment of TAp73β. Furthermore, TAp73α counteracts TAp73β transcriptional activity on the *P57^{Kip2}* promoter and it inhibits TAp73β-induced expression of p57^{Kip2} in a dose-dependent manner. Moreover, TAp73β-induced p57^{Kip2} sensitizes HeLa cells to apoptosis through the mitochondrial apoptotic pathway.

In paper IV, we reveal that TAp73α, TAp73β and ΔNp73α transactivate *P21* or *BAX* promoters with different capabilities. We identified a functional cooperation between p53 and DBDmutp73α. The transcriptional ability of DBDmut p73α to transactivate the *P21* promoter seems to depend on the p53 status of the cells. DBDmutp73α and p53 physically interact with each other and they stabilize each other's expression. Moreover, DBDmut p73α is localized both in the cytoplasm and nucleus, and co-expression of p53 promotes the nuclear localization of DBDmut p73α. Furthermore, co-expression of p53 promotes recruitment of DBDmut p73α to the *P21* promoter.

Finally, different isoforms of p73 influences cell life and death decisions differently upon drug treatments and this activity seems to depend on the context of the cell and its isoform-specific transcriptional activities. Furthermore, p53 family members have functionally cooperating networks that influence each other's transcriptional activities.

In our future studies, we will extend our investigations on the pro- and anti-apoptotic effects of p73 isoforms upon drug treatment in various cell lines based on the context of the cell. The potential role of p73 as a target for cancer treatment will be investigated by analysis of its expression level and its effect on different drug treatments in different patient materials. p73 isoform-specific interacting partners will be identified in various cells based on the cell context. Furthermore, influence of identified interacting partners over the pro- and anti-apoptotic functions of p73 will be investigated. The possible mechanisms, which influence the stabilization of both p53 and DBDmut p73α will be investigated based on the context of the cell. Future studies will focus on identification of other pro- and anti-apoptotic transcriptional target genes of p73. Furthermore, involvement of these target genes in mediating cell life and death decision will be investigated. Moreover, we will extend our studies *in vivo* and a group of mice will be transplanted with TAp73α and TAp73β overexpressing tumors, drug or mock-treated, and tumor growth will be monitored over time.

12. Acknowledgements

My thesis work was done at the Institute of Environmental Medicine and Cancer Centrum Karolinska, Karolinska Institute, Stockholm, Sweden. I would like to convey my appreciation to all people who motivated and helped me during my doctoral studies.

First, my supervisor, **Bertrand Joseph,** I admire your profound scientific knowledge, supervision skills and great interest in science. I am glad that I got an opportunity to associate with you and gain knowledge from you. You always inspired me through your conversations and your professional approach towards work. Your optimism and dedication towards the laboratory research deeply motivated me and influenced the way I look at science. Your motivating assurances always helped me to get through the disappointments and failures of the profession. How can I not say that I got a fantastic supervisor, not just because for motivating me, but for sharing my success with all our group members with champagne, for saying ‘’don’t forget to go home’ and inviting me for several delicious dinners at your home. I will miss not only nice food, wine, cognac, but also fantastic company. Furthermore, I must say my heartful thanks to **Bettina** for her warm welcome into your home during dinners, and my love for the cute **Nils** and **Astrid** whom I wish bright futures.

My co-supervisor **Hans Jörnvall**, it was pleasure for having you as my co-supervisor. Thanks for your support during my PhD studies. I admire your scientific skills and optimism. Thanks for welcoming me with a smile, for being patient and for listening to me attentively. I appreciated your quick email responses, precise messages and reminders of the schedules. Thanks for allowing me to present my work in your group and for a fantastic feedback. Thanks for sharing your amazing travel stories and your personal interests.

Boris Zhivotovsky, thanks for sharing your ideas and organizing fantastic blue group meetings. I am always amazed at your memory to cite the authors including the years during scientific discussions.

Sandra Ceccatelli, thanks for being a mentor to my PhD studies and providing me an opportunity to present my work in your group and for valuable suggestions.

Ulrika Nyman, I wonder about your organization skills. You are a fantastic colleague to work with. Thanks for the scientific discussions and valuable suggestions in thesis writing. Thanks for helping

me to learn science. I appreciate your cooperation and for letting me use the facilities of the Ludwig Institute.

Karolin Rommel, thank you for being there always to share fun and emotions, for the motivation and suggestions that you gave me to smoothen my PhD journey. I so miss our CCK coffees and funny discussions. I wish you all the best with your doctoral studies and for your colourful future.

Pinelopi Vlachos, It was a pleasure to share several of my childhood stories with you. It was nice to have good times in the office while learning Greek words, Efxaristo (Ha ha. I remember the pronunciation, but I searched in Google for its spelling).

Jens Füllgrabe, thanks for sharing good times in the lab and during the conferences. Good luck with your PhD.

Johanna Rodhe, thanks for helping me in the lab whenever my transfections were not working and for your patience when things went wrong during my learning.

Xianli Shen, thank you for good times in the lab and during the conferences.

Edel Kavanagh, thanks for having drinks together and nice walks in the beautiful forests and Walborg fires warmth in Stockholm and for helping in the lab.

Miguel A. Burguillos, thanks for sharing your views during group meetings. Good luck with your supervision.

Violaine Moumou and **Katrin Hönig,** thanks for the joyful times and I wish good luck with your aspirations.

Martin Tran, thank you for interesting scientific discussions. I wish you a best of luck with your PhD studies.

Vitaliy Kaminskyya and **Shubhranshu Debnath,** thank you for being open to discuss science and personal life. I appreciate you guys for your friendly attitude and your scientific interests.

Chanisa Uemait, thanks for your warm wishes and fantastic communications, though we don't understand each other's languages. I know we did not understand most of the words, but it was never an obstacle to figure out what we were trying to say.

Praveen Rangineni, thanks for always being there like a brother. Brunnsviken funny stories, snow barbeques and typical Duvbo Indian dances are fantastic. I will miss them. A coffee can change a lot. Yes it did. Ha ha, ask our Indian folks.

Maria Gansvik, thanks for welcoming always to your home and for the delicious food and mid-summer day and New year celebrations and birthday cakes. Leila, thanks for cute smiles and for your secret curiosity, I don't know if you remember it, but I will never forget.

Harish Potu, thanks you for encouragement and motivation for undiluted positive criticism. We have not met for seven years. Time is funny. Looking forward to eat your special Indian curries.

Kranthi, Jyothi and Kiran, thanks guys for having good and bad times, for sharing and caring, and for everything we had. Good luck with everything.

Suman Vemula, Siripurapu Rajitha Rani, Sreenivas Reddy Mamidi and Amulya thanks for being a part of my happy moments in Stockholm, for cooking delicious Indian food, and making several days special, as well as for birthday cakes, with happy mood.

Narasimha Rao Boga, the love guru, Thanks for sharing your philosophical views, Lappis lake parties, Stockholm library conversations, Indian folk singings and field works.

Srikanth Sathu, Sattu bhai, thanks for letting me to be part of a lot of good times. The times we cooked, sung, danced were wonderful. I wouldn't like to miss your instant stories for the stories and funny lyrics. I wish you all the best.

Cecilia Nordfors and **Nathalie Grün,** thanks you girls, for nice conversations in CCK as well as during parties.

Maja Lindh, thank you for nice dinners and fantastic conversations. All the best in your dream career of saving the lives of patients.

Arindam Majumdar, thanks for drinks and philosophical and cross continental views. Bank balance theory of emotions is very interesting.

Theocharis Panaretakis, thanks for warm greetings and for your best wishes.

Ran Ma, thank you for the nice evenings with drinks and Chinese foods. It's nice to have someone screaming around and talking about some random things. Good luck with your PhD.

Sara Mijwel, **Laura Venskutonyte, Alexandra Alvarsson, Dudi Warsito, Anne Baunbæk Pedersen, Salam khan, Aravindh Subramani** and **Nathalie Edvinsson**, thank you all for sharing ideas and good times.

Swarnalatha and Rambabu, Deepa, Ramu, Bhargav, Bharath, Niranjan Komandla and **Josefin Malmström** and **Malin Lidholm,** thanks for sharing happy moments. Good luck with your aspirations.

My family

నాయినమ్మా, తాతయ్యా , అమ్మా, నాన్నా, అన్నయ్యా ఇంకా తమ్ముడూ, ఈ కుటుంబంలో పుట్టడం నా అదృష్టంగా ఎప్పుడూ భావిస్తాను. నా ఇచ్ఛను, మరియు ఇలా సాగిన నా పయనాన్ని స్వేచ్ఛగా మన్నించినందుకు, మీ అందరికీ నా కృతజ్ఞతలు.

నాయినమ్మా, తాతయ్యా, మీ మనుమడిగా మీకు తగినంతగా అందుబాటులో లేకపోయినందుకు నన్ను మన్నిస్తారు కదూ!!!!!.

భావోద్వేగాల్లో ఎల్లవేళలా తోడుగా ఉన్నందుకు ప్రదీప్, ప్రభంజన్, దిలీప్, సందీప్, మీకు నా ధన్యవాదములు.

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