U.PORTO
FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

U.PORTO
INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

Studying p53 family proteins: search for small molecule modulators

Sara Gomes Moreira

Dissertação de Mestrado apresentada à Faculdade de Ciências da Universidade do Porto, Instituto de Ciências Biomédicas Abel Salazar
Bioquímica

2013

MSc
2.º CICLO
FCUP
ICBAS
2013
U.PORTO
Studying p53 family proteins: search for small molecule modulators
Sara Gomes Moreira
FC

U.PORTO
FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

Studying p53 family proteins: search for small molecule modulators

Sara Gomes Moreira
Mestrado em Bioquímica
Departamento de Química e Bioquímica
2013

Orientador
Lucília Saraiva, FFUP

Coorientadores
Helena Vasconcelos e Raquel Lima, IPATIMUP

U.PORTO

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR
UNIVERSIDADE DO PORTO

U.PORTO

FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, ______/______/_________

MSc

Acknowledgements

First and foremost, I would like to express my deepest gratitude to my supervisor, Prof. Lucília Saraiva, not only for giving me the opportunity to work with her, but also for her constant support, guidance, and valuable lessons. Prof. Lucília always pushed me to work harder and aim higher, helping me grow both as a Scientist and as a Person. For this I will always be truly thankful.

I would also like to acknowledge my co-supervisors, Prof. Helena Vasconcelos and Dr. Raquel Lima, for contributing to my thesis with valuable insights that largely enriched this work.

The present thesis was integrated in the PhD project of my colleague Mariana Leão, to whom I would like to thank with all my heart, along with the rest of my lab family, Cláudia Bessa, Joana Soares, Clara Pereira, and Cláudia Maciel. Thank you for making me feel welcome, and for the constant support and selflessness. Although in distinct ways, I consider each of you as a role model. It has been a great pleasure working alongside each of you, and I hope to continue doing so for a long time.

I would also like to acknowledge Prof. Alberto Inga, from CIBIO, for his important contributions to the work presented in this thesis.

To Prof. Luís Vieira, for always being available to listen, help and encourage me. Throughout the past years, Prof. Luís was much more than a teacher, and I am truly thankful for all the support and kind words.

To Bruno Vale Fernandes, thank you for all your patience and encouraging words, that made me believe in myself when I was filled with doubts, and for making me laugh when I needed it the most. I hope to do the same for you very soon.

To Lumi, it was a pleasure to, once again, be your lab neighbour. Thank you for the long talks and for brightening the hardest situations with well needed laughs. Your hard work and motivation are an example to me, and I know you are meant for great things.

To Pedro Melo, Emiliana César, and Mariana Pereira. For all our little moments throughout the past five years. I know all of you will achieve great things and I wish you the best of luck.

This work was supported by FCT (Fundação para a Ciência e a Tecnologia) through REQUIMTE (PEst-C/EQB/LA0006/2011), and FEDER funds through the COMPETE program under the project FCOMP-01-0124-FEDER-015752 (PTDC/SAU-FAR/110848/2009).

Abstract

The p53 protein is a transcription factor with a well-known tumour suppressor activity, which is inactivated in most human cancers due to mutation or to the negative regulation by MDM2 and MDMX. Human cells encode two other p53-related proteins, p63 and p73, which can induce the transcription of p53 target genes or to have distinct physiological functions from p53 in cancer. Based on this, the pharmacological regulation of p53 family proteins has been considered a promising therapeutic strategy against cancer. However, the high complexity of the p53 family-pathway, particularly the simultaneous expression of several isoforms in a same mammalian cell, has hampered the knowledge of the biology and pharmacology of these proteins.

To circumvent this issue, in the present work, the yeast *Saccharomyces cerevisiae* (without orthologues of p53 family proteins) was used as a simplified cellular system for an independent analysis of individual p53 family proteins. Particularly, using a previously developed yeast-based p53-MDM2 screening assay, α-mangostin and gambogic acid, two xanthones with well-known antitumor properties, were identified as potential inhibitors of the p53-MDM2 interaction. In a previous study, this yeast-based assay also led to the identification of potential p53 activators with a flavonoid scaffold, which molecular mechanism was studied, in this work, in human colon tumour cell lines with and without p53. The tested flavonoids exhibited potent growth inhibitory activities against the two human tumour cells, suggesting that besides p53 other molecular targets may be involved in their activities. Moreover, with the yeast model, relevant data about the role of p53 family proteins on cell proliferation and death were revealed.

Additionally, in this work, new yeast assays were developed, which may represent relevant tools for future studies of p53 family proteins, particularly for the identification of new therapeutic opportunities against cancer. In fact, the inhibitory effect of MDMX on p53 was reproduced in yeast, for the first time, which led to the development of a yeast-based assay for the screening of p53-MDMX interaction inhibitors. Moreover, with the identification of *ACT1* as a possible yeast endogenous p53 target gene, this work opened the way to a new simplified yeast p53 transactivation assay, which may be used for the analysis of the p53 transcriptional activity, instead of the traditional artificial yeast transactivation reporter assays.

As a whole, the yeast research performed in this thesis confirmed the enormous potential of this cell model towards the study of human proteins. Additionally, with this work, new ways were opened and several relevant questions were raised in the p53 family research field.

Resumo

A proteína p53 é um fator de transcrição com atividade supressora tumoral, a qual se encontra inativada na maioria dos tumores humanos, devido a mutações ou à inibição pelas proteínas MDM2 e MDMX. As células humanas codificam duas proteínas relacionadas com a p53, p63 e p73, as quais podem induzir a transcrição de genes-alvo da p53, ou desempenhar, no cancro, funções fisiológicas distintas das da p53. Assim, a modulação farmacológica das proteínas da família p53 tem sido considerada uma estratégia promissora no tratamento do cancro. Contudo, a complexidade das vias de sinalização das proteínas da família p53, em particular a expressão de várias isoformas na mesma célula, tem dificultado a compreensão das propriedades biológicas e farmacológicas destas proteínas.

Para contornar esta limitação, neste trabalho, utilizou-se a levedura *Saccharomyces cerevisiae* (sem ortólogos das proteínas da família p53), como um modelo celular simplificado para uma análise individual das proteínas da família p53. Em particular, utilizando-se um modelo previamente estabelecido pelo grupo para a pesquisa de moduladores da interação p53-MDM2, identificou-se a α-mangostina e o ácido gambógico, duas xantonas com propriedades anti-tumorais, como potenciais inibidores da interação p53-MDM2. Anteriormente, este modelo permitiu a identificação de flavonóides como potenciais ativadores da p53, cujo mecanismo molecular foi estudado, neste trabalho, em linhas tumorais de carcinoma do cólon, com e sem p53. Os flavonóides inibiram o crescimento de ambas as linhas tumorais com uma potência semelhante, sugerindo o envolvimento de outros alvos celulares para além da p53. Além disso, com o modelo da levedura, são revelados conhecimentos importantes acerca do papel das proteínas da família p53 na proliferação e morte celular.

Adicionalmente, neste trabalho, foram implementados novos modelos de levedura, que se revelam muito promissores no estudo das proteínas da família p53, em particular na identificação de novas oportunidades terapêuticas contra o cancro. De facto, com a reprodução do efeito inibitório da MDMX na p53, pela primeira vez, em levedura, implementou-se um modelo de pesquisa de inibidores da interação p53-MDMX. Além disso, a identificação do gene *ACT1* de levedura como um possível alvo da p53 poderá permitir a implementação de um ensaio simplificado para o estudo da atividade transcricional da p53, em substituição dos artificiais atualmente disponíveis.

No geral, a investigação desenvolvida no âmbito desta tese confirmou o enorme potencial da levedura como um organismo modelo para o estudo de proteínas humanas. Além disso, com este trabalho, novos caminhos e diversas questões foram levantadas na área de investigação das proteínas da família p53.

Table of Contents

List of figures

List of tables

Abbreviations

3,7DHF	3,7-dihydroxyflavone
ABP140	Actin binding protein 140
AMPK	Adenosine monophosphate activated protein kinase
Apaf-1	Apoptosis protease-activating factor 1
Bax	Bcl-2 associated X protein
Bid	BH3 interacting-domain death agonist
C1	4-benzyloxy-2’,4’-dihydroxychalcone
C2	2’,4’-dihydroxy-3,4,5-trimethoxy-3’-propylchalcone
C3	2’,4’-dihydroxy-4-methoxy-3’-propylchalcone
C4	3,4,4’,5,6’-pentamethoxy-2’-prenyloxychalcone
C5	3,4,5-trimethoxy-4’,6’-bis(methoxymethoxy)-3’-prenylchalcone
CAK	Cdk-activating kinase
Cdk	Cyclin-dependent kinase
CFU	Colony-forming unit
cyt *c*	Cytochrome *c*
DBD	DNA-binding domain
DMSO	Dimethyl sulfoxide
FADD	Fas-associated protein with death domain
FASAY	Functional analysis of separated alleles
GA	Gambogic acid
GFP	Green fluorescent protein
HTS	High throughput screening
mTOR	Molecular target of rapamycin
OD	Oligomerization domain
p53RE	p53 responsive element
PFT-α	Pifthrin-α
Pgk1p	Phosphoglycerate kinase
PI	Propidium iodide
PKC	Protein kinase C
PUMA	p53 upregulated modulator of apoptosis
Rb	Retinoblastoma protein
ROS	Reactive oxygen species
SAM	Sterile-alpha motif
TAD	Transactivation domain
TID	Transactivational inhibitory domain

TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
wt	Wild-type
αMG	α-Mangostin

1 Introduction

1. Introduction

1.1. The p53 family of proteins

The p53 protein was discovered more than 30 years ago as a protein interacting with the oncogenic T antigen from SV40 virus (Lane and Crawford, 1979; Linzer and Levine, 1979). This well-known tumour suppressor protein acts as an inducible sequence-specific DNA-binding transcription factor, playing a fundamental role in the maintenance of genomic integrity and normal cell growth. When activated in response to stress stimuli, such as DNA damage, oncogene activation, hypoxia or nutrient deprivation, p53 triggers a cascade of cellular responses in order to prevent the replication of damaged DNA or the proliferation of genetically altered cells that could lead to tumour formation. Due to its tumour preventive activity, p53 has been called "the guardian of the genome" (Lane, 1992; Wei et al., 2012).

Almost 20 years after the discovery of p53, two *TP53*-related genes, *TP63* and *TP73* (encoding p63 and p73, respectively), were identified (Kaghad et al., 1997; Yang et al., 1998). Although p53 was the first member of this group of proteins to be discovered, mainly due to its essential role in carcinogenesis, phylogenetic studies suggested that the p53 family derived from the triplication of a p63/p73-like ancestral gene was likely involved in the preservation of genomic stability in germ cells (Dotsch et al., 2010; Petre-Lazar et al., 2007; Rutkowski et al., 2010). p53, p63 and p73 share remarkable structural similarities, which led to the initial speculation that these proteins might have identical functions. However, accumulated data have suggested that unlike p53, p63 and p73 have a major role in the regulation of embryonic development [reviewed in (Deyoung and Ellisen, 2007)].

An increasing number of studies have also suggested that p53 family proteins regulate many vital biological processes, and alterations of their activities may often underlie mechanisms of malignant transformation and even affect tumour response to therapy. In fact, p53 inactivation promotes tumourigenesis by enhancing tumour cell growth and resistance to chemotherapy and radiotherapy. p53 is inactivated in the vast majority of human tumours, and *TP53* is the most mutated gene in human cancer (Hollstein et al., 1996; Hollstein et al., 1991). Although mutations in the *TP63* and *TP73* genes are less common, the p63 and p73 proteins appear to also play important roles in many human tumours through both p53-dependent and independent pathways. The contribution of p63 and p73 to tumourigenesis is complex since multiple isoforms of each protein are simultaneously expressed in human cells (Kovalev et al., 1998).

1.1.1. Structural organization

The *TP53, TP63* and *TP73* genes are located on chromosomes 17p13.1, 3q27-29 and 1p36.2-3, respectively (Wei et al., 2012). Through activation of different promoters and alternative splicing, each gene gives rise to multiple protein isoforms. The specific functions of each isoform are still not clearly established. The *TP63* and *TP73* genes have two promoters, generating proteins that can be categorized into two main groups (Fig. 1): i) proteins containing an N-terminal acidic transactivation domain (TAD), termed TA isoforms, resulting from the P1 promoter (located upstream of exon 1) and ii) N-terminally truncated proteins lacking the TAD, called ΔN isoforms, arising from transcription from the P2 promoter (located between exons 3 and 4). In addition, in the case of p73, other ΔN isoforms have been described (ΔN'p73, ΔEx2p73 and ΔEx2-3p73) to be produced through P1 activation and alternative splicing at the 5' end (Sayan et al., 2007; Vilgelm et al., 2008; Yang et al., 1998). Regarding the *TP53* gene, it includes an additional promoter P1' (located upstream of exon 1), which gives rise to proteins containing the full TAD, while the transcription initiated in the P1 promoter (located within exon 1) produces proteins lacking the first 40 amino acid residues (Δ40p53). On the other hand, proteins produced through activation of the P2 promoter (between exons 4 and 5) lack the first 133 amino acids (Δ133p53) (Wei et al., 2012).

Besides the activation of different promoters, additional diversity is generated by alternative splicing at the 3' end, which gives rise to splice variants traditionally named with letters from the Greek alphabet. Eight splice variants have been described for p53 (α, β, γ, δ, ε, ζ, Δp53 and ΔE6), five for p63 (α, β, γ, δ and ε) and nine for p73 (α, β, γ, δ, ε, θ, ζ, η and η1) (Fig. 1). The transactivation and apoptotic potential of p63 and p73 (TA isoforms) vary greatly depending on the isoform. However, they are generally considered to be less efficient than p53. p63γ and p73γ are the isoforms that most closely resemble full-length p53. In fact, p63γ has been shown to be as potent as p53 regarding the induction of gene transcription and apoptosis, while the most potent p73 isoform appears to be p73β. Little is known about the functions of the remainder isoforms, since most studies have been focused on the α, β and γ isoforms [reviewed in (Allocati et al., 2012)].

Alternative splicing combined with alternative promoter usage results in the production of a large number of isoforms. Theoretically, the *TP53* gene can produce at least 20 isoforms, while *TP63* can produce at least 10, and *TP73* more than 40, although not all have been experimentally confirmed [reviewed in (Wei et al., 2012)].

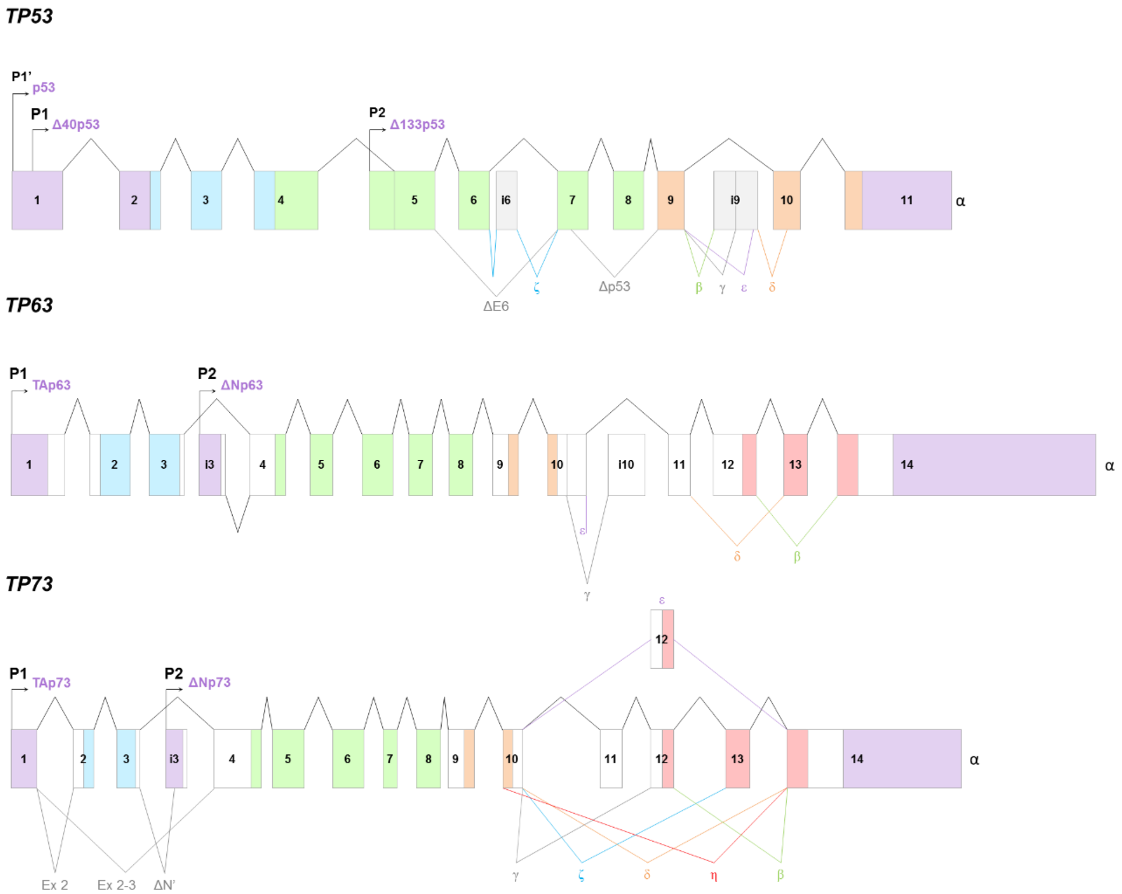


Fig. 1 – Architectures of human *TP53*, *TP73*, and *TP63* genes. Transcription of p53 family genes is controlled by two promoters, P1 and P2. P2 gives rise to N-terminally truncated isoforms, lacking all or part of the TAD. *TP53* possesses an additional promoter. Alternative splicing at the C-terminus leads to the generation of a variety of different isoforms of each protein [adapted from (Wei et al., 2012)].

All p53 family proteins possess three conserved structural domains: a DNA-binding domain (DBD), an oligomerization domain (OD) involved in the tetramerization of these proteins, required for their activity, and a transactivation domain (TAD) [Fig. 2; reviewed in (Allocati et al., 2012)]. The DBD presents the highest degree of homology among the three members, with over 60% amino acid identity between p53 and p63/p73, and about 85% amino acid identity between p63 and p73. This suggests a central role for the transcriptional activity among the range of functions attributed to p53 family proteins. Regarding the OD, the amino acid identity between p53 and p63/p73 is only about 30%, perhaps explaining why p63 and p73 are unable to form hetero-oligomeric complexes with wild-type (wt) p53. On the other hand, the ODs homology between p63 and p73 is approximately 60%. Therefore, p63 and p73 are able to form heterotetramers, although with less efficiency than homotetramers (Davison et al., 1999). p63 and p73 have an additional domain at the C-terminus termed sterile-alpha motif (SAM) (Fig. 2), which is responsible for protein-protein interactions. This type of motif can be found in a wide range of proteins involved in developmental regulation, also being implicated in transcriptional repression and in lipid membrane binding (Thanos and Bowie, 1999). In the alpha isoforms of p63 and p73, an additional post-SAM region has been identified and designated transactivational inhibitory domain (TID). TID is thought to be involved in the inhibition of the transactivating function of these proteins through intra- or intermolecular associations with the TAD. In fact, the p63 and p73 proteins appear to be less potent in transactivation and apoptosis induction, and their potency can be restored through deletion of the TID (Serber et al., 2002; Vilgelm et al., 2008).

Fig. 2 – Schematic representation of p53 family protein structure. p53 family proteins possess a transactivation domain (TAD), a DNA-binding domain (DBD) and an oligomerization domain (OD). p63 and p73 have an additional domain at the C-terminal, named sterile-alpha domain (SAM) [adapted from (Allocati et al., 2012)].

1.1.2. Molecular targets and signalling pathways

Low levels of p53 can be found in unstressed cells due to the constant proteasomal degradation of this protein (Olsson et al., 2007). Upon exposure to a variety of stress signals, p53 undergoes post-translational modifications, which lead to its stabilization and activation. Under these conditions, p53 accumulates in the nucleus, where it transcriptionally regulates a large number of downstream genes involved in cell cycle and cell death, among other cellular processes. Most p53 functions result from its binding to specific DNA sequences, called p53-responsive elements (p53REs), and consequent transcriptional activation of target genes involved in key cellular processes. Nevertheless, it is widely recognized that p53 can also influence several cellular processes through transcription-independent mechanisms [reviewed in (Alsafadi et al., 2009)].

Many functions attributed to p53 are shared by p63 and p73 (TA isoforms). This can be explained by the ability of these proteins to transactivate an overlapping array of target genes, since these proteins are able to bind to p53REs [reviewed in (Vilgelm et al., 2008)]. In fact, p63 and p73 have been shown to interact with p53REs in reporter assays. Chromatin immunoprecipitation experiments also demonstrated the ability of endogenous p63 and p73 to bind to p53-regulated promoters (Costanzo et al., 2002; Perez et al., 2007; Tomkova et al., 2006). In contrast, the ΔN isoforms of p63 and p73, which lack the TAD, are usually described as having a dominant-negative effect on TA isoforms (Alsafadi et al., 2009). Two non-mutually exclusive mechanisms have been proposed to explain this dominant negative effect: promoter competition and heterocomplex formation (Nakagawa et al., 2002; Stiewe et al., 2002; Vilgelm et al., 2008; Zaika et al., 2002). In promoter competition, the ΔN isoforms would compete with the TA isoforms for their target gene promoters, thus preventing TA-mediated transcriptional activity. Heterocomplex formation could be explained by the presence of the same OD in ΔN isoforms, which allows the formation of transcriptionally inactive hetero-oligomers with TA variants. In a given cellular context, the net effects of p53 family proteins are dependent on the TA/ΔN expression ratio. It has been described that both p53 and p73 can regulate the expression of ΔNp73 through direct activation of the P2 promoter of p73 (Grob et al., 2001; Kartasheva et al., 2002). A p53 binding element has been identified within the P2 promoter of *TP63*, which allows p53 to induce the expression of ΔNp63 (Harmes et al., 2003). Therefore, auto-regulatory feedback loops are established for the fine control of the TA/ΔN expression ratio, and consequently of the expression levels of p53 family target genes. ΔN isoforms appear to integrate a feedback mechanism to prevent an exacerbated activity of TA isoforms. However, current data suggest that these isoforms may have other functions. In fact, both ΔNp63 and ΔNp73 appear to exert their own transcriptional activity, mediated by additional transactivation domains (Dohn et al., 2001; Liu et al., 2004; Wu et al., 2003).

Cell cycle

One of the most important cellular processes regulated by p53 family proteins is the cell cycle. Cell cycle comprises four stages: Gap1 (G1; in which cells increase in size), synthesis (S; in which DNA replication occurs) and Gap2 (G2; in which cells continue to grow), collectively designated interphase, and the last phase, mitosis (M), corresponding to the nuclear division. Cell cycle checkpoints regulate the progression of cells through each stage of the cell cycle, in order to prevent the transmission of damaged genetic material to the daughter cells. There are four cell cycle checkpoints: G1/S, intra-S, G2/M and the spindle assembly checkpoint [reviewed in (Nurse, 2000)]. These cell cycle checkpoints are regulated by cyclin-dependent kinase complexes, which are protein complexes formed though association of an inactive catalytic subunit, cyclin-dependent kinase (Cdk), with a regulatory subunit, cyclin. Since Cdk levels are usually constant throughout the cell cycle, the regulation of cell cycle progression depends on variations of the levels of each cyclin (Morgan, 1995). In response to a variety of stress signals, the p53 family proteins can induce cell cycle arrest by inhibiting the G1/S or G2/M transitions (Fig. 3) [reviewed in (Harms et al., 2004)], as will be explained below.

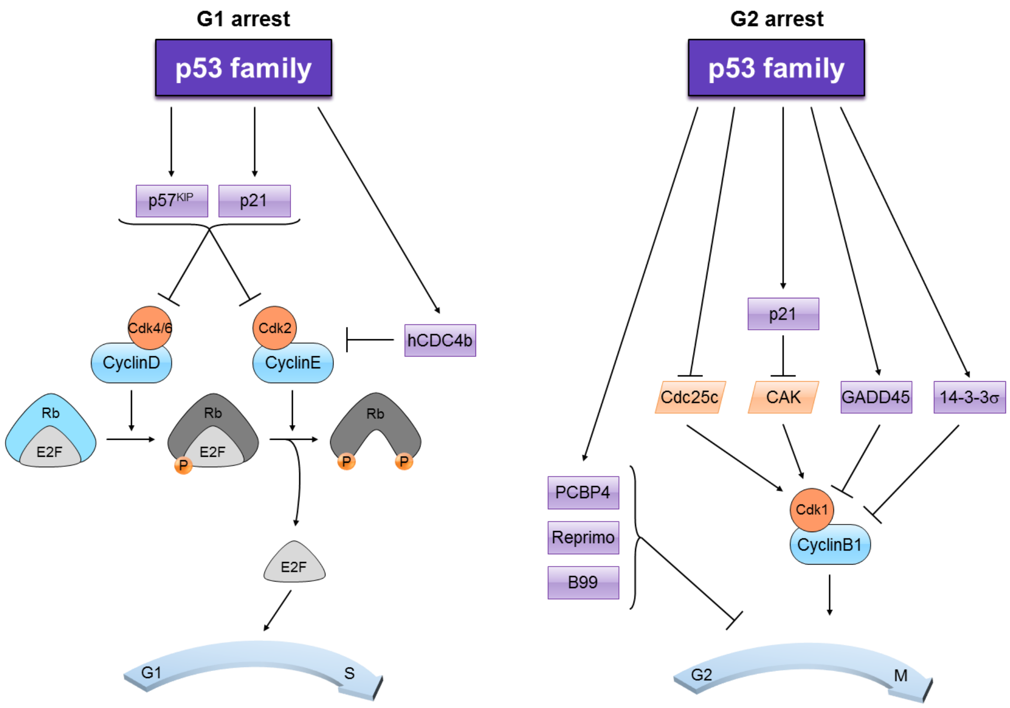


Fig. 3 – Regulation of the cell cycle by p53 family proteins. p53 family proteins induce cell cycle arrest by inducing the expression of several proteins, resulting in the inhibition of the G1/S and G2/M transitions. [CAK: Cdk-activating kinase; Cdk: cyclin-dependent kinase; GADD45: growth arrest and DNA-damage inducible protein; PCBP4: poly(rC) binding protein 4; Rb: retinoblastoma protein). Adapted from (Harms et al., 2004)].

During G1 phase, the expression of cyclin D is stimulated (Stevens and La Thangue, 2003). This cyclin forms complexes with either Cdk4 or Cdk6, which can then target the retinoblastoma protein (Rb) for phosphorylation. Once phosphorylated, Rb releases the E2F transcription factor, which leads to the transcription of genes required for S-phase entry (Fig. 3, left panel). One of the target genes of E2F is cyclin E which forms a complex with Cdk2, that phosphorylates and consequently inhibits Rb, leading to the establishment of a positive feedback loop (Koff et al., 1992; Weinberg, 1995; Zetterberg et al., 1995). In response to cellular stress (and probably to specific developmental signals), p53 family proteins induce the transcription of p21, a Cdk inhibitor that binds to cyclin E/Cdk2 and cyclin D/Cdk4/6 complexes, thus preventing the G1/S transition (Chen et al., 1996; el-Deiry et al., 1993). Although p21 is the major effector of p53 family-mediated G1 arrest, there are other proteins involved in this process that are upregulated by p53, such as hCDC4b, a component of the Skp, Cullin, F-box containing ubiquitin ligase complex, that targets cyclin E for degradation, thus inhibiting the S-phase entry (Kimura et al., 2003). Interestingly, $p57^{KIP}$, a Cdk inhibitor involved in the induction of G1 arrest, has been described as a unique target of p73 (Balint et al., 2002).

Entry in mitotisis is regulated by the cyclin B1/Cdk1 complex, which is activated through the combined activity of Cdc25c phosphatase (which removes phosphate groups that compromise the activity of the complex) and Cdk activating kinase (CAK; which adds phosphate groups on specific points required for activation of the complex) (Kishimoto and Okumura, 1997) (Fig. 3, right panel). p21, a major transcriptional target of p53, participates in G2 arrest through association with the cyclin B1/Cdk1 complex and inhibition of its phosphorylation by CAK (Smits et al., 2000). The growth arrest and DNA damage inducible protein (GADD45) was the first identified p53-target gene. It binds to Cdk1 preventing its binding to cyclin B1, and, consequently, the formation of the protein complex necessary for mitotic entry, which results in G2 arrest (Zhan et al., 1999). p53 is also able to induce the expression of other proteins involved in G2 arrest, namely the poly(rC) binding protein 4 (PCBP4; a RNA-binding protein), Reprimo (a highly glycosylated protein that is thought to play a role in cyclinB1/Cdk1 localization), and B99 (a protein with microtubule localization) (Ohki et al., 2000; Utrera et al., 1998; Zhu and Chen, 2000). The activity of the cyclin B1/Cdk1 complex is also modulated by subcellular localization, since during interphase it is kept in the cytoplasm through nuclear export. Both p53 and p73 are able to induce the expression of 14-3-3σ, a scaffold protein involved in G2 arrest, by promotion of cyclinB1/Cdk1 nuclear export. Through this mechanism, 14-3-3σ inhibits the cyclinB1/Cdk1 complex, by physically separating it from its target proteins (Hermeking et al., 1997). In addition, p53 was reported to promote G2 arrest through repression of the expression of proteins essential for mitotic entry, namely Cdk1, cyclinB1 and Cdc25c (Krause et al., 2000; Taylor et al., 2001; Yun et al., 1999).

Apoptosis

Apoptosis is a programmed cell death process characterized by nuclear condensation and fragmentation, mitochondrial swelling, and membrane blebbing. This type of cell death occurs without inflammation. There are two main apoptotic pathways: the extrinsic (or death receptor pathway) and the intrinsic (or mitochondrial pathway) (Fig. 4) [reviewed in (MacFarlane, 2003; Newmeyer and Ferguson-Miller, 2003)].

Briefly, the extrinsic pathway consists on the activation of cell death receptors located at the plasma membrane, such as KILLER/DR5 and Fas. The activation of these receptors leads to their trimerization and consequent clustering of the intracellular death domain, which subsequently recruits the protein adaptor FADD (Fas-associated protein with death domain) through homotypic death domain interactions. The death effector domain of FADD then recruits caspase-8, forming the death-inducing signalling complex (DISC).

On the other hand, the intrinsic pathway involves alterations of the mitochondrial membrane potential. These alterations can be caused by the translocation of Bcl-2 family proteins to the mitochondria. Bax (Bcl-2 associated X protein) translocates from the cytosol to the inner mitochondrial membrane where it facilitates the release of cytochrome *c* (cyt *c*). Similarly, PUMA (p53 upregulated modulator of apoptosis) interacts with Bcl-2 and Bcl-xL, inducing the cyt *c* release from the mitochondrial membrane (Yu et al., 2001). Interestingly, Bid (BH3 interacting domain death agonist) appears to function as a bridge between the extrinsic and intrinsic pathways. It is cleaved by caspase-8, thus exposing an N-terminal glycine that is subsequently myristoylated. The modified Bid then translocates to the mitochondria, where it participates in the intrinsic apoptotic pathway (Li et al., 1998; Zha et al., 2000). Noxa has also been shown to localize to the mitochondria where it interacts with anti-apoptotic members of the Bcl-2 family, resulting in the efflux of cyt *c* (Seo et al., 2003). Once released to the cytoplasm, cyt *c* combines with Apaf-1 (apoptosis protease-activating factor 1) and the initiator caspase-9, forming the apoptosome.

Both these pathways result in the activation of executioner caspases (caspases-3, -6 and -7) which will then cleave nuclear and cytoskeletal structure proteins, leading to apoptosis.

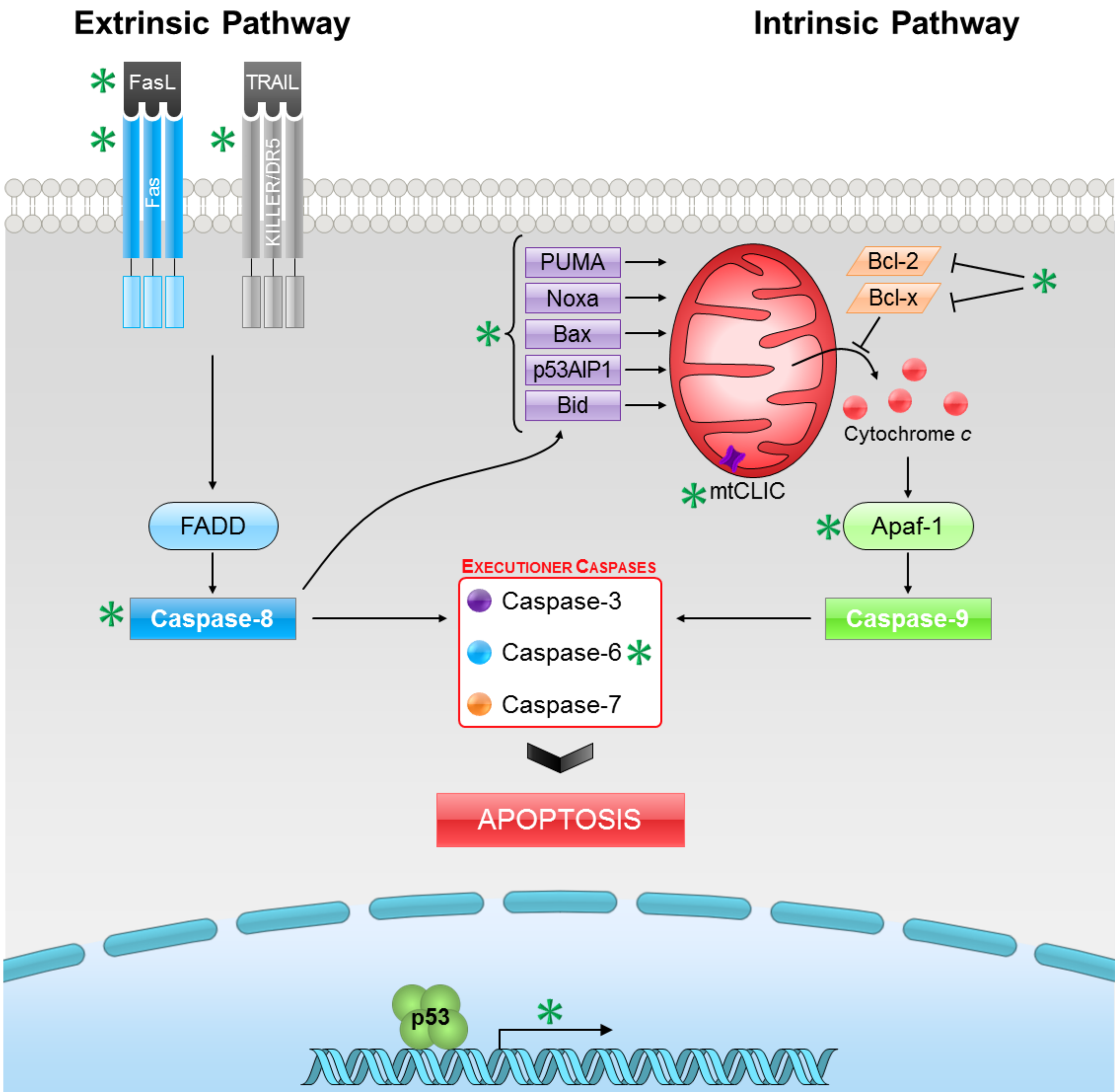


Fig. 4 – Regulation of intrinsic and extrinsic apoptotic pathways by p53. p53 induces the expression of proteins involved in both the intrinsic and the extrinsic pathways of the apoptotic cell death. p53 can also induce apoptosis through a transcription-independent pathway, through its direct translocation to the mitochondria, where it interacts with members of the Bcl-2 family. The asterisks refer to p53 transcriptional activity. [Apaf-1: apoptotic protease activating factor 1; Bax: Bcl-2 associated X protein; Bid: BH3 (Bcl-2 homology domain 3) interacting domain death agonist; FADD: Fas-associated protein with death domain; mtCLIC: mitochondrial chloride intracellular channel; p53AIP: p53-regulated apoptosis inducing protein 1; PUMA: p53-upregulated modulator of apoptosis; TRAIL: TNF (tumour necrosis factor)-related apoptosis-inducing ligand. Adapted from (Ryan, 2011)].

The p53 protein is able to induce apoptosis through the extrinsic pathway by upregulating two cell death receptors, KILLER/DR5 and Fas, as well as the ligand for Fas, FasL (Fukazawa et al., 1999; Owen-Schaub et al., 1995; Takimoto and El-Deiry, 2000). *CASP8*, the gene that encodes caspase-8, is another transcriptional target of p53 (Liedtke et al., 2003).

In addition, p53 family proteins may up-regulate several proteins involved in the intrinsic apoptotic pathway. These include pro-apoptotic proteins from the Bcl-2 family, such as Bax, Noxa, PUMA and Bid (Miyashita and Reed, 1995; Nakano and Vousden, 2001; Oda et al., 2000a; Sax et al., 2002). Furthermore, p53 is able to promote apoptosis through transcriptional repression of the anti-apoptotic members of the Bcl-2 family, namely Bcl-2 and Bcl-xL, which stabilize the mitochondrial membrane potential (Sugars et al., 2001). Both p53 and p73 induce the expression of p53AIP1 (p53-apoptosis inducing protein 1), which localizes to the mitochondria where it interacts with Bcl-2 to facilitate the release of cyt *c* and the consequent apoptosis induction through the intrinsic pathway (Costanzo et al., 2002; Oda et al., 2000b). p53 also upregulates mtCLIC/CLIC4 (mitochondrial chloride intracellular channel), an organellular chloride channel protein that reduces mitochondrial membrane potential (Fernandez-Salas et al., 2002). Additionally, p53 induces the expression of intracellular regulators of the intrinsic apoptotic pathway, namely Apaf-1 and caspase-6 (Fortin et al., 2001; MacLachlan and El-Deiry, 2002).

Besides its transcriptional function, accumulating data have shown that p53 itself can translocate to the mitochondria where it induces apoptosis through a transcription-independent mechanism (Manfredi, 2003). Haupt and colleagues (1995) described that a mutant variant of p53, which failed to upregulate gene expression, retained the ability to induce apoptosis, thus suggesting the existence of a transcription-independent mechanism of action of the p53 protein. Since then, it has been described that a fraction of p53 translocates to the mitochondria in tumour cells undergoing p53-dependent apoptosis (Marchenko et al., 2000). Furthermore, Mihara and colleagues (2003) have shown that targeting p53 to the mitochondria is sufficient for apoptosis induction, and have provided evidence that supports the formation of complexes between p53 and the anti-apoptotic proteins Bcl-2 and Bcl-xL. The formation of such complexes results in the release of the pro-apototic members of the Bcl-2 family, Bax and Bak (Bcl-2 antagonist/killer), and consequent permeabilization of outer mitochondrial membrane and cyt *c* release.

The production of reactive oxygen species (ROS) can also facilitate apoptosis. Although ROS are by-products of normal mitochondrial function, high levels of ROS have been associated with p53-induced apoptosis, although the exact mechanism has not been elucidated yet (Fleury et al., 2002).

Autophagy

Autophagy is a conserved catabolic mechanism consisting on the lysosomal degradation of intracellular components of cells. Through autophagy new metabolic substrates are provided to the cell, favouring its adaptation to stressful conditions and its survival [reviewed in (Yang and Klionsky, 2010)]. However, uncontrolled or excessive autophagy can lead to cell death [reviewed in (Mizushima et al., 2008)]. p53 can either activate or inhibit autophagy, depending on its subcellular localization [reviewed in (Ryan, 2011)]. Once in the nucleus, p53 directly activates the adenosine monophosphate activated protein kinase (AMPK) pathway in response to stress signals, resulting in the inhibition of the molecular target of rapamycin (mTOR) pathway and subsequent induction of autophagy (Feng et al., 2005). Several p53 transcriptional targets encode pro-autophagic modulators, including AMPK, death associated protein kinase 1 (DAPK-1), damage-regulated autophagy modulator 1 (DRAM1), pro-apoptotic Bcl-2 family proteins [Bad (Bcl-2 associated death promoter), Bax, Bnip3 (Bcl-2/adenovirus E1B 19 kDa protein-interacting protein 3), and PUMA], sestrin 2 (SESN2), and tuberous sclerosis protein 2 (TSC2) (Sui et al., 2011). Besides p53, p73 can also activate DRAM, thus contributing to autophagy induction (Crighton et al., 2007). Interferon stimulated exonuclease gene 20 kDa-like 1 (ISG20L1), which modulates genotoxic stress-induced autophagy, has been recently described as a p53 and p73 target (Eby et al., 2010).

In the cytoplasm, p53 is able to inhibit autophagy through a transcription-independent mechanism. In contrast to its nuclear effects, in the cytoplasm, p53 inhibits AMPK and activates mTOR, although the molecular mechanism is still not fully understood (Tasdemir et al., 2008).

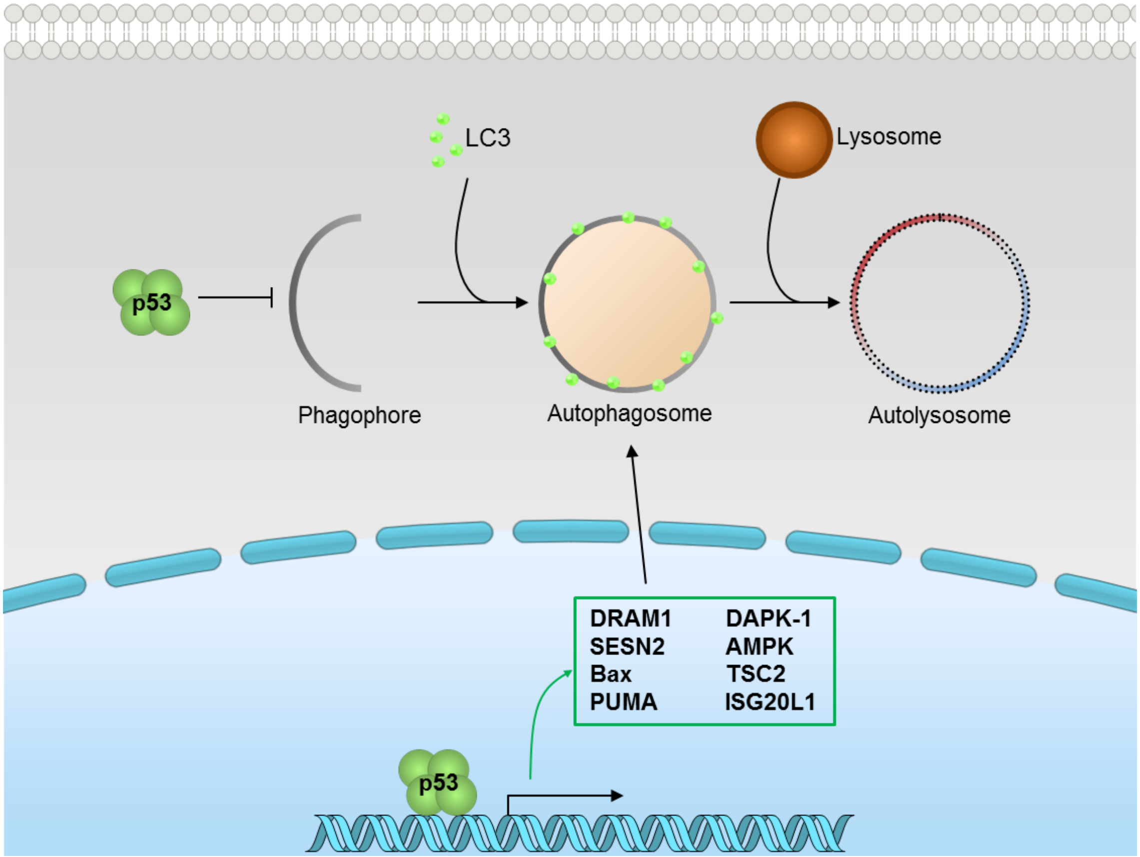


Fig. 5 – p53 distinctly regulates autophagy depending on its subcellular localization. In the nucleus, p53 induces the expression of proteins that favour autophagy. In the cytoplasm, p53 inhibits autophagy through a transcription-independent mechanism [AMPK: adenosine monophosphate activated protein kinase; Bax: Bcl-2 associated X protein; DAPK: death associated protein kinase 1; DRAM1: damage-related autophagy modulator; ISG20L1: Interferon stimulated exonuclease gene 20 kDa-like 1; PUMA: p53 upregulated modulator of apoptosis; SESN2: sestrin 2; TSC2: tuberous sclerosis protein 2. Adapted from (Ryan, 2011)].

1.2. Endogenous modulators of p53 family proteins

As referred above, the p53 family of proteins plays fundamental roles in the regulation of cellular proliferation and death. These cellular processes must be tightly regulated, since their disruption can lead to apoptosis related-diseases, resulting either from an excessive (degenerative disorders) or a deficient (neoplastic disorders) cell death (Reinhardt and Schumacher, 2012).

The main endogenous modulators of p53 family proteins are MDM2 and MDMX (also called MDM4), which are oncoproteins found to be deregulated in many human tumours (Wade et al., 2013). These proteins share a high level of structural similarity and are thought to derive from the duplication of an ancestral gene (Momand et al., 2011). Both MDM2 and MDMX bind to a short α-helical stretch on the TAD of p53 through their N-terminal hydrophobic region, which is highly conserved (Böttger et al., 1999). Therefore, both MDM2 and MDMX are able to inhibit the p53 activity by blocking the TAD (Kussie et al., 1996; Momand et al., 1992) (Fig. 6). MDM2 and MDMX possess a zinc finger and an acidic region in their central portion, followed by a C-terminal RING finger domain, involved in homo- and heterodimerization. The two latter domains are essential for the E3 ubiquitin ligase activity in MDM2 (Lenos and Jochemsen, 2011). This RING finger domain mediates the interaction with an E2 ubiquitin conjugating enzyme, thereby bringing it into close proximity to its substrate proteins for ubiquitin conjugation [reviewed in (Glickman and Ciechanover, 2002)]. In fact, MDM2 is able to inhibit the p53 activity through ubiquitylation, either in the form of a homodimer or of a MDM2/MDMX heterodimer (Dolezelova et al., 2012; Uldrijan et al., 2007). In spite of the extensive sequence homology between its RING finger domain and that of MDM2, MDMX has no detectable E3 ubiquitin ligase activity *in vivo* (Kawai et al., 2003; Meulmeester et al., 2003). However, Badciong and colleagues (2002) have reported that, *in vitro,* MDMX is capable of ubiquitylating p53, albeit with very low efficiency.

When the levels of MDM2 are low, monoubiquitylation of p53 is favoured, leading to nuclear export and consequent inhibition of p53 transcriptional activity. Under these circumstances, monoubiquitylated p53 can localize to the mitochondria where it is deubiquitylated by the ubiquitin-specific protease-7 (USP7). Once deubiquitylated, p53 is able to induce apoptosis through a transcription-independent mechanism. Therefore, low levels of MDM2 are able to promote p53-mediated apoptosis (Marchenko et al., 2007). On the other hand, high levels of MDM2 favour polyubiquitylation of p53, which results in its proteasomal degradation (Fig. 6). This degradation mechanism is responsible for the maintenance of low levels of p53 in unstressed cells (Lee and Gu, 2010). Furthermore, under such unstressed conditions, it has been proposed that the MDM2/MDMX heterodimer may function as a more active ubiquitin ligase. In fact, heterodimer formation has been described to stabilize MDM2, and also to provide an extended motif for interaction with the E2 ubiquitin conjugating enzyme (Linke et al., 2008).

MDMX interaction with the TAD of p53 also prevents its interaction with p300, a protein involved in p53 activation through acetylation of certain lysine residues at the C-terminal. In this way, MDMX may indirectly stimulate MDM2-mediated p53 inhibition, since these lysine residues are also targeted for ubiquitylation by MDM2 (Lee and Gu, 2010).

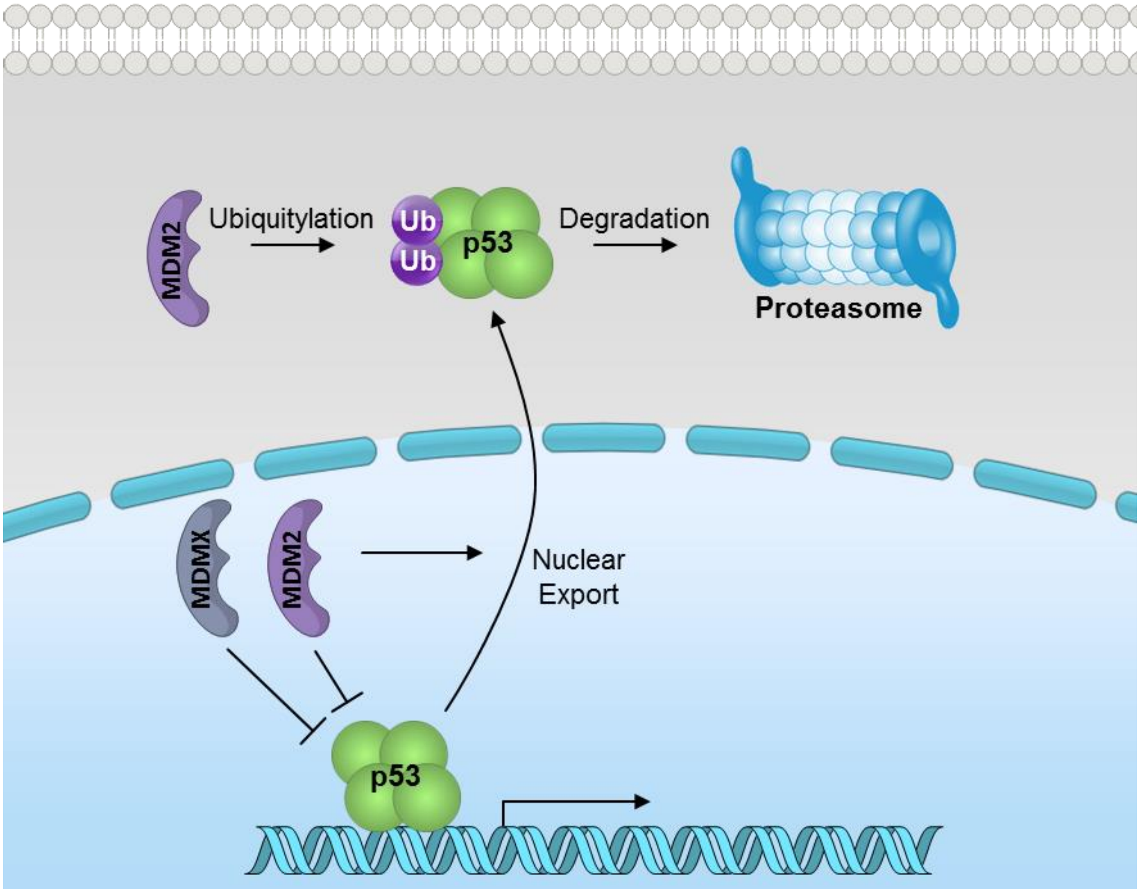


Fig. 6 – Regulation of p53 by the negative modulators MDM2 and MDMX. Both MDM2 and MDMX bind to and inhibit p53 transcriptional activity. MDM2 is also able to promote p53 nuclear export and ubiquitylation, which results in proteasomal degradation (Ub: ubiquitin).

Because of the structural similarities between p53 and its homologues, p63 and p73, it is plausible that MDM2 and MDMX might regulate these proteins in the same way as they regulate p53. In fact, it has been described that all three p53 family members encode a consensus sequence related to the MDM2 binding domain (Kallen et al., 2009; Kussie et al., 1996; Popowicz et al., 2007). Several groups attempted to identify and characterize these possible interactions, but results have often been controversial. Indeed, although it is widely accepted that both MDM2 and MDMX bind to and inhibit p73, albeit not inducing its degradation (Balint et al., 1999; Ongkeko et al., 1999; Wang et al., 2001; Zeng et al., 1999), data regarding a presumable interaction between these proteins and p63 is still contradictory. For example, it has been described that neither MDM2 nor MDMX are able to bind to p63 (Little and Jochemsen, 2001; Wang et al., 2001). However, it has also been reported that p63 can interact with MDM2, but instead of the inhibitory effect observed with p53, this interaction results in an increase of p63 transcriptional activity (Calabro et al., 2002). On the other hand, it has also been suggested that both MDM2 and MDMX are able to downregulate p63 transcriptional activity, with equivalent efficiency, although not by inducing its degradation (Kadakia et al., 2001). Furthermore, according to this work, MDM2, but not MDMX, seemed to be able to induce the nuclear export of p63, thus inhibiting the p63-mediated apoptosis. More recently, Zdzalik and colleagues (2010) suggested that both p63 and p73 were able to interact with MDM2 and MDMX. This work predicted that p63 and p73 interact more strongly with MDMX than with MDM2, and that the binding affinities of MDM2 and MDMX for p53 and p73 were similar and stronger than those determined for p63. However, it is important to refer that for these determinations, the authors did not use the full-length proteins, but rather peptides containing the regions presumably involved in the interactions.

Both MDM2 and MDMX are tightly regulated by several post-translational modifications that can either promote or inhibit their activities, and are also subjected to transcriptional regulation [reviewed in (Wade et al., 2010)]. Both *MDM* genes contain two promoters, P1 and P2, which give rise to identical transcripts. While P1 (located upstream of the first exon) controls the production of basal levels of these proteins, P2 (located within exon 1) is highly regulated and is responsible for the inducible expression of MDM2 and MDMX (Barak et al., 1994; Manfredi, 2010; Zauberman et al., 1995). Indeed, MDM2 and MDMX expression can be induced by mitogens through activation of the P2 promoter (Gilkes et al., 2008; Ries et al., 2000). The P2 promoter of the *MDM2* gene has been found to contain a p53RE through which p53 is able to increase the MDM2 expression levels, thus establishing an auto-regulatory negative feedback loop, presumably as a way to prevent an exacerbated p53 activity (Wu et al., 1993). For a long time, it was thought that p53 was unable to induce the expression of MDMX. However, Wei and colleagues (2006) reported the existence of a p53RE within the first intron of the *MDMX* gene. Later, it was reported that p53 could, in fact, induce the transcription of *MDMX* (Li et al., 2010), through the P2 promoter (Phillips et al., 2010), thus establishing another negative regulatory feedback loop.

Although some details about the modulation of p53 family proteins by MDM2 and MDMX are still not fully understood, it seems clear that the ratio between MDM2 and MDMX strongly determines the outcome of p53 stability. Moreover, it is also known that perturbations in this network have clear implications in tumourigenesis, representing promising opportunities for cancer therapy (Lenos and Jochemsen, 2011; Wade et al., 2013).

1.3. Targeting p53 family proteins in cancer therapy

Since p53 family proteins control many of the processes that ultimately determine cell proliferation, it is not surprising that these proteins play a fundamental role in cancer development and progression. In fact, *TP53* is mutated in approximately 50% of all human tumours. Missense mutations within the DBD are the most frequently observed (Martinez, 2010). In tumours harbouring wt p53, p53 regulated signalling pathways are often inactivated due to overexpression of the p53 endogenous modulators MDM2 and MDMX (Brown et al., 2009). Furthermore, p53 is thought to become inactivated at some point of the progression of every tumour, since p53 inactivation has been described as a necessary condition for the maintenance of the tumour phenotype (Martins et al., 2006; Ventura et al., 2007; Xue et al., 2007). The relevance of p53 in tumourigenesis is also documented by the Li-Fraumeni syndrome, a rare disease resulting from germline mutations in the *TP53* gene and characterized by an extremely high susceptibility for the development of tumours (Malkin, 1993). Similarly, *TP53* deletion in mice increases susceptibility for cancer development, without causing any major developmental defect (Donehower et al., 1992).

Besides its importance in tumour development, a functional p53 pathway is essential for an effective response to the anti-cancer drugs that trigger apoptosis by inducing DNA damage. (Chen et al., 2010).

Due to their striking structural similarities with p53, it was initially hypothesized that p63 and p73 would function as classic tumour suppressors. However, the role of these proteins in tumourigenesis is still not fully understood. In spite of this, overexpression of specific isoforms of p63 and p73 has been described in some human tumours, and in some cases even related to an unfavourable prognosis [reviewed in (Deyoung and Ellisen, 2007)]. In p63$^{+/-}$ heterozygous mice, the susceptibility to tumour development is increased (Flores et al., 2005), while *TP63* deletion is related to profound alterations of the skin and epithelial appendages, truncated limbs and craniofacial alterations, as well as premature death after birth (Mills et al., 1999). Although rare, p63 mutations in humans are also related to developmental defects rather than cancer susceptibility [reviewed in (van Bokhoven and Brunner, 2002)]. However, p63 overexpression, particularly of ΔNp63α, has been demonstrated in up to 80% of primary head and neck squamous cell carcinomas (Sniezek et al., 2004; Weber et al., 2002). Furthermore, loss of p63 expression is observed in a subset of invasive bladder carcinomas, and it appears to be related to invasion, metastasis, and thus, poor prognosis (Park et al., 2000; Urist et al., 2002).

In mice, deletion of *TP73* results in premature death as a result of inflammation and recurring infections. Additionally, these animals suffer from various neurological abnormalities (Yang et al., 2000). Concerning to tumour susceptibility, TAp73$^{-/-}$ mice are tumour prone, displaying genomic instability, while ΔNp73 knockouts show increased sensitivity to DNA-damaging agents (Tomasini et al., 2008; Wilhelm et al., 2010). Indeed, it has been described that, in humans, the DNA damage induced by specific chemotherapeutic agents results in TAp73 activation, which appears to be fundamental for triggering apoptosis (Gong et al., 1999; Yuan et al., 1999). Overexpression of N-terminally truncated isoforms of p73 has been described in many types of human cancer, and appears to be related to the formation of metastases, poor response to chemotherapeutic drugs, and unfavourable patient prognosis (Dominguez et al., 2006; Wager et al., 2006). Increased expression of TA isoforms of p73 has also been described in some tumours, which contradicts its role as a tumour suppressor. It is thought that, under specific circumstances, TAp73 isoforms may act as a tumour promoters, rather than a tumour suppressors, although the molecular mechanisms mediating these functions are still not elucidated (Vikhanskaya et al., 2007).

Hence, the contribution of p53 family proteins to cancer development appears to result from an interplay between different isoforms of its members. Additionally, whether p63 and p73 promote or inhibit tumourigenesis appears to depend on the predominant isoforms expressed in a given context. The understanding of how each protein functions at a molecular level is therefore of extreme importance for their potential targeting in anti-cancer therapy [reviewed in (Deyoung and Ellisen, 2007)].

Based on that exposed above, activation of p53 in tumour cells has been considered a promising anti-cancer therapeutic strategy

One strategy to achieve p53 reactivation in tumours is gene therapy. In fact, adenovirus-based p53 therapies, aiming the restoration of the *TP53* gene, have reached phase III clinical trials, demonstrating anti-tumour activity, without significant adverse effects. These results were extremely encouraging for the development of p53-target based therapies (Huang et al., 2009).

In addtion, the direct pharmacological activation of p53 is still of particular interest in the treatment of tumours expressing mutant variants of this protein. However, based on the conventional drug development process, p53 is not an ideal therapeutic target, since it is neither an enzyme nor a receptor. Furthermore, the forms of inactivated p53 in human tumours are extremely diverse, with more than 2000 types of mutations currently described. Despite the difficulties associated with the development of drugs targeting p53, recent technological advances, in association with the knowledge obtained over the past 30 years about p53 family proteins, make this a recent promising strategy for anti-cancer therapy [reviewed in (Chen et al., 2010)]. In fact, some promising small molecules able to reactivate mutant p53 have been developed, such as PRIMA-1

(Bykov et al., 2002) and CP-31398 (Rippin et al., 2002), the latter having been also described as an activator of wt p53 (Wang et al., 2003).

The pharmacological reactivation of p53, in tumours harbouring a wt p53, may also be achieved through inhibition of its interaction with either MDM2 or MDMX. In fact, recent advances in the development of small molecule inhibitors of protein-protein interactions have opened the way for a new class of drug targets. Moreover, the successful development of p53-MDM2 interaction inhibitors confirms the feasibility of this therapeutic approach (Vassilev, 2004; Vassilev et al., 2004). So far, inhibitors of the p53-MDM2 interaction have adequate safety profiles and are able to selectively induce cancer cell death (Shangary and Wang, 2009). Nutlin-3a is a small molecule that fits the p53-binding pocket in MDM2, thus inhibiting its interaction with p53, which leads to p53 stabilization and activation. This compound triggers cell cycle arrest and apoptosis, and was able to reduce tumour growth in a nude mice xenograft model (Vassilev et al., 2004). Currently, there are two ongoing phase I clinical trials, where RG7112 (a derivative of nutlin-3a, also named RO5045337) is being tested in patients with myelogenous leukemia (ClinicalTrials.gov identifiers: NCT01635296 and NCT01677780; last access 12th August 2013). RITA is another example of a p53-MDM2 interaction inhibitor, but unlike nutlin-3a, it binds to p53 instead of MDM2. RITA inhibits the p53 interaction with MDM2 both *in vitro* and *in vivo*, and induces apoptosis in various human cells expressing wt p53 (Issaeva et al., 2004). Regarding the p53 interaction with MDMX, SJ-172550, which binds reversibly to MDMX, was the first described inhibitor. This compound was shown to effectively reduce the growth of retinoblastoma cells that overexpress MDMX (Reed et al., 2010).

Activation of pro-apoptotic isoforms of p63 and p73 may also be a promising strategy for anti-cancer therapy. However, further studies are still required in order to fully understand how these proteins control cellular proliferation and death. A small molecule named RETRA has been shown to increase the p73 expression in cells with mutant p53, leading to a p53-like tumour suppression; nevertheless, its molecular mechanism is still unclear (Kravchenko et al., 2008).

1.4. Yeast model in the study of p53 family proteins

The p53 family proteins participate in extremely complex signal transduction pathways, which are still not fully elucidated in mammalian cells, in part due to the coexistence of many isoforms of each protein. One strategy to overcome this issue is the use of simpler model organisms, such as the yeast *Saccharomyces cerevisiae,* in which the role of each protein isoform can be analysed individually without interference from other proteins.

There are several advantages in the use of this eukaryotic organism as a model to study disease-related proteins, including its ease of manipulation, short generation time and compliance to genetic manipulation. In fact, *S. cerevisiae* has greatly contributed to the elucidation of basic cellular processes related to cell cycle, cell death, and organelle biosynthesis, among many others. Moreover, the heterologous expression of human proteins in yeast (commonly referred to as “humanized yeast”) has provided important clues about the pathways in which they are involved, due to the conservation of human cell signalling networks and protein interactions in this organism. Yeast has in fact highly contributed to the elucidation of the molecular mechanisms underlying the pathobiology of several human diseases [reviewed in (Pereira et al., 2012a; Pereira et al., 2012b)].

No orthologues of p53 family proteins or of their endogenous modulators, MDM2 and MDMX, have been identified in *S. cerevisiae* (Smardova et al., 2005; Yousef et al., 2008). Therefore, through heterologous expression, it has been possible to study the activity of individual human proteins of the p53 family, in a simplified cellular background, without the interference of other members of this family of proteins, which often display similar or even overlapping functions.

Several yeast-cell based assays have been developed in recent years, for functional, molecular and pharmacological studies of p53 family proteins. One of the first techniques to be developed was the yeast FASAY assay, which consists of a functional analysis of the separated alleles of *TP53* in yeast in order to evaluate the p53 activity in cancer cells, as described (Ishioka et al., 1993). Using an improved version of the FASAY assay, developed by Flaman et al. (1995), it was verified that some *TP53* mutations led to the partial loss of the p53 transactivation function (Kovvali et al., 2001). The more recently developed dual-luciferase functional assay is another type of yeast-based transactivation assay (Fig. 7) (Andreotti et al., 2011). This transactivation assay uses a luciferase reporter gene containing a p53RE placed upstream of a minimal promoter (pCYC1), which is integrated as a single copy at a chromosomal *locus* of yeast cells expressing p53. In this way, when wt p53 is activated, it will bind to the p53RE, inducing the transcription of the luciferase reporter gene and the synthesis of luciferase. Therefore, the p53 transcriptional activity may then be assessed through quantification of the light emitted, which is directly proportional to the luciferase oxidative activity. Recently, this dual-luciferase transactivation assay was adapted to the high throughput screening (HTS) of small molecules and co-factors [e.g. MDM2 and 53BP1 (p53-binding protein 1)] modulators of p53 (Andreotti et al., 2011). In spite of all the advantages of this yeast-based assay, it is an artificial (since it relies on the insertion of a human reporter gene containing a p53RE in yeast cells) and expensive cell system for HTS.

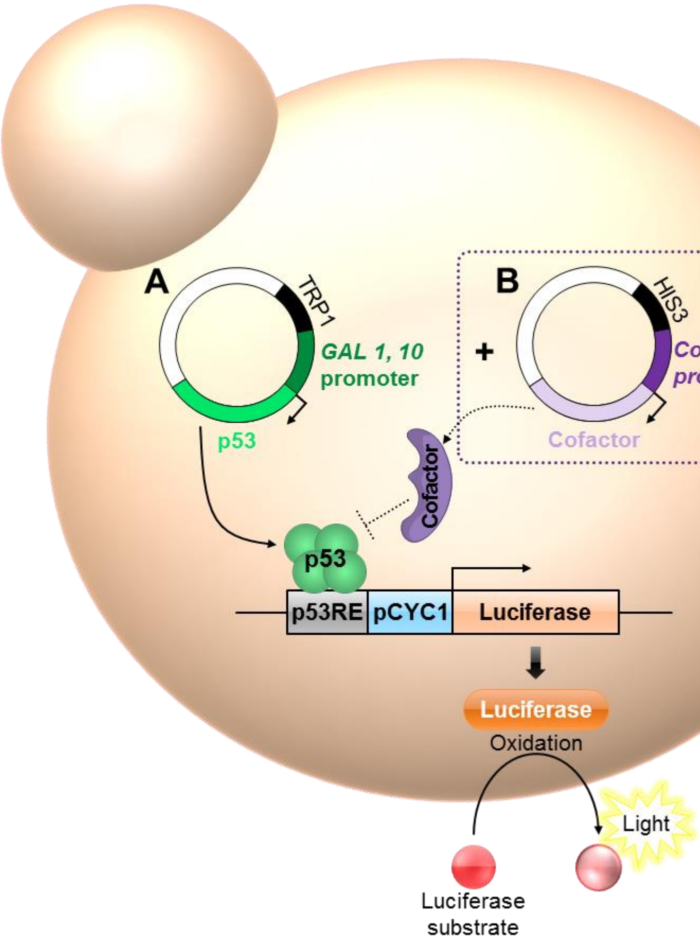


Fig. 7 – Yeast-based p53 luciferase transactivation assay using cells (A) expressing human wt p53 or (B) co-expressing human wt p53 and a cofactor. This assay utilizes the luciferase reporter gene. While a common minimal promoter (pCYC1) controls low-level constitutive expression of the reporter gene, p53-dependent expression of the luciferase is achieved through a specific p53RE placed upstream of the minimal promoter. The p53 transcriptional activity is evaluated by quantification of the luciferase activity, which is directly proportional to the light units measured in a plate reader [adapted from (Pereira et al., 2012b)].

Studies with such model systems have demonstrated that human wt p53 also acts as a sequence-dependent transcription factor in yeast (Coutinho et al., 2011; Fields and Jang, 1990; Scharer and Iggo, 1992). Also, they allowed to verify that the heterologous expression of this protein in yeast causes a mild growth inhibition, associated with increased apoptosis (Hadj Amor et al., 2008) or, depending on the *S. cerevisiae* strain used, with S-phase cell cycle arrest (Coutinho et al., 2009; Nigro et al., 1992).

Yeast has been also used to study the regulation of p53 by protein kinase C (PKC) isoforms. Particularly, in a recent work from our group, the effect of different PKC isoforms on wt p53 activity was studied (Coutinho et al., 2009). In this work, it was shown that, in the absence of a stress stimulus, PKCα reduced the p53 toxic effects in yeast (growth inhibition and S-phase cell cycle arrest), while the novel PKCδ and ε isoforms enhanced the p53 activity through p53 phosphorylation at Ser376-378 residues. For PKCζ, no effects on p53 activity were observed. These conclusions, obtained for PKCδ and ε, were further confirmed by a subsequent study performed by our group in yeast cells exposed to the apoptotic stimulus, H_2O_2 (Coutinho et al., 2011). In this study, it was shown that while PKCα and ζ did not affect the p53 activity, PKCδ and ε stimulate the p53 transcription-dependent and -independent apoptotic pathway through phosphorylation at Ser376-378. With this study, the conservation in yeast of a p53 transcription-independent mechanism, characterized by mitochondrial translocation of p53, was revealed for the first time. Additionally, further insights about the mitochondrial p53 translocation were provided. Particularly, PKCδ and ε were identified for the first time as possible inducers of the p53 translocation to the mitochondria (Fig. 8).

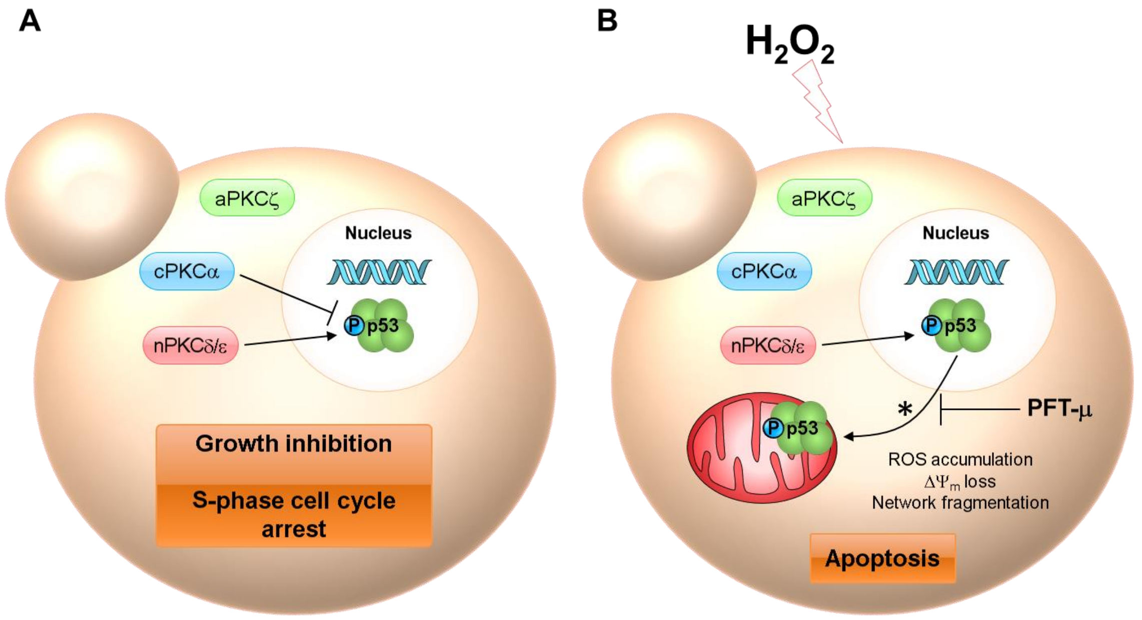


Fig. 8 – Human wt p53 is distinctly regulated by PKC isoforms. The expression of wt p53 in *S. cerevisiae* caused cell growth inhibition associated with S-phase cell cycle arrest. (A) In the absence of an apoptotic stimulus: cPKCα negatively regulated the p53 growth-inhibitory effect, leading to an increase in the percentage of cells arrested in G2/M phase; nPKCδ and ε stimulated the p53-induced growth inhibition, which was accompanied by a marked increase on p53 phosphorylation at Ser376-378 residues and in the percentage of cells arrested in S-phase. aPKCζ did not interfere with p53 effect. (B) In the presence of an apoptotic stimulus: a differential regulation of p53-mediated apoptosis was obtained by PKCα, δ, ε and ζ. Contrary to PKCα and ζ, which did not interfere with p53 activity, PKCδ/ ε markedly increase the p53 activity, leading to an increase of a set of mitochondrial alterations, through p53 phosphorylation at Ser376-378. In addition, PKC δ and ε stimulated mitochondrial p53 translocation (*), an effect reverted by pifithrin-μ (PFT-μ; a selective inhibitor of mitochondrial p53 translocation).

Furthermore, it was shown MDM2 is also able to interact with endogenous yeast pathways in order to promote p53 ubiquitylation and subsequent degradation (Di Ventura et al., 2008). In fact, as in mammalian cells, when co-expressed with wt p53, MDM2 markedly reduced the p53 activity in yeast, an effect abolished by the inhibitor of the p53-MDM2 interaction, nutlin-3a (Leao et al., 2013). Using this yeast phenotypic assay, a new inhibitor of the p53-MDM2 interaction with a xanthone scaffold (LEM1) was discovered, and its molecular mechanism was further confirmed in human tumour cells (Leao et al., 2013).

Accumulating evidence have been, therefore, provided showing the suitability of the yeast model system for the study of molecular mechanisms of p53 family proteins, and for the pharmacological screening of modulators of p53 family proteins as potential anti-cancer drugs.

1.5. Aims of research

With the present project, it was intended:

a) To search for new inhibitors of the p53-MDM2 interaction, using a previously developed yeast-based p53-MDM2 screening assay;

b) To validate, in human tumour cell lines, the molecular mechanism of action of potential activators of p53 identified in a previous work using the yeast-based p53-MDM2 screening assay;

c) To develop a new yeast-based p53-MDMX screening assay for the search of inhibitors of the p53-MDMX interaction;

d) To identify an endogenous p53 target gene in order to develop a simplified and cost-effective yeast p53 transcription assay, as an alternative to the artificial yeast reporter strains, for the analysis of the impact of mutants, cofactors and small molecules on p53 transcriptional activity;

e) To study the role of p63, ΔNp63 and p73 in cell proliferation and death using the yeast cell system.

2 Materials and Methods

2. Materials and Methods

2.1. Compounds

Compounds used as controls included: nutlin-3a (Alexis Biomedicals) and SJ-172550 (Sigma-Aldrich), known inhibitors of the interaction of p53 with MDM2 and MDMX, respectively (Reed et al., 2010; Vassilev et al., 2004); pifthrin-α (PFT-α; Sigma-Aldrich), a selective inhibitor of p53 transcriptional activity (Gudkov and Komarova, 2005); and doxorubicin (Sigma-Aldrich), a conventional chemotherapeutic agent. Rapamycin (Sigma-Aldrich), an inhibitor of mTOR, was used for autophagy induction in yeast (Noda and Ohsumi, 1998).

α-mangostin (αMG) was extracted from the mangosteen fruit of *Garcinia mangostana* L. (Clusiaceae) (Fig. 9) by Professor José Pedraza-Chaverri (from Facultad de Química, Universidad Nacional Autónoma de México), as previously described (Marquez-Valadez et al., 2009). Gambogic acid (GA; Fig. 9) and 3,7-dihydroxyflavone (3,7DHF; Fig. 12) were purchased from Sigma-Aldrich. The chalcones 4-benzyloxy-2',4'-dihydroxychalcone (C1), 2',4'-dihydroxy-3,4,5-trimethoxy-3'-propylchalcone (C2), 2',4'-dihydroxy-4-methoxy-3'-propylchalcone (C3), 3,4,4',5,6'-pentamethoxy-2'-prenyloxychalcone (C4), 3,4,5-trimethoxy-4',6'-bis(methoxymethoxy)-3'-prenylchalcone (C5) (Fig. 12), were synthesized by Professor Honorina Cidade (CEQUIMED-UP), as previously described (Neves et al., 2012a; Neves et al., 2012b).

All the referred compounds were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich).

2.2. Plasmids

The yeast expression vectors used in the yeast assays were: pGADT7-(*LEU2*) encoding human MDM2 under the control of the *ADH1* constitutive promoter (kindly provided by Dr. Xue-Min Zhang from National Center of Biomedical Analysis, China); pGADT7-(*LEU2*) encoding human MDMX under the control of the *ADH1* constitutive promoter (kindly provided by Dr. Martin Scheffner from University of Konstanz, Germany); pLS89-(*TRP1*) encoding human wt p53 or human mutant p53 V122A under the control of the *GAL1-10* inducible promoter (kindly provided by Dr. Richard Iggo from the Swiss Institute for Experimental Cancer Research, Switzerland); pLS76-(*LEU2*) encoding human mutant p53 R280K or R273H, under the control of the constitutive *ADH1* promoter; pRS314-(*TRP1*) encoding human TAp63α or ΔNp63α under the control of the *GAL1-10* inducible promoter (kindly provided by Dr. Gilberto Fronza from Istituto Nazionale per la Ricerca sul Cancro, Italy); pRS314-(*TRP1*) encoding human TAp73α under the control of the *GAL1-10* inducible promoter (kindly provided by Dr. Alberto Inga from Centre for Integrative Biology, Italy); the yeast integrative ABP140-3xGFP-(*LEU2*) plasmid (kindly provided by Dr. Isabelle Sagot from Institut de Biochimie et Génétique Cellulaires, France) was used in actin depolarization experiments.

2.3. Yeast strains, transformation, and growth conditions

The yeast *Saccharomyces cerevisiae* strains used were: CG379 (α ade5 his7-2 leu2-112 trp1-289α ura3-52 [Kil-O], Yeast Genetic Stock Center, University of California, USA), W303 (α ura3-1 leu2-3, 112 his3-11, 15 trp1-1 ade2-1 can1-100; Yeast Genetic Stock Center, University of California, USA), W303 atg1Δ::KanMX4 (Yorimitsu et al., 2007; kindly provided by Dr. Daniel Klionsky from University of Michigan, Life Sciences Institute, USA), and W303 atg5Δ::KanMX4 (Kissova, et al. 2004; kindly provided by Dr. Stefan Manon from CNRS, UMR5095, Université de Bordeaux, France). Yeast cells were transformed using the standard lithium acetate method (Ito et al., 1983) and were routinely grown in minimal selective medium with 2% (w/w) glucose, 0.7 % (w/w) yeast nitrogen base without amino acids (Difco), and all the amino acids required for yeast growth (50 μg/mL) except leucine or tryptophan (for single expression systems), or except leucine and tryptophan (for co-expression systems). For expression of human proteins, cells were diluted to 0.05 OD_{600} in selective induction medium, in which glucose was replaced by 2% (w/w) galactose and 2% (w/w) raffinose. Yeast cells were then incubated at 30 °C, under continuous orbital shaking (200 rpm) for approximately 30 h (for single expression systems) or 42 h (for co-expression systems), corresponding to the time required by control yeast (transformed with the empty vectors) to achieve 0.5 OD_{600}. Yeast cell growth was analysed by counting the number of colony-forming units (CFU) after 2 days incubation at 30 °C on Sabouraud Dextrose Agar plates (Liofilchem).

2.4. Western blot analysis

To analyse protein expression in yeast, samples were lysed with Cellytic™ Y Cell Lysis Reagent (Sigma-Aldrich) in the presence of EDTA-free protease inhibitor cocktail (Sigma-Aldrich). Following whole protein quantification, using the Bio-Rad Protein Assay (Bio-Rad) according to manufacturer’s instructions, proteins (40 μg) were electrophoresed on 10% SDS-PAGE and transferred to an Amersham nitrocellulose membrane (GE Healthcare). Membranes were blocked with 5% milk and probed with the following antibodies: mouse monoclonal anti-p53 (DO-1; Santa Cruz Biotechnology) for the detection of wt and mutant forms of p53; mouse monoclonal anti-p63 (4A4; Santa Cruz Biotechnology) for TAp63α and ΔNp63α detection; rabbit polyclonal anti-p73 (AB7284; Milipore) for TAp73α detection; rabbit polyclonal anti-MDMX (A300; Bethyl Laboratories); rabbit polyclonal anti-Atg8p (y-117; Santa Cruz Biotechnology); rabbit polyclonal anti-actin (C11; Santa Cruz Biotechnology). Membranes were, then, probed with either anti-mouse or anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology). For loading control, membranes were stripped and reprobed with a mouse anti-yeast phosphoglycerate kinase (Pgk1p) antibody (Molecular Probes). The signal was detected by chemiluminescence with the ECL Amersham kit (GE Healthcare). Band intensities were quantified using the Bio-Profil Bio-1D++ software (Vilber-Lourmat).

2.5. Effect of compounds on yeast cell growth

To evaluate the effect of α-mangostin and gambogic acid, on yeast cell growth, yeast cells co-expressing human wt p53 and MDM2 [obtained in previous work (Leao et al., 2013)] or p53 R280K alone, were incubated in selective induction medium with 10 μM nutlin-3a, 1 – 100 μM α-mangostin (αMG) or gambogic acid (GA), or with 0.1% DMSO only, for approximately 42 h (for analysis of the effect on the p53-MDM2 interaction) or 30 h (for analysis of the effect on p53 R280K), at 30 °C, under continuous shaking. As a positive control, yeast cells were incubated, in the same conditions, with nutlin-3a. Yeast cell growth was analysed by CFU counts, as described above.

2.6. Yeast cell cycle analysis

Yeast cell cycle progression was examined by flow cytometric analysis of DNA content using Sytox Green Nucleic Acid stain (Invitrogen), as previously described (Fortuna et al., 2001). Briefly, 1×10^7 cells were fixed overnight in 70% (v/v) ethanol, at 4 °C. Cells were then washed with sodium citrate buffer and incubated for 3 h, first with 250 μg/mL RNase A (Sigma-Aldrich), then with 1 mg/mL Proteinase K (Sigma-Aldrich), and finally stained with 10 μM Sytox Green Nucleic Acid stain (Invitrogen) for approximately 12 h. For the flow cytometric analysis the FACSCalibur flow cytometer (BD Biosciences) and the CellQuest software (BD Biosciences) were used. Yeast cell cycle phases were identified and quantified using ModFit LT software (Verity Software House Inc.).

2.7. Cell death assays

To assess plasma membrane integrity, transformed *S. cerevisiae* CG379 cells were incubated with 5 µg/mL PI (Molecular Probes) for 10 minutes at room temperature, and the fluorescence analysed by flow cytometry using the FACSCalibur flow cytometer (BD Biosciences) and the CellQuest software (BD Biosciences). To monitor DNA fragmentation in yeast, the TUNEL assay was carried out using the *In Situ Cell Death Detection Kit*, Fluorescein (Roche Applied Science) as previously described (Saraiva et al., 2006). Samples were observed under a fluorescence microscope (Eclipse E400 fluorescence microscope, Nikon) under appropriate filter setting.

For the apoptotic cell death analysis, transformed *S. cerevisiae* CG379 cells, previously grown in minimal selective medium to 0.3 OD_{600}, were treated with 1.5 and 5 mM H_2O_2 for 1 h at 30 °C under continuous shaking. Cell death was assessed by counting the number of CFU/mL after 1 day incubation at 30 °C on Sabouraud Dextrose Agar plates. For each transformed yeast cells, the percentage of dead cells was estimated considering 100% survival (0% death) as the number of CFU obtained with cells incubated in the same conditions without H_2O_2.

2.8. Autophagy analysis

For the autophagy analysis, *S. cerevisiae* wt W303, W303 atg1Δ, and W303 atg5Δ cells expressing a p53 family member and control yeast were grown in induction selective medium for 30 h, and cell growth was analysed by counting the number of CFU. Additionally, transformed *S. cerevisiae* CG379 cells expressing a p53 family member and control yeast were grown in induction selective medium to 0.35 OD_{600} before treatment with 0.2 µg/mL rapamycin for 4 h at 30 ºC. The viability of rapamycin-treated yeast cells was assessed by CFU counts, considering the number of CFU obtained with yeast cells incubated with DMSO only as 100%.

2.9. Analysis of actin depolarization

Yeast actin organization was analysed using yeast expressing wt p53 and control yeast (transformed with the empty vector) co-transformed with the yeast integrative

ABP140-3xGFP-(*LEU2*) plasmid. Fluorescence microscopic examinations were performed with an inverted laser-scanning confocal microscope (Olympus FV 1000/IX81) equipped with a 60× magnification objective lens (Olympus PlanApo 60x/1.42 NA, oil). GFP was excited with the 488 nm Multi-line Argon laser. The emitted fluorescence was detected at 505-520 nm using the scanner of the confocal microscope. The area of labelling was analysed using the FluoView Advanced Software (ASW, v.1.4; Olympus).

2.10. Effect of compounds on the *in vitro* cell growth of human tumour cell lines

The effect of 3,7-dihydroxyflavone (3,7DHF) and chalcones C1 – C5 was evaluated on a human colon carcinoma cell line with (HCT116 $p53^{+/+}$) and without (HCT116 $p53^{-/-}$) p53 using the protein-binding dye sulforhodamine B to assess cell growth (Vichai and Kirtikara, 2006). Briefly, cells were plated in 96-well plates at a density of 5×10^3 cells/well, in RPMI-1640 medium with ultragltamine I (Lonza) supplemented with 5% fetal bovine serum (Gibco), and allowed to adhere overnight in a humidified incubator at 37 °C with 5% CO_2 in air. Cells were further incubated for 48 hours with five serial dilutions of each compound (150, 50, 16.67, 5.56 and 1.85 μM). Doxorubicin and nutlin-3a were used as a positive controls. The effect of the vehicle solvent on the growth of these cell lines was also evaluated, by exposing untreated control cells to the maximum concentration of DMSO used in each assay (0.075%). Following incubation, cells were fixed *in situ* with ice cold 10% trichloroacetic acid, washed, and stained with sulforhodamine B. Unbound stain was washed with 1% acetic acid and the bound stain was solubilized in 10mM Tris Base. Finally, optical density was measured at 510 nm using a microplate reader (Synergy HT; Biotek). The GI_{50} values (concentration resulting in 50% inhibition of cell growth) were determined for each compound from the plotted results.

2.11. Statistical analysis

Data were analysed statistically using the SigmaStat 3.5 software. Differences between means were tested for significance using the unpaired Student’s *t*-test ($P < 0.05$).

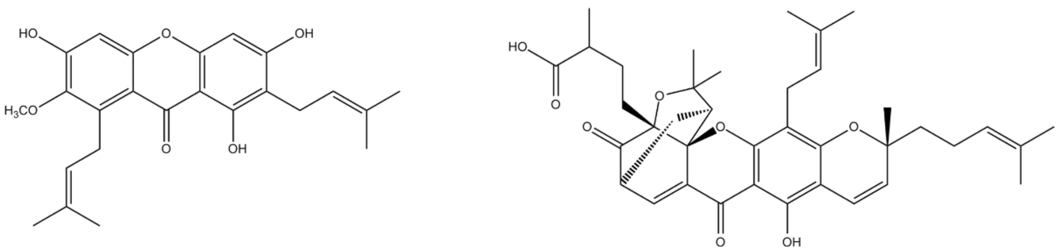
3 Results

3. Results

3.1. Searching for small molecule activators of p53 activity

3.1.1. Inhibitors of the p53-MDM2 interaction

In a previous study, our group identified an inhibitor of the p53-MDM2 interaction with a xanthone scaffold (Leao et al., 2013). Based on this, it was hypothesized that α-mangostin (αMG) and gambogic acid (GA) (Fig. 9), two xanthones with well-known potent anti-tumour properties, could also modulate the p53-MDM2 interaction.



αMG GA

Fig. 9 – Chemical structures of α-mangostin (αMG) and gambogic acid (GA).

To test this hypothesis, a yeast model previously developed by our group (Leao et al., 2013) was used. In this yeast phenotypic assay, the expression of human wt p53 alone causes growth inhibition associated with S-phase cell cycle arrest (Fig. 10A, C), as previously described (Coutinho et al., 2009). Furthermore, although the expression of human MDM2 alone did not interfere with cell growth and cell cycle progression, its co-expression with wt p53 significantly reduced the p53-dependent growth inhibition and cell cycle arrest (Fig. 10A, C). In this assay, nutlin-3a, a known inhibitor of the p53-MDM2 interaction (Vassilev et al., 2004), was used as a positive control. As previously described (Leao et al., 2013) nutlin-3a was able to abolish the inhibitory effect of MDM2 on p53-induced growth inhibition and cell cycle arrest (Fig. 10A, C), thus supporting the effectiveness of this yeast model to search for inhibitors of the p53-MDM2 interaction.

Therefore, using this assay, the effects of 1 – 100 μM of αMG and GA on the p53-MDM2 interaction were evaluated. From the concentration-response curves obtained (Fig. 10B), the concentration of 10 μM was selected for both xanthones as the lowest concentration at which a significant reversion of the MDM2 effect was obtained, while not affecting the growth of control yeast (transformed with the empty vectors). At a concentration of 10 µM, similarly to nutlin-3a, both xanthones were able to re-establish the p53-induced growth inhibition and S-phase cell cycle arrest in yeast cells co-expressing p53 and MDM2, while having no effect on control yeast, or on yeast expressing p53 or MDM2 alone (Fig. 10A, C).

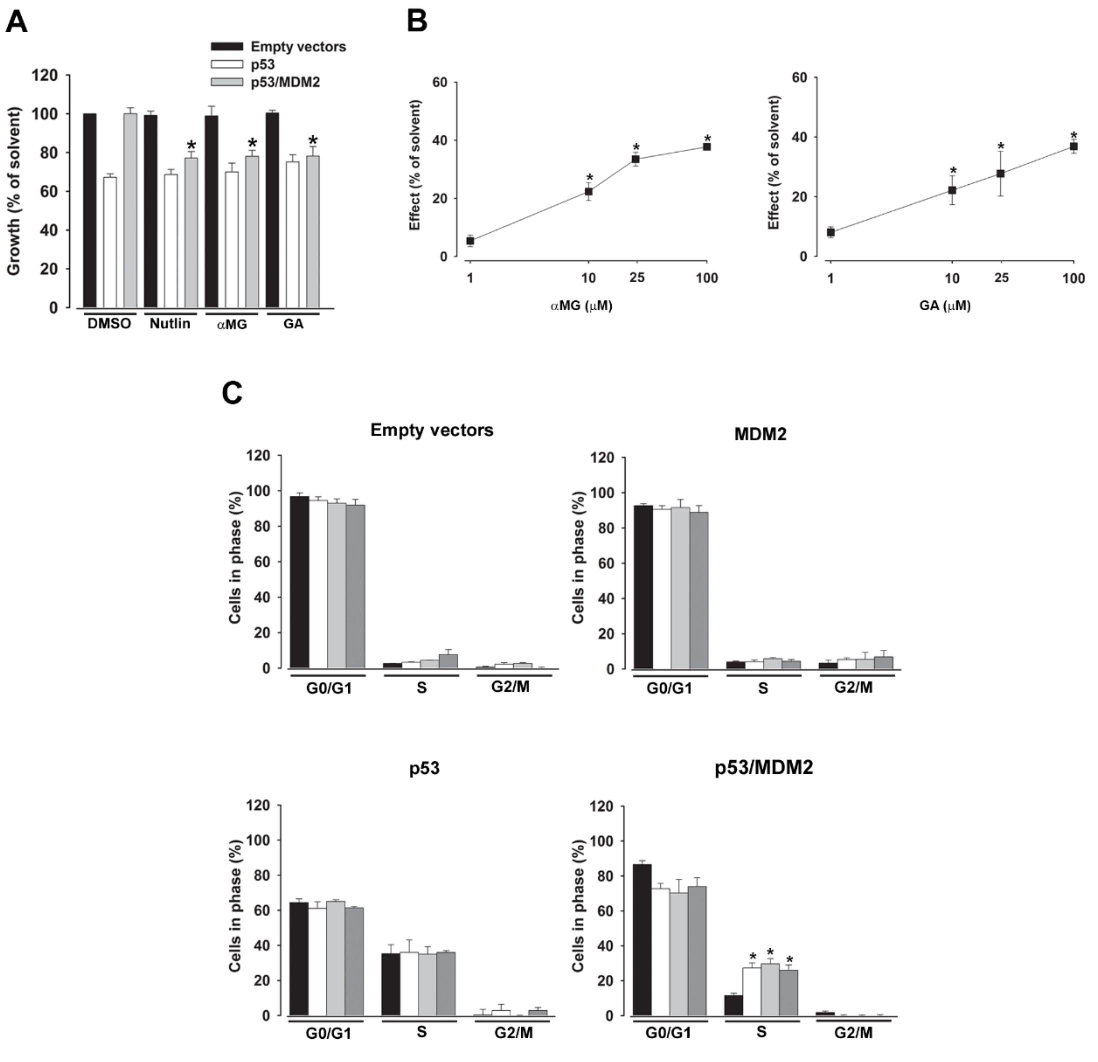


Fig. 10 – α-Mangostin (αMG) and gambogic acid (GA) revert the inhibitory effect of MDM2 on p53-induced growth inhibition and S-phase cell cycle arrest in yeast. (A) Effects of 10 µM nutlin-3a and 10 µM αMG and GA on the growth of control yeast, yeast expressing only p53 and yeast co-expressing p53 and MDM2. Results were estimated considering the number of CFU obtained with control yeast (empty vectors) as 100% growth. The growth of yeast expressing MDM2 alone treated with compounds was approximately 100%, therefore it is represented by the control yeast. Data are represented as mean ± SEM of five independent experiments; values obtained with yeast co-expressing p53 and MDM2 treated with the compound were significantly different from DMSO only (**P* < 0.05). **(B)** Effects of 1-100 µM αMG and GA on the reduction of p53-induced growth inhibition by MDM2 in yeast co-expressing p53 and MDM2. Data are represented as mean ± SEM of four independent experiments; values significantly different from DMSO only (**P* < 0.05). **(C)** Effects of 10 µM nutlin-3a and 10 µM αMG and GA on the reversion of p53-induced S-phase cell cycle arrest by MDM2; data are represented as mean ± SEM of two independent experiments; values significantly different from DMSO only (**P* < 0.05).

In previous work, it was shown that αMG was cytotoxic against a human adenocarcinoma cell line (MDA-MB-231) which harbours the mutant variant of p53, R280K (Shibata et al., 2011). Based on this, the activity of both αMG and GA on mutant p53 R280K was also evaluated using this yeast-based growth assay. In this assay, contrary to wt p53, the expression of p53 R280K mutant (confirmed by Western blot analysis; Fig. 11A) had no effect on yeast cell growth, when compared to control yeast (Fig. 11B). As such, activators of this mutant variant of p53 would re-establish the wt p53-dependent growth inhibition. The results obtained showed that neither αMG nor GA were able to affect the growth of yeast cells expressing p53 R280K (Fig. 11B).

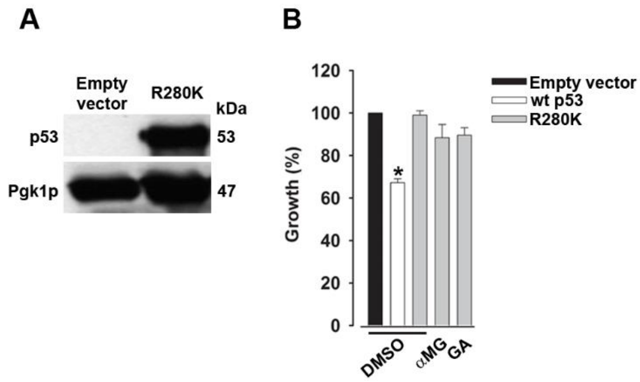


Fig. 11 – α-Mangostin (αMG) and gambogic acid (GA) do not interfere with the activity of mutant p53. (A) Expression of human mutant p53 (R280K) in yeast was confirmed by Western blot analysis. Pgk1p was used as loading control. Immunoblots are representative of two independent experiments. **(B)** Effects of 10 µM αMG and GA on the growth of yeast expressing mutant p53. Results are plotted setting as 100% growth the number of CFU obtained with control yeast (empty vector). Data are represented as mean ± SEM of four independent experiments; values significantly different from control yeast (**P* < 0.05).

3.1.2. Activators of p53

In a previous work performed by our group, in which a library of flavonoids (from CEQUIMED-UP) was screened, using the yeast-based p53-MDM2 assay (Leao et al., 2013), six compounds emerged as potential activators of wt p53: 3,7DHF and chalcones C1 – C5 (Fig. 12).

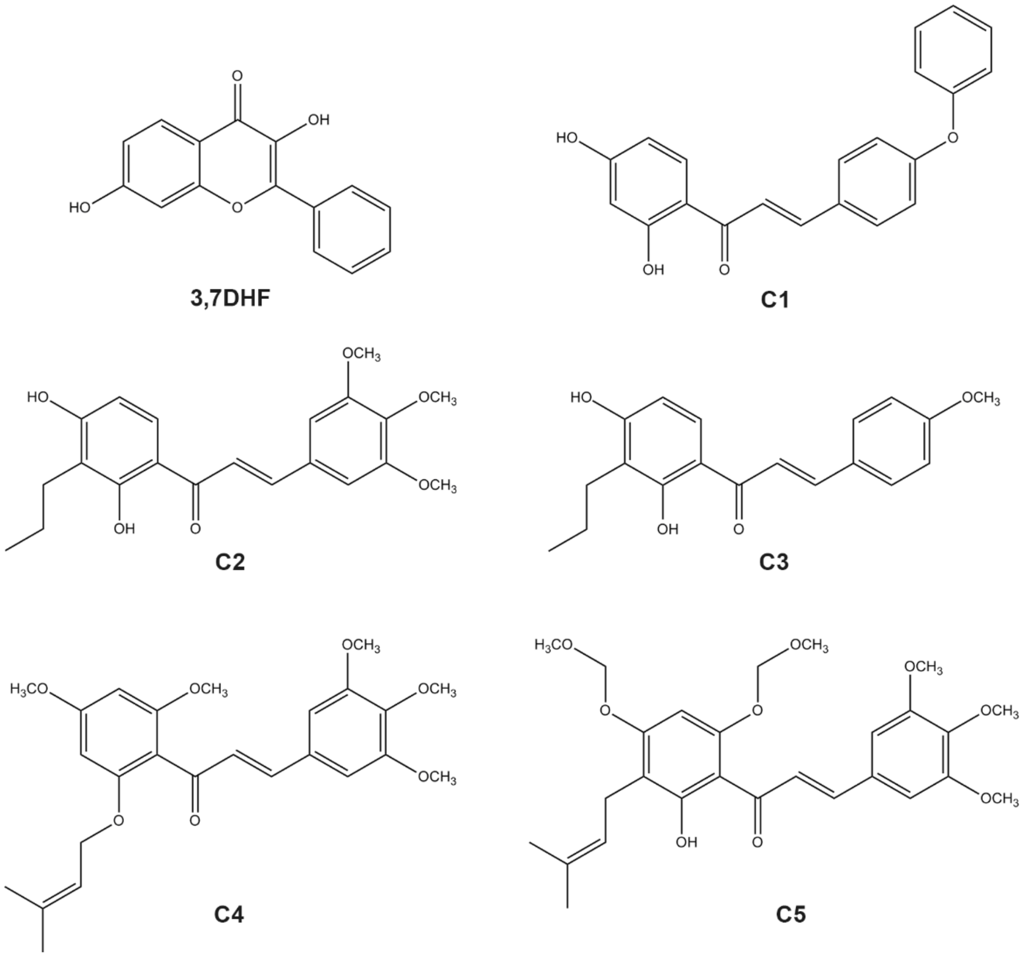


Fig. 12 – Chemical structures of the six flavonoids tested. 3,7-dihydroxyflavone (3,7DHF), 4-benzyloxy-2',4'-dihydroxychalcone (C1), 2',4'-dihydroxy-3,4,5-trimethoxy-3'-propylchalcone (C2), 2',4'-dihydroxy-4-methoxy-3'-propylchalcone (C3), 3,4,4',5,6'-pentamethoxy-2'-prenyloxychalcone (C4), 3,4,5-trimethoxy-4',6'-bis(methoxymethoxy)-3'-prenylchalcone (C5).

In order to confirm if the mechanism of action of these compounds involved p53 activation, their effects on the growth of human colon carcinoma cell lines with (HCT116 $p53^{+/+}$) and without (HCT116 $p53^{-/-}$) wt p53 were assessed by the sulforhodamine B assay. A concentration-dependent response curve was obtained for each compound, in both cell lines, and the concentration that caused 50% of cell growth inhibition (GI_{50}) was determined as previously described (Monks et al., 1991) (Table 1). In general, all the tested flavonoids exhibited potent anti-proliferative effects against the two human colon carcinoma cell lines studied. In spite of this, contrary to nutlin-3a and doxorubicin [positive controls, both of which have been described to act via p53 (Fritsche et al., 1993; Vassilev et al., 2004)], the GI_{50} values determined for the six flavonoids were similar for both cell lines, suggesting they were not dependent on the p53 status. This indicated a non p53-dependent cell growth inhibitory effect for all the tested flavonoids.

Table 1 – GI_{50} values obtained for the tested flavonoids in colon carcinoma cell lines with or without wt p53.

	GI_{50} (μM)	
Compounds	**HCT116 $p53^{+/+}$**	**HCT116 $p53^{-/-}$**
3,7DHF	36.3 ± 1.4	39.7 ± 1.9
C1	9.57 ± 0.78	8.63 ± 0.56
C2	4.63 ± 0.59	3.13 ± 0.26
C3	24.3 ± 4.8	15.7 ± 2.4
C4	4.00 ± 0.36	3.73 ± 0.27
C5	2.50 ± 0.19	2.40 ± 0.33
Doxorubicin	0.136 ± 0.029	0.450 ± 0.042*
Nutlin-3a	3.67 ± 0.34	24.0 ± 0.7*

Nutlin-3a and doxorubicin were used as positive controls. Data are represented as mean ± SEM of three independent experiments performed in duplicate. Values significantly different from HCT116 $p53^{+/+}$ (*$P < 0.05$)

3.2. Development of a yeast-based assay to search for inhibitors of the p53-MDMX interaction

In order to study the regulatory effect of MDMX on p53 activity in yeast, the human MDMX was expressed in these cells either alone or combined with human wt p53 (Fig. 13A). Similarly to MDM2 (Leao et al., 2013), although the single expression of MDMX in yeast did not interfere with the cell growth and cell cycle progression, its co-expression with p53 significantly reduced the p53-induced growth inhibition (Fig. 13B, DMSO) and S-phase cell cycle arrest (from 18.0 ± 1.2%, obtained with cells expressing only p53, to 5.9 ± 1.9%, n = 2, P < 0.05; Fig. 13C, DMSO).

The efficiency of this assay for the screening of inhibitors of the p53-MDMX interaction, was assessed by testing the known inhibitor of the p53-MDMX interaction, SJ-172550 (Reed et al., 2010). In this assay, inhibitors of the p53-MDMX interaction would abolish or significantly reduce the negative effect of MDMX on p53 activity. As expected, 20 µM SJ-172550 significantly reduced the negative effect of MDMX on p53-induced growth inhibition (Fig. 13B) and S-phase cell cycle arrest (from 5.9 ± 1.9%, obtained with DMSO only, to 15.8 ± 1.5%, n = 2, P < 0.05; Fig. 13C), without interfering with the activity of p53 and MDMX when expressed alone (Fig. 13B, C). In fact, after 42 h treatment with 20 µM SJ-172550, approximately 83% of the p53-induced growth inhibition and S-phase cell cycle arrest were re-established. Higher concentrations of SJ-172550 did not improve the reversion of the MDMX effect on p53-induced growth inhibition (Fig. 13D). Furthermore, from 50 µM SJ-172550, a cytotoxic effect on control yeast (empty vectors) was observed (Fig. 13E). On the other hand, SJ-172550 concentrations lower than 20 µM did not significantly reduce the negative effect of MDMX on p53 induced growth inhibition (Fig. 13D). Additionally, for 0.1 – 20 µM, SJ-172550 had no effect on the growth of yeast expressing p53 alone (Fig. 13E).

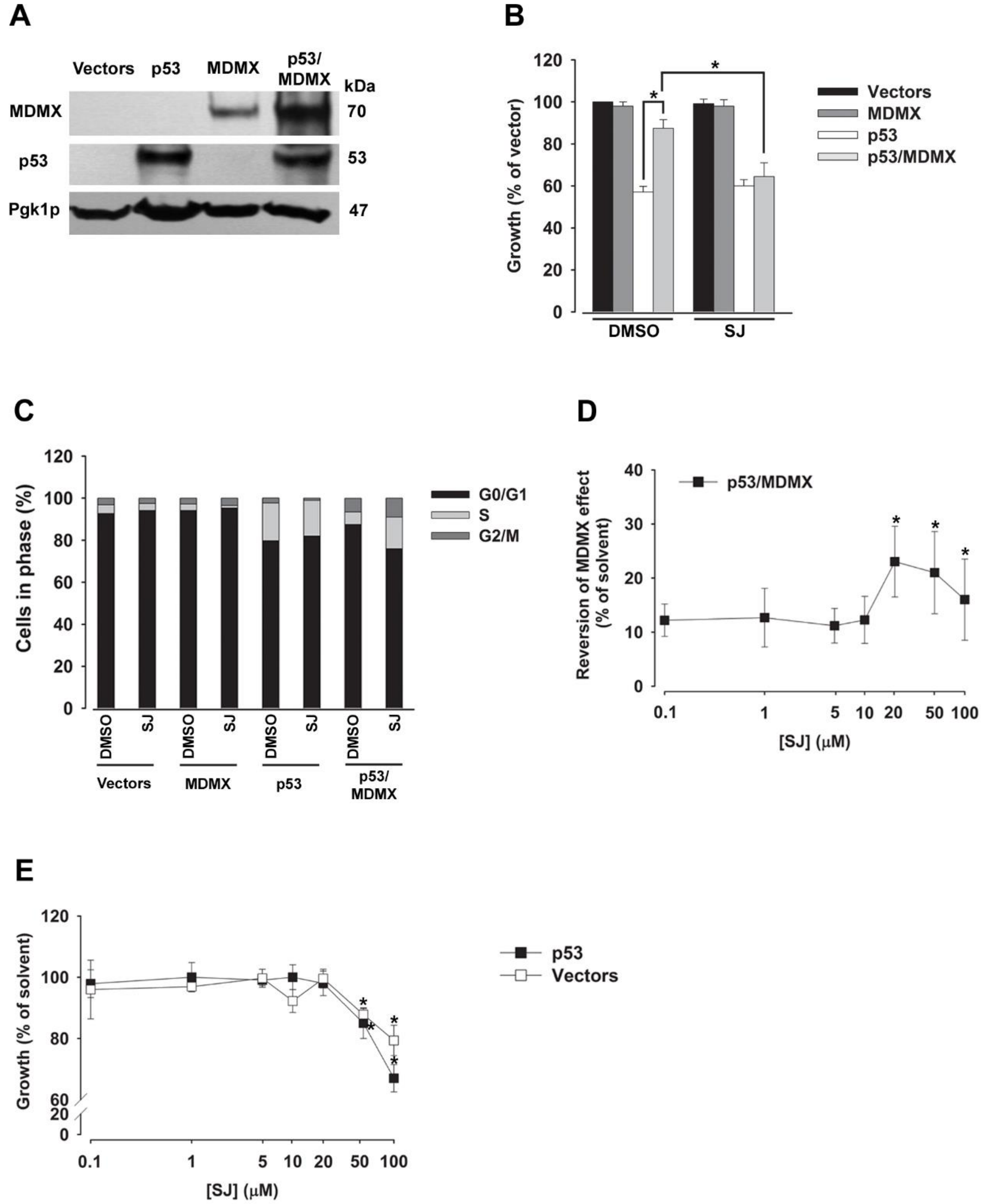


Fig. 13 – MDMX inhibits the impact of p53 on the growth of yeast, an effect abolished by SJ-172550. (A) Expression of human wt p53 and/or human MDMX was confirmed by Western blot analysis; Pgk1p was used as loading control; immunoblots are representative of two independent experiments. **(B)** Effect of 20 µM SJ-172550 on the growth of control yeast, yeast expressing only p53 or MDMX and yeast co-expressing p53 and MDMX. Results were plotted setting the number of CFU obtained with control yeast incubated with DMSO only as 100% growth. Data are represented as mean ± SEM of five independent experiments; significantly different values are indicated (*P < 0.05). **(C)** Effect of 20 µM SJ-172550 on the yeast cell cycle progression of control yeast, yeast expressing only p53 or MDMX and yeast co-expressing p53 and MDMX. Datarepresents the mean of two independent experiments. **(D, E)** Concentration-response curves for the reversion of MDMX effect by SJ-172550 in yeast co-expressing p53 **(D)** and the effects of SJ-172550 on the growth of control yeast (empty vectors) and yeast cells expressing p53 alone **(E)**; Yeast cell growth was analysed by CFU counts after incubation in the presence of 0.1 – 100 µM SJ-172550 or DMSO only. Data are represented as mean ± SEM of four independent experiments; values significantly different from DMSO only (*P < 0.05).

3.3. wt p53 interferes with actin protein expression levels in yeast

In the present work, it was verified that the growth inhibition caused by expression of human wt p53 in yeast (Fig. 14A, B; see also Fig. 17) was associated with a marked increase of actin protein levels, when compared to control yeast (empty vector) (Fig. 14C). On the other hand, the expression in yeast of the p53 R273H mutant (Fig. 14A), which is known for its reduced transcriptional activity in comparison to wt p53 both in mammalian cells (Maslon and Hupp, 2010) and in a yeast reporter construction (Scharer and Iggo, 1992), caused no increase of actin protein levels (Fig. 14C). Accordingly, R273H expression did not interfere with yeast cell growth (Fig. 14B; see also Fig. 17) and cell cycle progression (Fig. 18A). Interestingly, when the toxic p53 V122A mutant, which has been reported to have higher cytotoxic and transcriptional activity than wt p53 (Inga and Resnick, 2001), was expressed in yeast (Fig. 14A), its higher inhibitory effect on yeast cell growth (Fig. 14B) was accompanied by a higher increase of actin protein levels (Fig. 14D), when compared to wt p53.

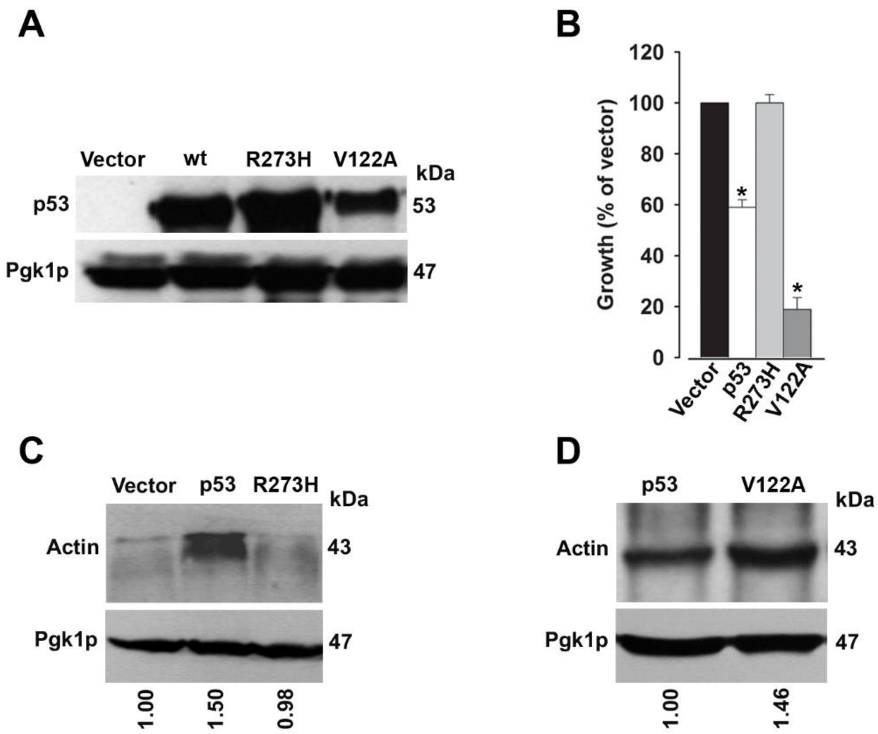


Fig. 14 – Contrary to R273H, wt p53 and V122A increase actin protein levels. (A) Expression of human wt p53, R273H or V122A in yeast was confirmed by Western blot analysis. **(B)** Effect of wt p53, R273H and V122A on yeast cell growth was analysed by CFU counts; results were plotted setting the number of CFU obtained with control yeast (empty vector) as 100% growth; data are represented as mean ± SEM of five independent experiments; values significantly different from control yeast (*P < 0.05). **(C, D)** The effect of wt p53, R273H and V122A on actin protein levels was analysed by Western blot. Immunoblots represent one of two experiments; Pgk1p was used as loading control.

The effect of pifthrin-α (PFT-α), a known inhibitor of the p53 transcriptional activity (Gudkov and Komarova, 2005), on the actin expression levels of yeast cells expressing wt p53, was also assessed. The results showed that when yeast cells expressing wt p53 were treated with 20 µM PFT-α, a prominent reduction of p53-induced growth inhibition of 41.7 ± 4.3% (*n* = 4) was observed, as previously reported (Coutinho et al., 2011), which was accompanied by a significant reduction of the actin protein levels when compared to cells treated with DMSO only (Fig. 15A).

Since it has been previously shown that MDM2 (Leao et al., 2013) and MDMX also inhibited the toxic effect of p53 in yeast, the impact of these two negative regulators on p53-induced actin protein levels was also analysed. When co-expressed with wt p53, both MDM2 (Fig. 15B) and MDMX (Fig. 15C) led to a visible reduction of actin protein levels. Interestingly, the treatment of yeast cells co-expressing p53 and MDM2 with 10 µM nutlin-3a (the known inhibitor of p53-MDM2 interaction), reverted this inhibitory effect of MDM2, re-establishing the p53-induced actin protein levels (Fig. 15B). Similarly to the effect of nutlin-3a on MDM2 co-expressing cells, 20 µM SJ-172550 also re-established the p53-induced actin protein levels in yeast cells co-expressing p53 and MDMX (Fig. 15C). Together, these results may suggest that *ACT1* (the gene that encodes actin) is an endogenous transcriptional target of p53, in yeast.

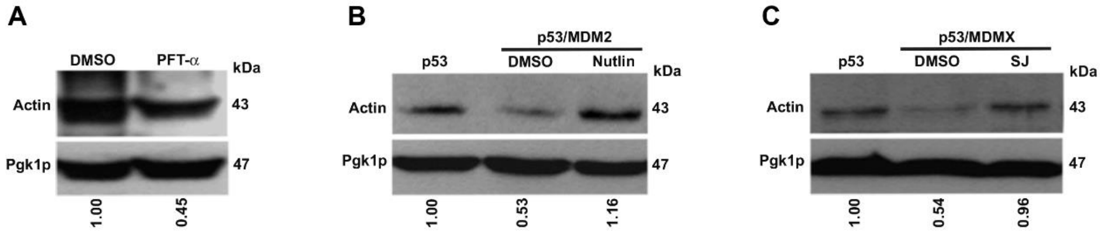


Fig. 15 – Reduction of actin protein levels by chemical (PFT-α) and natural (MDM2 and MDMX) inhibitors of p53 activity. (A) Effect of PFT-α on actin protein levels was analysed by Western blot; yeast cells expressing wt p53 were grown in selective induction medium for approximately 30 h in the presence of 20 µM PFT-α or DMSO only. **(B)** Effect of MDM2 on actin protein levels was analysed by Western blot; yeast cells co-expressing human wt p53 and/or MDM2 and control yeast (empty vectors) were grown in selective induction medium for approximately 42 h in the presence of 10 µM nutlin-3a or DMSO only. **(C)** Effect of MDMX on actin protein levels was analysed by Western blot; yeast cells co-expressing human wt p53 and/or MDMX and control yeast (empty vectors) were grown in selective induction medium for approximately 42 h in the presence 20 µM SJ-172550 or DMSO only. Immunoblots are representative of two experiments; Pgk1p was used as loading control.

The effect of p53 on the yeast actin cytoskeleton organization was also assessed in this work. For that, yeast cells expressing wt p53 alone were co-transformed with a yeast integrative vector encoding the green fluorescent protein (GFP) fused to the actin binding protein 140 (ABP140), as previously described (Sagot et al., 2006). By analysing the yeast actin organization by fluorescence microscopy, it was possible to observe that wt p53 expression caused a marked actin cytoskeleton depolarization, after 30 h incubation in induction selective medium. In fact, contrary to the diffuse GFP cellular background corresponding to the staining of actin cables observed in control yeast (vector), highly depolarized actin patches confined to regions near the plasma membrane could be visualized in a high number of yeast cells expressing wt p53 (Fig. 16B, C).

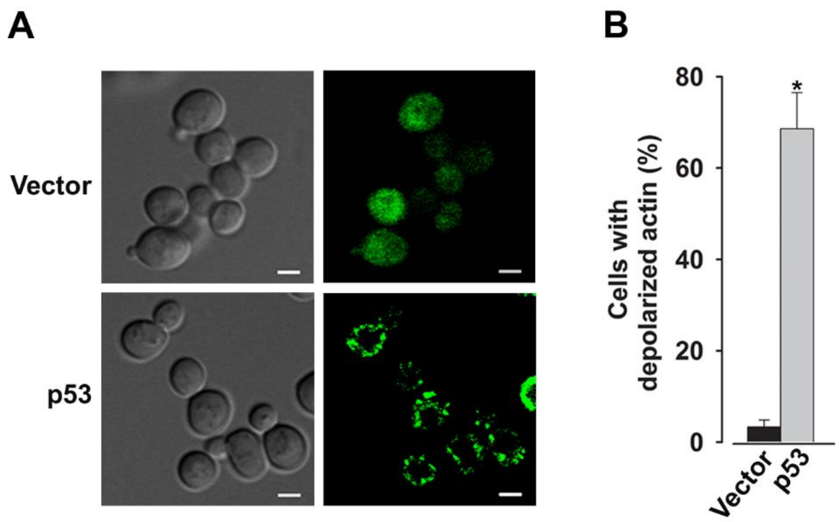


Fig. 16 – Expression of p53 in yeast induces actin depolarization. (A) Effect of p53 on yeast actin organization was analysed by fluorescence microscopy using yeast expressing wt p53 or control yeast co-transformed with the yeast integrative ABP140-3xGFP plasmid, incubated in selective induction medium for 30 h. Bars represent 2 µm. **(B)** Quantification of actin depolarization was estimated by counting at least 300 cells per sample. Data are mean of two independent experiments; value significantly different from control yeast (**P* < 0.05).

3.4. Effect of p53 family proteins on yeast cell proliferation and death

In the present work, the individual expression of the human alpha isoforms of p63, ΔNp63 and p73 in yeast was confirmed by Western blot analysis (Fig. 17A). For p63α and ΔNp63α, protein bands of 52 and 72 kDa, respectively, were obtained as has been previously described for their expression in human cell lines (Kommagani et al., 2009). As referred above (in section 3.3), contrary to the p53 R273H mutant (used as negative control), the expression of wt p53 in yeast caused a marked yeast growth inhibition (Fig. 17B, C). Similarly to wt p53, p63, ΔNp63 and p73 induced yeast growth inhibition (Fig. 17B, C). Nevertheless, with the exception of ΔNp63, the effects on yeast cell growth observed with p63 and p73 were different from that observed with wt p53 (Fig. 17B, C). In fact, at 30 h incubation, a higher growth inhibitory effect was observed with p63, (72.9%) when compared to wt p53 (53.3%) (Fig. 17C). On the contrary, a lower growth inhibitory effect was obtained with p73α (40.4%) when compared to wt p53 (53.3%) (Fig. 17C).

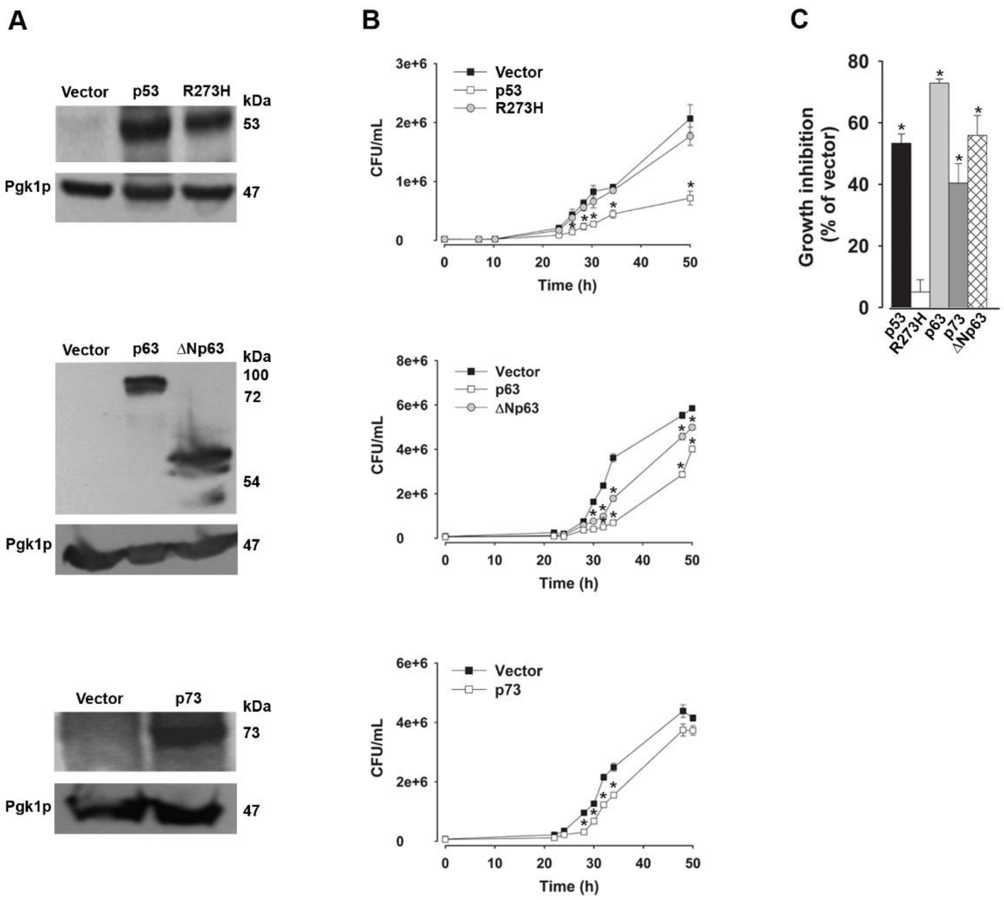


Fig. 17 – Yeast cell growth inhibition induced by human p53 family members. Yeast cells individually expressing human wt p53, R273H, p63, ΔNp63, p73 and control yeast (empty vector) were grown in induction selective medium for up to 50 h for growth curves experiments or for 30 h (time required by control yeast to achieve 0.5 OD_{600}) in all other experiments. **(A)** Expression of human wt p53, R273H, p63, ΔNp63 and p73 in yeast was confirmed by Western blot analysis. Pgk1p was used as a loading control. Immunoblots are representative of two experiments. **(B)** Growth curves were obtained by CFU counts. Representative data of two independent experiments with six replicates. Values significantly different from control yeast (**P* < 0.05). **(C)** Growth inhibition induced by wt p53, R273H, p63, ΔNp63 and p73. Results were estimated considering the number of CFU obtained with control yeast as 100%. Results are represented as mean ± SEM of six independent experiments. Values significantly different from control yeast (**P* < 0.05).

Moreover, similarly to wt p53, and contrary to p53 R273H, p63-, ΔNp63- and p73-induced growth inhibition was also associated with an arrest in cell cycle progression. In fact, both ΔNp63 and p63 induced S-phase cell cycle arrest (22.7% for ΔNp63, and 34.4% for p63), similarly to that observed for wt p53 (33.9%) (Fig. 18). However, a significant increase in the percentage of cells arrested in G1-phase (53.3%), instead of S-phase, was observed for p73, when compared to control yeast (39.5%) (Fig. 18). Together, the results obtained revealed distinct effects of p53 family members on the growth and cell cycle progression of yeast cells.

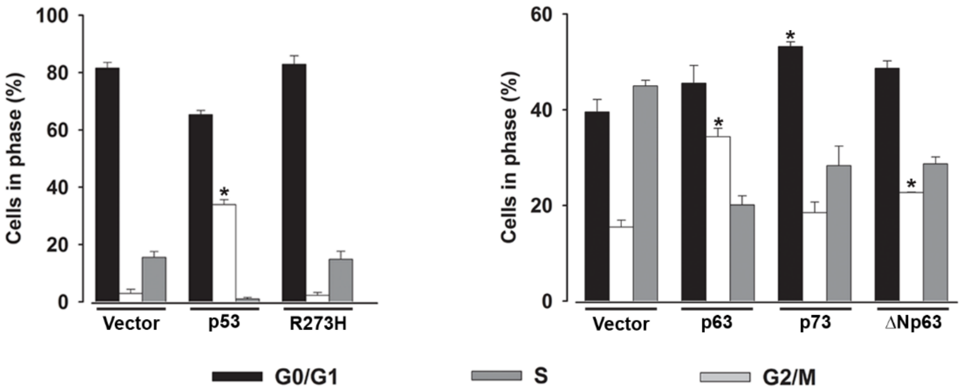


Fig. 18 – Yeast cell cycle arrest induced by human p53 family members. Yeast cells individually expressing human wt p53, R273H, p63, ΔNp63, p73 and control yeast (empty vector) were grown in induction selective medium for 30 h; wt p53, p63α and ΔNp63α induced S-phase cell cycle arrest and p73 induced G1-phase cell cycle arrest. Mutant p53 R273H did not interfere with yeast cell cycle progression. Data are mean ± SEM of two independent experiments; values significantly different from control yeast (**P* < 0.05).

Moreover, the expression of p63, ΔNp63 or p73 in yeast also seemed to increase of actin expression levels, when compared to the effect of the empty vector (Fig. 19A), as observed for wt p53 (Fig. 14C). This suggested that p63, ΔNp63 and p73 may induce the transcription of the *ACT1* gene, although possible indirect effects cannot be discarded at this stage.

The effect of p63, ΔNp63 or p73 was also evaluated on yeast actin depolarization (Fig. 19B). Similarly to the previously observed for wt p53, p63, ΔNp63 and p73 significantly increased the percentage of cells with depolarized actin. In spite of this, the effects of these p53 family members were not as pronounced as those observed with wt p53.

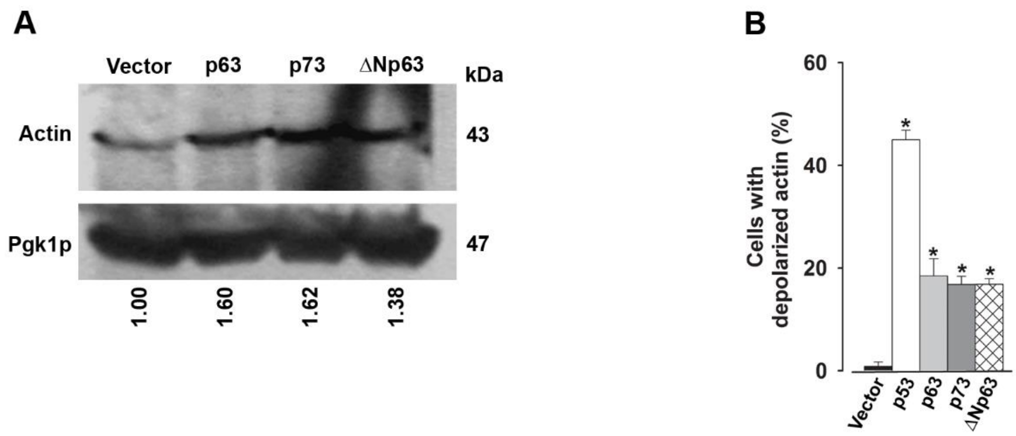


Fig. 19 – Effects of p63, ΔNp63, p73 on actin depolarization and protein expression levels. (A) Actin protein levels were analysed by Western blot. Pgk1p was used as a loading control. Immunoblots are representative of two independent experiments. **(B)** Quantification of actin depolarization was estimated by counting at least 300 cells per sample; data are represented as mean ± SEM of two independent experiments; values significantly different from control yeast (*$P < 0.05$).

A previous work performed by our group has shown that yeast growth inhibition induced by wt p53 was not associated with a necrotic or an apoptotic cell death (Coutinho et al., 2009). In the present work, by analysing two cell death markers, namely loss of plasma membrane integrity [necrotic cells; PI positively stained (PI+) cells], and DNA fragmentation (apoptotic cells; TUNEL positive cells), it was verified that, similarly to wt p53, the yeast cell growth inhibition induced by p63, ΔNp63, and p73 was not associated with an increase in the percentage of necrotic and apoptotic cells, when compared to control yeast (empty vector) (Fig. 20A).

To further investigate the activity of p53 family proteins on apoptosis, yeast cells individually expressing wt p53, p53 R273H, p63, ΔNp63, and p73 and were treated with a known yeast apoptotic inducer, H_2O_2 (Madeo et al., 1999). As previously reported for wt p53 (Coutinho et al., 2011), the results obtained in this work showed that, upon 1 h treatment with 1.5 and 5 mM H_2O_2, none of the studied p53 family members significantly increased the percentage of H_2O_2-induced cell death, when compared to control yeast (empty vector; Fig. 20B).

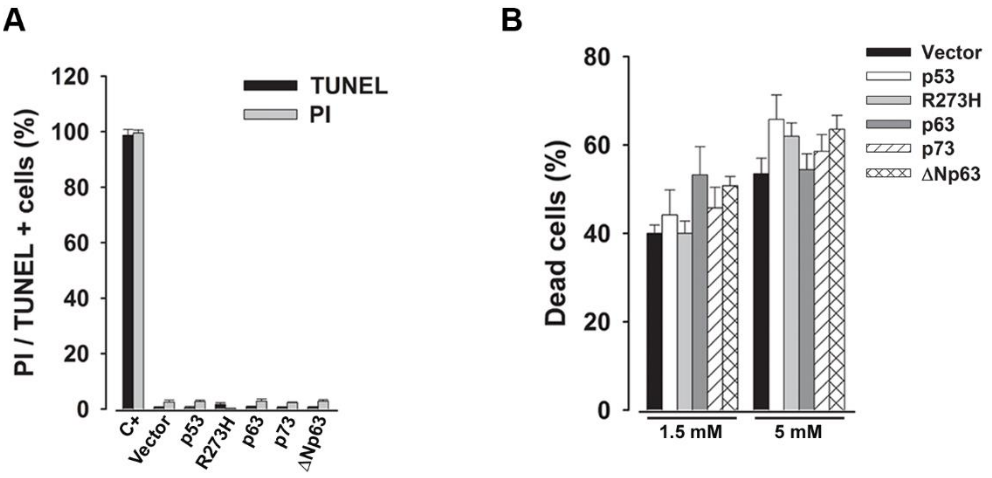


Fig. 20 – p53 family members do not induce yeast apoptotic cell death. Yeast cells individually expressing human wt p53, R273H, p63, p73, ΔNp63 and control yeast (empty vector) were grown in induction selective medium for 30 h. **(A)** Cells with DNA fragmentation (TUNEL +) and necrotic cells (PI +) obtained with yeast cells expressing wt p53, p63, p73, ΔNp63 and control yeast . Yeast cells treated with DNAse were used as positive control (C+). Presented are mean ± SEM of four independent experiments. **(B)** Percentage of dead cells obtained with yeast cells expressing wt p53, R273H, p63, p73, ΔNp63 and control yeast after treatment with 1.5 or 5 mM H_2O_2 for 1 h at 30 ºC. The percentage of dead cells was assessed by CFU counts, considering 100% survival (0% death) as the number of CFU obtained with H_2O_2-untreated cells. Presented are mean ± SEM of four independent experiments. In **(A)** and **(B)**, values were not significantly different from control yeast (vector): $P > 0.05$.

The possible involvement of an autophagic process in yeast growth inhibition induced by wt p53, p63, ΔNp63 and p73 was then investigated. This was possible by using the *S. cerevisiae* strains wt W303, W303 atg1Δ and W303 atg5Δ individually expressing each of these human proteins or the mutant p53 R273H (as negative control). The W303 atg1Δ and W303 atg5Δ strains lack atg1p and atg5p, respectively, which are proteins required for the autophagosome formation, their deletion leading to a defective macroautophagy (George et al., 2000; Kametaka et al., 1996; Matsuura et al., 1997). The single expression of wt p53, p53 R273H, p63, ΔNp63, or p73 in yeast was confirmed by Western blot analysis (data not shown). Similarly to what was observed in the previously described studies (using the CG379 yeast strain), when wt p53 was expressed in wt W303 strain, it induced a marked inhibition of yeast growth (of approximately 54%), when compared to control yeast (empty vector) (Fig. 21A). However, a pronounced decrease of wt p53-induced growth inhibition was observed when this protein was expressed in W303 atg1Δ (of about 25%) and W303 atg5Δ (of about 40%) strains (Fig. 21A). On the contrary, R273H did not significantly interfere with the yeast cell growth in any of the studied strains (Fig. 21A). Similarly to what was observed for wt p53, the p63-, p73- and ΔNp63-induced growth inhibition observed in wt W303 strain was also markedly reduced when these proteins were expressed in W303 atg1Δ and W303 atg5Δ strains (Fig. 21A): p63 (about 17% for atg1Δ and 26% for atg5Δ); p73 (about 36% for atg1Δ and 15% for atg5Δ); ΔNp63 about 20% for atg1Δ and 26% for atg5Δ). The difference in the effects observed in strains which have mutations in autophagy related genes, when comparing to the wt strain seemed to indicate a possible involvement of autophagy in the mechanism of action. This was further supported by analysing the expression levels of Atg8p in CG379 strain. The expression of Atg8p (a yeast homologue of the mammalian protein LC3, one of the proteins required for autophagosome formation) has been shown to increase when autophagy is induced (Kirisako et al., 1999). As expected, the results indicated that expression of wt p53, p63, ΔNp63 and p73 increased the Atg8p expression levels (Fig. 21B). Altogether, these results suggest that the observed yeast growth inhibitory effect caused by wt p53, p63, ΔNp63 and p73 could be related to an autophagic process.

In order to clarify whether the growth inhibition induced by p53 family members is associated with autophagic cell death, CG379 yeast cells expressing wt p53, p63, ΔNp63 or p73 were treated with rapamycin, an inhibitor of mTOR and, therefore, inducer of autophagy in yeast (Noda and Ohsumi, 1998), and its effect on yeast cell viability was assessed. After 4 h treatment with 0.2 µg/mL rapamycin, it was observed that expression of wt p53 in yeast stimulated rapamycin-induced yeast loss of viability, when compared to control yeast (Fig. 21C). Although in a lower degree than the observed for wt p53, p63, ΔNp63, and p73 also stimulated rapamycin-induced yeast loss of viability. Interestingly, contrary to that observed in cell proliferation, no differences were observed between the effects obtained with p63, ΔNp63, and p73 on rapamycin-induced loss of cellular viability.

These results indicated an involvement of p53 family proteins on an autophagic process. Furthermore, the increase of rapamycin-induced yeast loss of viability caused by expression of p53 family proteins suggests that the yeast growth inhibition induced by these proteins could be related to autophagic death.

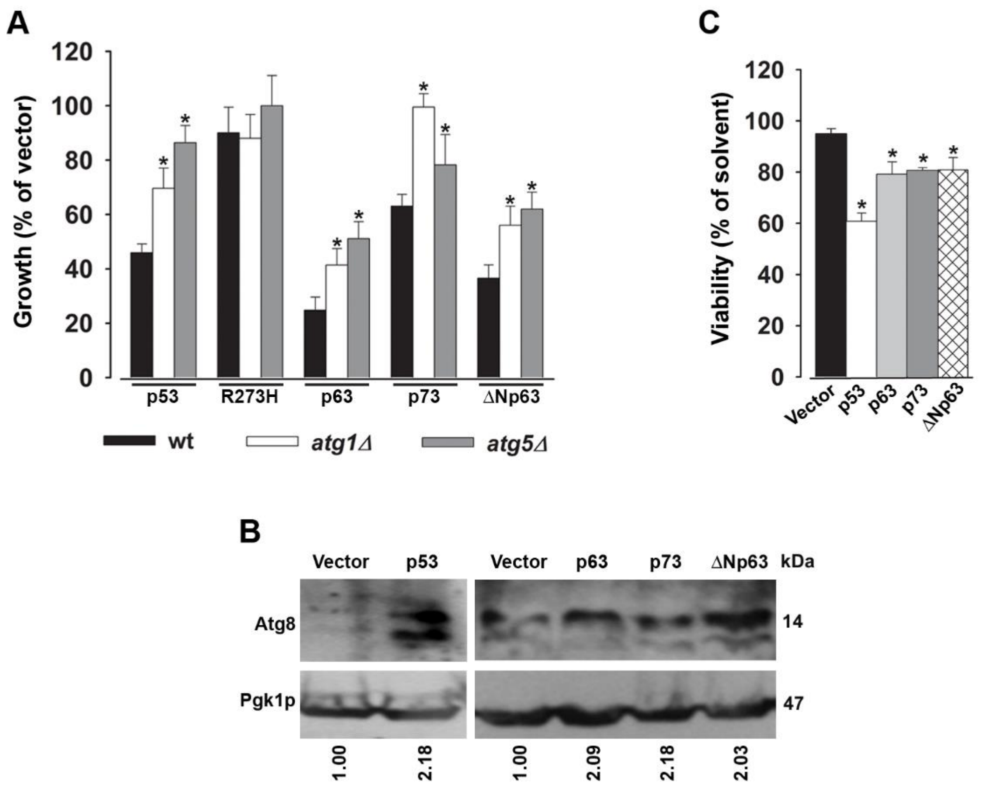


Fig. 21 – p53 family members induce yeast autophagic cell death. CG379, wt W303, W303 atg1Δ and W303 atg5Δ yeast strains individually expressing human wt p53, R273H, p63, p73 or ΔNp63, and control yeast (empty vector) were grown in induction selective medium for 30 h **(A)** Effect of p53 family proteins on wt W303, W303 atg1Δ and W303 atg5Δ cell growth was analysed by CFU counts, considering the number of CFU obtained with control yeast as 100%. Results are represented as mean ± SEM of four independent experiments; values significantly different from wt W303 (*$P < 0.05$). **(B)** Atg8p protein levels, in CG379 yeast cells expressing a p53 family protein, were analysed by Western blot. Pgk1p was used as a loading control. Immunoblots represent one experiment. Presented in the quantification of immunoblots are the values of one experiment. **(C)** CG379 yeast cells expressing a p53 family protein were treated with 0.2 µg/mL rapamycin for 4 h. The percentage of viability was assessed by CFU counts, considering the number of CFU obtained with cells incubated with DMSO only as 100%. Presented are mean ± SEM of four independent experiments. Values significantly different from control yeast (*$P < 0.05$).

4 Discussion

4. Discussion

4.1. α-Mangostin and gambogic acid as potential inhibitors of the p53-MDM2 interaction

α-Mangostin (αMG) and gambogic acid (GA) are naturally occurring prenylated xanthones with a well-known potent anti-tumour activity both *in vitro* [reviewed in (Chantarasriwong et al., 2010; Pedraza-Chaverri et al., 2008)], and *in vivo* (Chitchumroonchokchai et al., 2013; Zhao et al., 2008). In spite of this, the molecular mechanism of action of these compounds remains controversial. In fact, a correlation between their anti-tumour properties and the activation of a p53-dependent pathway has been suggested by several authors (Aisha et al., 2012; Gu et al., 2008; Rong et al., 2009). Particularly, αMG was shown to increase the p53 transcriptional activity (Aisha et al., 2012), and to induce apoptosis associated with an increase of the protein levels of Bax, a p53 target gene (Kaomongkolgit et al., 2011). Moreover, it was described that GA induced apoptosis and cell cycle arrest in tumour cells expressing wt p53 (Gu et al., 2008). On the other hand, a recent report showed that αMG induced apoptosis in a mouse model carrying a p53 mutation (Shibata et al., 2011). Additionally, it was also shown that GA induced apoptosis and cell cycle arrest in human tumour cells lacking p53 (Rong et al., 2009).

In a previous study, our group identified an inhibitor of the p53-MDM2 interaction with a xanthone scaffold (Leao et al., 2013). Based on this, it was hypothesized that αMG and GA, could also target the p53-MDM2 interaction.

In the present work, it was shown that, as nutlin-3a, both αMG and GA were able to re-establish the p53 activity in yeast by inhibiting the negative effect of MDM2 on p53. This was consistent with an inhibition of the p53-MDM2 interaction by αMG and GA. Furthermore, neither of the xanthones had any effect on the growth of yeast cells expressing wt p53 alone, or a mutant form of p53. These results were further supported by the fact that studies carried out, under the scope of this work, at Prof. Inga’s Lab (Centre for Integrative Biology, CIBIO, University of Trento, Italy), using a dual-luciferase yeast p53 transactivation assay [described in (Andreotti et al., 2011)] showed that both xanthones (as nutlin-3a) significantly increased the p53 transcriptional activity by inhibiting the negative effect of MDM2 on p53. Interestingly, other studies performed at Prof Inga´s lab have confirmed these results in breast adenocarcinoma cell lines and computational studies performed at Prof. Madalena Pinto’s Lab (CEQUIMED-UP) suggested that both αMG and GA were able to interact with MDM2. Nevertheless, since those studies were performed by others they were not included in this thesis. Together, these results suggested that αMG and GA were potential inhibitors of the p53-MDM2 interaction.

As a whole, the results obtained in this work and by collaborators revealed, for the first time, that αMG and GA are potential inhibitors of the p53-MDM2 interaction. The computational docking studies indicated that, similarly to nutlin-3a, αMG and GA have the potential to bind to MDM2, therefore acting as direct inhibitors of MDM2. These results provide new clues and open new perspectives into the mode of action of these xanthones in human tumour cells with wt p53. Moreover, with the confirmation of the results obtained in yeast, a proof-of-concept for the effectiveness of the yeast-based assay to search for inhibitors of the p53-MDM2 interaction is provided.

4.2. Flavonoids as potential activators of p53

Flavonoids represent another group of compounds widely recognized for their potent anti-tumour activity (Boumendjel et al., 2009; Lopez-Lazaro, 2002). In general, and depending on the cell line studied, the growth inhibitory effects of flavonoids, such as 3,7-dihydroxyflavone, have been associated with the induction of cell cycle arrest and apoptosis (Monasterio et al., 2004).

In the present work, five chalcones (a sub-class of flavonoids synthesised by the CEQUIMED-UP group) and 3,7-dihydroxyflavone, which had emerged as potential p53 activators from a yeast-based screening assay previously performed by our group, were tested in human colon carcinoma cell lines with and without p53. As expected, all the tested flavonoids presented a potent anti-proliferative activity in both cell lines studied. However, no significant differences were observed in the response of both cell lines to these flavonoids, which suggested that they had a p53-independent mechanism of action. In fact, besides p53, the tested flavonoids may probably act on other cellular targets absent in yeast, particularly on another p53 family protein, such as p73, expressed in both tested tumour cell lines. Therefore, additional studies should be performed in order to further investigate the precise molecular mechanism of action of these compounds.

4.3. Develoment of a yeast model for funtional and pharmacological studies of the p53-MDMX interaction

As previously referred, the pharmacological modulation of p53 is a promising therapeutic strategy in the treatment of cancer [reviewed in (Chen et al., 2010)]. Approximately 50% of all human tumours have wt p53, which is often inactivated due to the overexpression of its endogenous negative regulators MDM2 and MDMX (Brown et al., 2009). Several efforts have been focused on the therapeutic inhibition of the p53-MDM2 interaction, but to date few inhibitors of the p53-MDMX interaction have been identified [reviewed in (Zhao et al., 2013)]. Nevertheless, inhibition of the p53-MDMX interaction would be particularly useful in the treatment of tumours overexpressing MDMX. In fact, MDMX overexpression has been described in 18 – 19% of breast, lung, and colon cancers (Danovi et al., 2004), 50% of head and neck squamous carcinomas (Valentin-Vega et al., 2007), and 65% of retinoblastomas (Laurie et al., 2006). Moreover, despite being structurally related proteins with the ability to inhibit the p53 transcriptional activity, as referred above, MDM2 and MDMX play multifaceted and non-redundant roles in modulating p53. Therefore, the development of inhibitors of the interaction both of MDM2 and MDMX with p53 has emerged as a promising therapeutic strategy against tumours retaining wt p53 [reviewed in (Pei et al., 2012; Shadfan et al., 2012; Wade et al., 2013)]. However, it has been shown that inhibitors of the p53-MDM2 interaction, such as nutlin-3a, were largely ineffective against tumours overexpressing MDMX (Wade and Wahl, 2009). Recently, Reed and colleagues (2010) identified the first small molecule inhibitor of the p53-MDMX interaction, called SJ-172550. However, its weak cellular effect and other limitations for its further development as a selective MDMX inhibitor (Bista et al., 2012) have made the need for the discovery of new and more effective small molecule inhibitors of the p53-MDMX interaction an urgent area of research. Therefore, the development of new screening approaches that may improve and expedite this search would certainly contribute to achieve such a goal.

In the present work, the human p53-MDMX regulatory pathway was reconstituted in yeast for the first time. This allowed the development of a simplified and cost-effective yeast-based growth inhibition assay, which can be used for the screening of potential inhibitors of the p53-MDMX interaction. Additionally, it can be easily adapted to the high-throughput screening (HTS) of large chemical libraries, since it is based on simple measurements of yeast cell growth by optical density.

Overall, the simplified cell model developed under the scope of this thesis can be used for functional and molecular studies of the complex p53-MDMX network. Most importantly, it can be of great help as a first-line screening of potential inhibitors of the p53-MDMX interaction, which may then be tested in more complex and physiologically accurate cell models. The use of this assay, in the first stage of the drug discovery process, would certainly reduce the costs and expedite the identification of new inhibitors of the p53-MDMX interaction.

4.4. Development of a simplified yeast p53 transcriptional assay based on the identification of a yeast endogenous p53 target gene

In a previous work performed in human cells, Comer and colleagues (1998) showed that the p53-induced growth inhibition and cell cycle arrest were associated with drastic changes in the actin cytoskeleton, and also with an increase of the smooth muscle α-actin mRNA levels. More recently, it was also reported that nutlin-3a was a potent inducer of smooth muscle α-actin expression in human cells (Secchiero et al., 2012). Together, these works showed that actin, with a crucial role in cell cycle progression [reviewed in (Heng and Koh, 2010)], was a direct p53 transcriptional target in human cells.

In the present work, it was shown that in yeast the p53-induced growth inhibition and cell cycle arrest were also associated with an increase of actin protein levels, which was modulated by chemical (PFT-α, nutlin-3a and SJ-172550) and natural (MDM2 and MDMX) regulators of p53 activity. Furthermore, it was shown that in yeast, p53 also induced actin depolarization. These results were further supported by studies carried out, under the scope of this work, at Prof. Inga's Lab, in which the actin mRNA levels were determined in yeast cells expressing p53 by quantitative-PCR. In accordance with the increase of actin protein levels, a significant 2-fold increase of actin mRNA levels was obtained, when compared to control yeast.

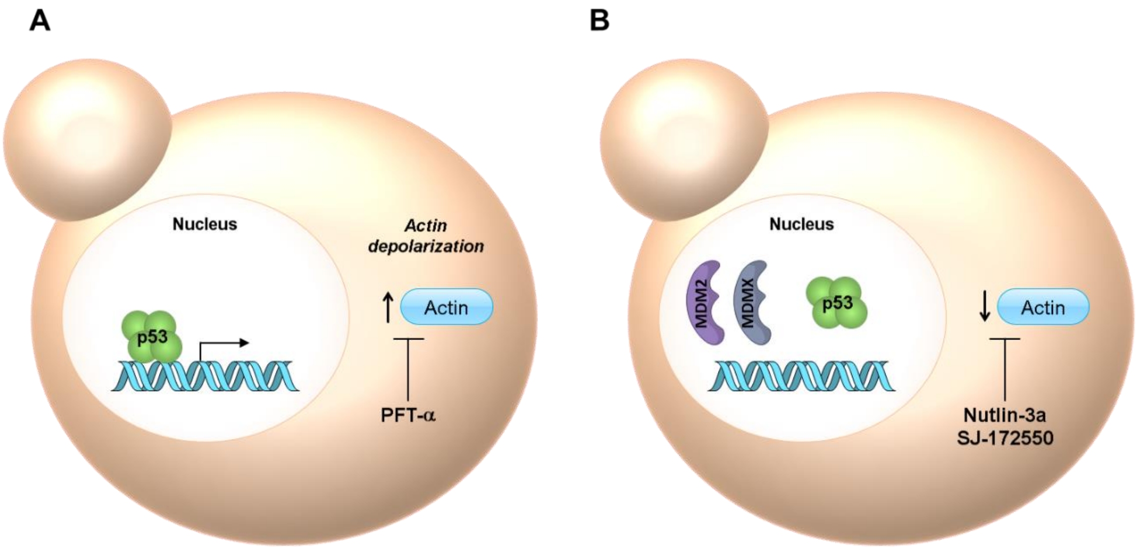


Fig. 22 – Identification of *ACT1* as a potential yeast endogenous p53 target gene. (A) Expression of p53 in yeast increases the actin protein levels and induces actin depolarization. This effect is reversed by PFT-α, an inhibitor of p53 transcriptional activity. **(B)** Co-expression of MDM2 or MDMX inhibits the p53-induced actin protein levels, an effect reversed by the inhibitors of the p53-MDM2 and p53-MDMX interactions, nutlin-3a and SJ-172550, respectively.

Altogether, with this work, relevant insights into the p53 regulatory pathway in yeast were revealed. Particularly, the results obtained strongly indicated that *ACT1* is a potential endogenous p53 transcriptional target in yeast.

Yeast p53 transactivation assays have been extensively used by several authors to study the impact of mutations, cofactors and small molecules on p53 transcriptional activity. However, these assays have been carried out using artificial yeast reporter constructs with response elements derived from human genes regulated by p53, such as *PUMA* and *BAX* [reviewed in (Pereira et al., 2012a; Pereira et al., 2012b)]. With the identification of *ACT1* as a possible endogenous p53 target gene in yeast a new simplified and cost-effective yeast p53 transactivation assay may be developed, which can be used for the analysis of the p53 transcriptional activity, instead of the traditional artificial yeast transactivation reporter assays.

4.5. Effect of p53 family proteins on yeast cell proliferation and death

The p53 family proteins regulate several important processes related to cell proliferation and death, thus being attractive therapeutic targets in cancer treatment [reviewed in (Wei et al., 2012)]. Nevertheless, the molecular and pharmacological profile of these proteins remains largely controversial [reviewed in (Vilgelm et al., 2008)]. Furthermore, very few small molecule modulators of p53 family proteins are currently available [reviewed in (Chung and Irwin, 2010)].

The high complexity of the p53 family pathway in mammalian cells led us to the development of yeast-based assays to study not only the biology, but also the pharmacology of these proteins, particularly to discover isoform-selective p53 family modulators. Therefore, in the present work, the p63-, ΔNp63- and p73-signalling networks were reconstituted in yeast, and the effects of these p53 family proteins on cell proliferation and death were analysed for the first time in yeast.

The results obtained showed that, in general, although with some differences in the intensity of their activities, p53, p63, ΔNp63 and p73 exhibited similar functions in yeast. In fact, similarly to p53, the p63, ΔNp63 and p73 proteins were also able to induce yeast growth inhibition associated with cell cycle arrest and probably autophagy. Although the stimulation of an autophagic cell death by TA isoforms of p63 and p73 had already been described in human cells (Eby et al., 2010) this work represents the first report for an induction of autophagy by ΔNp63. Further studies are underway, in our laboratory, in order to elucidate the cellular localization of p53 family proteins (associated to the induction of an autophagic cell death), as well as to identify possible yeast p53 family target genes involved in this mechanism. Surprisingly, like p53, none of the tested p53 family proteins interfered with the apoptotic cell death in yeast. This may be probably due to the absence of orthologues of human p53 target genes involved in apoptosis, particularly Bcl-2 family proteins, such as Bax, in yeast.

Moreover, similarly to p53, the p63, ΔNp63 and p73 proteins increased the actin expression levels and induced actin depolarization in yeast cells. In spite of this, the much lower degree of actin depolarization observed with p63, ΔNp63 and p73, when compared to p53, suggests a lesser involvement of these proteins on actin organization than that of p53. Most importantly, to our knowledge, to date, no data is available concerning a possible regulation of actin expression by p63, ΔNp63 and p73 in human cells. The results obtained in yeast therefore suggest that actin may be regulated by p53 family proteins.

It has been described that TA isoforms of p63 and p73 are able to induce the transcription of p53 target genes in mammalian cells, thus performing similar roles to p53. In fact, these proteins are well-known by their anti-proliferative activities in mammalian cells, which is consistent with the results obtained in the present work in the yeast model. However, since ΔN isoforms lack the N-terminal TAD, it has been proposed that these isoforms are transcriptionally inactive (Yang et al., 1998). In fact, ΔN isoforms are generally described as dominant-negative regulators of TA isoforms, being often associated with the promotion of cell proliferation [reviewed in (Alsafadi et al., 2009)]. Curiously, the present work, showed that ΔNp63 induced yeast growth inhibition, similarly to that observed with p53 and with TA isoforms of p63 and p73. In agreement with this, similar results were described by Dohn and colleagues (2001), which showed that ΔNp63α could induce apoptosis and cell cycle arrest in a p53-null H1299 human carcinoma cell line. The authors also showed that, in these cells, ΔNp63α was able to induce the transcription of the p53 target gene *CDKN1A* (which encodes p21), possibly through an alternative transactivation domain. The results obtained by Dohn and colleagues (2001) were further confirmed in yeast, suggesting the conservation of the ΔNp63α-pathway in this organism. Further studies will be performed in yeast in order to clarify the activities of ΔN isoforms.

As a whole, in the present work, a yeast-based assay was developed for each of the p53 family proteins. The independent analysis of the p53 family members in yeast allowed the study of the role of each protein in cell proliferation and death with new data on their functional and molecular profiles being provided. Further insights about the roles of p53 family proteins in cellular processes, particularly their involvement in autophagic cell death, have emerged from the exploitation of the yeast assays here developed. Additionally, with the anti-proliferative effects of p63, ΔNp63 and p73 in yeast, a cell-based phenotypic assay was developed, which may be used to study the impact of cofactors (such as MDM2 and MDMX) on their activities, as well as to the HTS of selective small molecule modulators of each p53 family member. Finally, with the identification of *ACT1* as a possible endogenous yeast target gene of p63, ΔNp63 and p73, new simplified and cost-effective yeast transactivation assays for the analysis of the transcriptional activity of these p53 family proteins may be developed, which can be used instead of the traditional artificial yeast transactivation reporter assays.

4.6. Final remarks

In the present work, valuable data about the biology and pharmacology of p53 family proteins was provided using the yeast model. New targets and pharmacological regulators of p53 family proteins were revealed. In addition, new yeast assays were developed, which may represent relevant tools for the further elucidation of the p53 family network, as well as for the identification of new therapeutic opportunities based on selective modulation of p53 family proteins.

The yeast research performed in this thesis confirmed the tremendous potential of this model system towards the study of human proteins. It is therefore anticipated that new promising discoveries may occur in the area of p53 family proteins using this model organism. In fact, as pointed above, new ways were opened and several relevant questions were raised with this work, some of them being currently under study in our laboratory, while others would be interesting to be investigated in the near future.

5 References

5. References

Aisha, A.F., K.M. Abu-Salah, Z. Ismail, and A.M. Majid. 2012. In vitro and in vivo anti-colon cancer effects of Garcinia mangostana xanthones extract. *BMC Complement Altern Med*. 12:104.

Allocati, N., C. Di Ilio, and V. De Laurenzi. 2012. p63/p73 in the control of cell cycle and cell death. *Exp Cell Res*. 318:1285-1290.

Alsafadi, S., S. Tourpin, F. Andre, G. Vassal, and J.C. Ahomadegbe. 2009. p53 family: at the crossroads in cancer therapy. *Curr Med Chem*. 16:4328-4344.

Andreotti, V., Y. Ciribilli, P. Monti, A. Bisio, M. Lion, J. Jordan, G. Fronza, P. Menichini, M.A. Resnick, and A. Inga. 2011. p53 transactivation and the impact of mutations, cofactors and small molecules using a simplified yeast-based screening system. *PLoS One*. 6:e20643.

Badciong, J.C., and A.L. Haas. 2002. MdmX is a RING finger ubiquitin ligase capable of synergistically enhancing Mdm2 ubiquitination. *J Biol Chem*. 277:49668-49675.

Balint, E., S. Bates, and K.H. Vousden. 1999. Mdm2 binds p73 alpha without targeting degradation. *Oncogene*. 18:3923-3929.

Balint, E., A.C. Phillips, S. Kozlov, C.L. Stewart, and K.H. Vousden. 2002. Induction of p57(KIP2) expression by p73beta. *Proc Natl Acad Sci U S A*. 99:3529-3534.

Barak, Y., E. Gottlieb, T. Juven-Gershon, and M. Oren. 1994. Regulation of mdm2 expression by p53: alternative promoters produce transcripts with nonidentical translation potential. *Genes Dev*. 8:1739-1749.

Bista, M., D. Smithson, A. Pecak, G. Salinas, K. Pustelny, J. Min, A. Pirog, K. Finch, M. Zdzalik, B. Waddell, B. Wladyka, S. Kedracka-Krok, M.A. Dyer, G. Dubin, and R.K. Guy. 2012. On the mechanism of action of SJ-172550 in inhibiting the interaction of MDM4 and p53. *PLoS One*. 7:e37518.

Böttger, V., A. Böttger, C. Garcia-Echeverria, Y.F. Ramos, A.J.v.d. Eb, A. Jochemsen, and D.P. Lane. 1999. Comparative study of the p53-mdm2 and p53-MDMX interfaces. *Oncogene*. 18:189-199.

Boumendjel, A., X. Ronot, and J. Boutonnat. 2009. Chalcones derivatives acting as cell cycle blockers: potential anti cancer drugs? *Curr Drug Targets*. 10:363-371.

Brown, C.J., S. Lain, C.S. Verma, A.R. Fersht, and D.P. Lane. 2009. Awakening guardian angels: drugging the p53 pathway. *Nat Rev Cancer*. 9:862-873.

Bykov, V.J., N. Issaeva, G. Selivanova, and K.G. Wiman. 2002. Mutant p53-dependent growth suppression distinguishes PRIMA-1 from known anticancer drugs: a statistical analysis of information in the National Cancer Institute database. *Carcinogenesis*. 23:2011-2018.

Calabro, V., G. Mansueto, T. Parisi, M. Vivo, R.A. Calogero, and G. La Mantia. 2002. The human MDM2 oncoprotein increases the transcriptional activity and the protein level of the p53 homolog p63. *J Biol Chem*. 277:2674-2681.

Chantarasriwong, O., A. Batova, W. Chavasiri, and E.A. Theodorakis. 2010. Chemistry and biology of the caged Garcinia xanthones. *Chemistry*. 16:9944-9962.

Chen, F., W. Wang, and W.S. El-Deiry. 2010. Current strategies to target p53 in cancer. *Biochem Pharmacol*. 80:724-730.

Chen, X., L.J. Ko, L. Jayaraman, and C. Prives. 1996. p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells. *Genes Dev*. 10:2438-2451.

Chitchumroonchokchai, C., J.M. Thomas-Ahner, J. Li, K.M. Riedl, J. Nontakham, S. Suksumrarn, S.K. Clinton, A.D. Kinghorn, and M.L. Failla. 2013. Anti-tumorigenicity of dietary alpha-mangostin in an HT-29 colon cell xenograft model and the tissue distribution of xanthones and their phase II metabolites. *Mol Nutr Food Res*. 57:203-211.

Chung, J., and M.S. Irwin. 2010. Targeting the p53-family in cancer and chemosensitivity: triple threat. *Curr Drug Targets*. 11:667-681.

Comer, K.A., P.A. Dennis, L. Armstrong, J.J. Catino, M.B. Kastan, and C.C. Kumar. 1998. Human smooth muscle alpha-actin gene is a transcriptional target of the p53 tumor suppressor protein. *Oncogene*. 16:1299-1308.

Costanzo, A., P. Merlo, N. Pediconi, M. Fulco, V. Sartorelli, P.A. Cole, G. Fontemaggi, M. Fanciulli, L. Schiltz, G. Blandino, C. Balsano, and M. Levrero. 2002. DNA damage-dependent acetylation of p73 dictates the selective activation of apoptotic target genes. *Mol Cell*. 9:175-186.

Coutinho, I., C. Pereira, G. Pereira, J. Goncalves, M. Corte-Real, and L. Saraiva. 2011. Distinct regulation of p53-mediated apoptosis by protein kinase Calpha, delta, epsilon and zeta: Evidence in yeast for transcription-dependent and -independent p53 apoptotic mechanisms. *Exp Cell Res*. 317:1147-1158.

Coutinho, I., G. Pereira, M. Leao, J. Goncalves, M. Corte-Real, and L. Saraiva. 2009. Differential regulation of p53 function by protein kinase C isoforms revealed by a yeast cell system. *FEBS Lett*. 583:3582-3588.

Crighton, D., J. O'Prey, H.S. Bell, and K.M. Ryan. 2007. p73 regulates DRAM-independent autophagy that does not contribute to programmed cell death. *Cell Death Differ*. 14:1071-1079.

Danovi, D., E. Meulmeester, D. Pasini, D. Migliorini, M. Capra, R. Frenk, P. de Graaf, S. Francoz, P. Gasparini, A. Gobbi, K. Helin, P.G. Pelicci, A.G. Jochemsen, and J.C. Marine. 2004. Amplification of Mdmx (or Mdm4) directly contributes to tumor formation by inhibiting p53 tumor suppressor activity. *Mol Cell Biol*. 24:5835-5843.

Davison, T.S., C. Vagner, M. Kaghad, A. Ayed, D. Caput, and C.H. Arrowsmith. 1999. p73 and p63 are homotetramers capable of weak heterotypic interactions with each other but not with p53. *J Biol Chem*. 274:18709-18714.

Deyoung, M.P., and L.W. Ellisen. 2007. p63 and p73 in human cancer: defining the network. *Oncogene*. 26:5169-5183.

Di Ventura, B., C. Funaya, C. Antony, M. Knop, and L. Serrano. 2008. Reconstitution of Mdm2-dependent post-translational modifications of p53 in yeast. *PLoS One*. 3:e1507.

Dohn, M., S. Zhang, and X. Chen. 2001. p63α and ΔNp63α can induce cell cycle arrest and apoptosis and differentially regulate p53 target genes. *Oncogene*. 20:3193-3205.

Dolezelova, P., K. Cetkovska, K.H. Vousden, and S. Uldrijan. 2012. Mutational analysis of Mdm2 C-terminal tail suggests an evolutionarily conserved role of its length in Mdm2 activity toward p53 and indicates structural differences between Mdm2 homodimers and Mdm2/MdmX heterodimers. *Cell Cycle*. 11:953-962.

Dominguez, G., J.M. Garcia, C. Pena, J. Silva, V. Garcia, L. Martinez, C. Maximiano, M.E. Gomez, J.A. Rivera, C. Garcia-Andrade, and F. Bonilla. 2006. DeltaTAp73 upregulation correlates with poor prognosis in human tumors: putative in vivo network involving p73 isoforms, p53, and E2F-1. *J Clin Oncol*. 24:805-815.

Donehower, L.A., M. Harvey, B.L. Slagle, M.J. McArthur, C.A. Montgomery, Jr., J.S. Butel, and A. Bradley. 1992. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature*. 356:215-221.

Dotsch, V., F. Bernassola, D. Coutandin, E. Candi, and G. Melino. 2010. p63 and p73, the ancestors of p53. *Cold Spring Harb Perspect Biol*. 2:a004887.

Eby, K.G., J.M. Rosenbluth, D.J. Mays, C.B. Marshall, C.E. Barton, S. Sinha, K.N. Johnson, L. Tang, and J.A. Pietenpol. 2010. ISG20L1 is a p53 family target gene that modulates genotoxic stress-induced autophagy. *Mol Cancer*. 9:95.

El-Deiry, W.S., T. Tokino, V.E. Velculescu, D.B. Levy, R. Parsons, J.M. Trent, D. Lin, W.E. Mercer, K.W. Kinzler, and B. Vogelstein. 1993. WAF1, a potential mediator of p53 tumor suppression. *Cell*. 75:817-825.

Feng, Z., H. Zhang, A.J. Levine, and S. Jin. 2005. The coordinate regulation of the p53 and mTOR pathways in cells. *Proc Natl Acad Sci U S A*. 102:8204-8209.

Fernandez-Salas, E., K.S. Suh, V.V. Speransky, W.L. Bowers, J.M. Levy, T. Adams, K.R. Pathak, L.E. Edwards, D.D. Hayes, C. Cheng, A.C. Steven, W.C. Weinberg, and S.H. Yuspa. 2002. mtCLIC/CLIC4, an organellular chloride channel protein, is increased by DNA damage and participates in the apoptotic response to p53. *Mol Cell Biol*. 22:3610-3620.

Fields, S., and S.K. Jang. 1990. Presence of a potent transcription activating sequence in the p53 protein. *Science*. 249:1046-1049.

Flaman, J.M., T. Frebourg, V. Moreau, F. Charbonnier, C. Martin, P. Chappuis, A.P. Sappino, I.M. Limacher, L. Bron, J. Benhattar, and et al. 1995. A simple p53 functional assay for screening cell lines, blood, and tumors. *Proc Natl Acad Sci U S A*. 92:3963-3967.

Fleury, C., B. Mignotte, and J.L. Vayssiere. 2002. Mitochondrial reactive oxygen species in cell death signaling. *Biochimie*. 84:131-141.

Flores, E.R., S. Sengupta, J.B. Miller, J.J. Newman, R. Bronson, D. Crowley, A. Yang, F. McKeon, and T. Jacks. 2005. Tumor predisposition in mice mutant for p63 and p73: evidence for broader tumor suppressor functions for the p53 family. *Cancer Cell*. 7:363-373.

Fortin, A., S.P. Cregan, J.G. MacLaurin, N. Kushwaha, E.S. Hickman, C.S. Thompson, A. Hakim, P.R. Albert, F. Cecconi, K. Helin, D.S. Park, and R.S. Slack. 2001.

APAF1 is a key transcriptional target for p53 in the regulation of neuronal cell death. *J Cell Biol*. 155:207-216.

Fortuna, M., M.J. Sousa, M. Corte-Real, C. Leao, A. Salvador, and F. Sansonetty. 2001. Cell cycle analysis of yeasts. *Current protocols in cytometry / editorial board, J. Paul Robinson, managing editor ... [et al.]*. Chapter 11:Unit 11 13.

Fritsche, M., C. Haessler, and G. Brandner. 1993. Induction of nuclear accumulation of the tumor-suppressor protein p53 by DNA-damaging agents. *Oncogene*. 8:307-318.

Fukazawa, T., T. Fujiwara, Y. Morimoto, J. Shao, M. Nishizaki, Y. Kadowaki, A. Hizuta, L.B. Owen-Schaub, J.A. Roth, and N. Tanaka. 1999. Differential involvement of the CD95 (Fas/APO-1) receptor/ligand system on apoptosis induced by the wild-type p53 gene transfer in human cancer cells. *Oncogene*. 18:2189-2199.

George, M.D., M. Baba, S.V. Scott, N. Mizushima, B.S. Garrison, Y. Ohsumi, and D.J. Klionsky. 2000. Apg5p functions in the sequestration step in the cytoplasm-to-vacuole targeting and macroautophagy pathways. *Mol Biol Cell*. 11:969-982.

Gilkes, D.M., Y. Pan, D. Coppola, T. Yeatman, G.W. Reuther, and J. Chen. 2008. Regulation of MDMX expression by mitogenic signaling. *Mol Cell Biol*. 28:1999-2010.

Glickman, M.H., and A. Ciechanover. 2002. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiol Rev*. 82:373-428.

Gong, J.G., A. Costanzo, H.Q. Yang, G. Melino, W.G. Kaelin, Jr., M. Levrero, and J.Y. Wang. 1999. The tyrosine kinase c-Abl regulates p73 in apoptotic response to cisplatin-induced DNA damage. *Nature*. 399:806-809.

Grob, T.J., U. Novak, C. Maisse, D. Barcaroli, A.U. Luthi, F. Pirnia, B. Hugli, H.U. Graber, V. De Laurenzi, M.F. Fey, G. Melino, and A. Tobler. 2001. Human delta Np73 regulates a dominant negative feedback loop for TAp73 and p53. *Cell Death Differ*. 8:1213-1223.

Gu, H., X. Wang, S. Rao, J. Wang, J. Zhao, F.L. Ren, R. Mu, Y. Yang, Q. Qi, W. Liu, N. Lu, H. Ling, Q. You, and Q. Guo. 2008. Gambogic acid mediates apoptosis as a p53 inducer through down-regulation of mdm2 in wild-type p53-expressing cancer cells. *Mol Cancer Ther*. 7:3298-3305.

Gudkov, A.V., and E.A. Komarova. 2005. Prospective therapeutic applications of p53 inhibitors. *Biochem and Biophys Res Commun*. 331:726-736.

Hadj Amor, I.Y., K. Smaoui, I. Chaabene, I. Mabrouk, L. Djemal, H. Elleuch, M. Allouche, R. Mokdad-Gargouri, and A. Gargouri. 2008. Human p53 induces cell death and downregulates thioredoxin expression in Saccharomyces cerevisiae. *FEMS Yeast Res*. 8:1254-1262.

Harmes, D.C., E. Bresnick, E.A. Lubin, J.K. Watson, K.E. Heim, J.C. Curtin, A.M. Suskind, J. Lamb, and J. DiRenzo. 2003. Positive and negative regulation of deltaN-p63 promoter activity by p53 and deltaN-p63-alpha contributes to differential regulation of p53 target genes. *Oncogene*. 22:7607-7616.

Harms, K., S. Nozell, and X. Chen. 2004. The common and distinct target genes of the p53 family transcription factors. *Cell Mol Life Sci*. 61:822-842.

Haupt, Y., S. Rowan, E. Shaulian, K.H. Vousden, and M. Oren. 1995. Induction of apoptosis in HeLa cells by trans-activation-deficient p53. *Genes Dev*. 9:2170-2183.

Heng, Y.W., and C.G. Koh. 2010. Actin cytoskeleton dynamics and the cell division cycle. *Int J Biochem Cell Biol*. 42:1622-1633.

Hermeking, H., C. Lengauer, K. Polyak, T.C. He, L. Zhang, S. Thiagalingam, K.W. Kinzler, and B. Vogelstein. 1997. 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. *Mol Cell*. 1:3-11.

Hollstein, M., B. Shomer, M. Greenblatt, T. Soussi, E. Hovig, R. Montesano, and C.C. Harris. 1996. Somatic point mutations in the p53 gene of human tumors and cell lines: updated compilation. *Nucleic Acids Res*. 24:141-146.

Hollstein, M., D. Sidransky, B. Vogelstein, and C.C. Harris. 1991. p53 mutations in human cancers. *Science*. 253:49-53.

Huang, P.I., J.F. Chang, D.H. Kirn, and T.C. Liu. 2009. Targeted genetic and viral therapy for advanced head and neck cancers. *Drug Discov Today*. 14:570-578.

Inga, A., and M.A. Resnick. 2001. Novel human p53 mutations that are toxic to yeast can enhance transactivation of specific promoters and reactivate tumor p53 mutants. *Oncogene*. 20:3409-3419.

Ishioka, C., T. Frebourg, Y.X. Yan, M. Vidal, S.H. Friend, S. Schmidt, and R. Iggo. 1993. Screening patients for heterozygous p53 mutations using a functional assay in yeast. *Nat Genet*. 5:124-129.

Issaeva, N., P. Bozko, M. Enge, M. Protopopova, L.G. Verhoef, M. Masucci, A. Pramanik, and G. Selivanova. 2004. Small molecule RITA binds to p53, blocks p53-HDM-2 interaction and activates p53 function in tumors. *Nat Med*. 10:1321-1328.

Ito, H., Y. Fukuda, K. Murata, and A. Kimura. 1983. Transformation of intact yeast cells treated with alkali cations. *J Bacteriol*. 153:163-168.

Kadakia, M., C. Slader, and S.J. Berberich. 2001. Regulation of p63 function by Mdm2 and MdmX. *DNA Cell Biol*. 20:321-330.

Kaghad, M., H. Bonnet, A. Yang, L. Creancier, J.C. Biscan, A. Valent, A. Minty, P. Chalon, J.M. Lelias, X. Dumont, P. Ferrara, F. McKeon, and D. Caput. 1997. Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. *Cell*. 90:809-819.

Kallen, J., A. Goepfert, A. Blechschmidt, A. Izaac, M. Geiser, G. Tavares, P. Ramage, P. Furet, K. Masuya, and J. Lisztwan. 2009. Crystal Structures of Human MdmX (HdmX) in Complex with p53 Peptide Analogues Reveal Surprising Conformational Changes. *J Biol Chem*. 284:8812-8821.

Kametaka, S., A. Matsuura, Y. Wada, and Y. Ohsumi. 1996. Structural and functional analyses of APG5, a gene involved in autophagy in yeast. *Gene*. 178:139-143.

Kaomongkolgit, R., N. Chaisomboon, and P. Pavasant. 2011. Apoptotic effect of alpha-mangostin on head and neck squamous carcinoma cells. *Arch Oral Biol*. 56:483-490.

Kartasheva, N.N., A. Contente, C. Lenz-Stoppler, J. Roth, and M. Dobbelstein. 2002. p53 induces the expression of its antagonist p73 Delta N, establishing an autoregulatory feedback loop. *Oncogene*. 21:4715-4727.

Kawai, H., D. Wiederschain, and Z.M. Yuan. 2003. Critical contribution of the MDM2 acidic domain to p53 ubiquitination. *Mol Cell Biol*. 23:4939-4947.

Kimura, T., M. Gotoh, Y. Nakamura, and H. Arakawa. 2003. hCDC4b, a regulator of cyclin E, as a direct transcriptional target of p53. *Cancer Sci*. 94:431-436.

Kirisako, T., M. Baba, N. Ishihara, K. Miyazawa, M. Ohsumi, T. Yoshimori, T. Noda, and Y. Ohsumi. 1999. Formation process of autophagosome is traced with Apg8/Aut7p in yeast. *J Cell Biol*. 147:435-446.

Kishimoto, T., and E. Okumura. 1997. In vivo regulation of the entry into M-phase: initial activation and nuclear translocation of cyclin B/Cdc2. *Prog Cell Cycle Res*. 3:241-249.

Kissova, I., M. Deffieu, S. Manon, and N. Camougrand. 2004. Uth1p is involved in the autophagic degradation of mitochondria. *J Biol Chem*. 279:39068-39074.

Koff, A., A. Giordano, D. Desai, K. Yamashita, J.W. Harper, S. Elledge, T. Nishimoto, D.O. Morgan, B.R. Franza, and J.M. Roberts. 1992. Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle. *Science*. 257:1689-1694.

Kommagani, R., M.K. Leonard, S. Lewis, R.A. Romano, S. Sinha, and M.P. Kadakia. 2009. Regulation of VDR by deltaNp63alpha is associated with inhibition of cell invasion. *J Cell Sci*. 122:2828-2835.

Kovalev, S., N. Marchenko, S. Swendeman, M. LaQuaglia, and U.M. Moll. 1998. Expression level, allelic origin, and mutation analysis of the p73 gene in neuroblastoma tumors and cell lines. *Cell Growth Differ*. 9:897-903.

Kovvali, G.K., B. Mehta, C.B. Epstein, and S.G. Lutzker. 2001. Identification of partial loss of function p53 gene mutations utilizing a yeast-based functional assay. *Nucleic Acids Res*. 29:E28.

Krause, K., M. Wasner, W. Reinhard, U. Haugwitz, C.L. Dohna, J. Mossner, and K. Engeland. 2000. The tumour suppressor protein p53 can repress transcription of cyclin B. *Nucleic Acids Res*. 28:4410-4418.

Kravchenko, J.E., G.V. Ilyinskaya, P.G. Komarov, L.S. Agapova, D.V. Kochetkov, E. Strom, E.I. Frolova, I. Kovriga, A.V. Gudkov, E. Feinstein, and P.M. Chumakov. 2008. small molecule RETRA suppresses mutant p53-bearing cancer cells through a p73-dependent salvage pathway. *Proc Natl Acad Sci U S A*. 105:6302-6307.

Kussie, P.H., S. Gorina, V. Marechal, B. Elenbaas, J. Moreau, A.J. Levine, and N.P. Pavletich. 1996. Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. *Science*. 274:948-953.

Lane, D.P. 1992. Cancer. p53, guardian of the genome. *Nature*. 358:15-16.

Lane, D.P., and L.V. Crawford. 1979. T antigen is bound to a host protein in SV40-transformed cells. *Nature*. 278:261-263.

Laurie, N.A., S.L. Donovan, C.S. Shih, J. Zhang, N. Mills, C. Fuller, A. Teunisse, S. Lam, Y. Ramos, A. Mohan, D. Johnson, M. Wilson, C. Rodriguez-Galindo, M. Quarto, S. Francoz, S.M. Mendrysa, R.K. Guy, J.C. Marine, A.G. Jochemsen, and M.A. Dyer. 2006. Inactivation of the p53 pathway in retinoblastoma. *Nature*. 444:61-66.

Leao, M., C. Pereira, A. Bisio, Y. Ciribilli, A.M. Paiva, N. Machado, A. Palmeira, M.X. Fernandes, E. Sousa, M. Pinto, A. Inga, and L. Saraiva. 2013. Discovery of a new small molecule inhibitor of p53-MDM2 interaction using a yeast-based approach. *Biochem Pharmacol*. 85:1234-1245.

Lee, J.T., and W. Gu. 2010. The multiple levels of regulation by p53 ubiquitination. *Cell Death Differ*. 17:86-92.

Lenos, K., and A.G. Jochemsen. 2011. Functions of MDMX in the modulation of the p53-response. *J Biomed Biotechnol*. 2011:876173.

Li, B., Q. Cheng, Z. Li, and J. Chen. 2010. p53 inactivation by MDM2 and MDMX negative feedback loops in testicular germ cell tumors. *Cell Cycle*. 9:1411-1420.

Li, H., H. Zhu, C.J. Xu, and J. Yuan. 1998. Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. *Cell*. 94:491-501.

Liedtke, C., N. Groger, M.P. Manns, and C. Trautwein. 2003. The human caspase-8 promoter sustains basal activity through SP1 and ETS-like transcription factors and can be up-regulated by a p53-dependent mechanism. *J Biol Chem*. 278:27593-27604.

Linke, K., P.D. Mace, C.A. Smith, D.L. Vaux, J. Silke, and C.L. Day. 2008. Structure of the MDM2/MDMX RING domain heterodimer reveals dimerization is required for their ubiquitylation in trans. *Cell Death Differ*. 15:841-848.

Linzer, D.I., and A.J. Levine. 1979. Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell*. 17:43-52.

Little, N.A., and A.G. Jochemsen. 2001. Hdmx and Mdm2 can repress transcription activation by p53 but not by p63. *Oncogene*. 20:4576-4580.

Liu, G., S. Nozell, H. Xiao, and X. Chen. 2004. DeltaNp73beta is active in transactivation and growth suppression. *Mol Cell Biol*. 24:487-501.

Lopez-Lazaro, M. 2002. Flavonoids as anticancer agents: structure-activity relationship study. *Curr Med Chem Anticancer Agents*. 2:691-714.

MacFarlane, M. 2003. TRAIL-induced signalling and apoptosis. *Toxicol Lett*. 139:89-97.

MacLachlan, T.K., and W.S. El-Deiry. 2002. Apoptotic threshold is lowered by p53 transactivation of caspase-6. *Proc Natl Acad Sci U S A*. 99:9492-9497.

Madeo, F., E. Frohlich, M. Ligr, M. Grey, S.J. Sigrist, D.H. Wolf, and K.U. Frohlich. 1999. Oxygen stress: a regulator of apoptosis in yeast. *J Cell Biol*. 145:757-767.

Malkin, D. 1993. p53 and the Li-Fraumeni syndrome. *Cancer Genet Cytogenet*. 66:83-92.

Manfredi, J.J. 2003. p53 and apoptosis: it's not just in the nucleus anymore. *Mol Cell*. 11:552-554.

Manfredi, J.J. 2010. The Mdm2-p53 relationship evolves: Mdm2 swings both ways as an oncogene and a tumor suppressor. *Genes Dev*. 24:1580-1589.

Marchenko, N.D., S. Wolff, S. Erster, K. Becker, and U.M. Moll. 2007. Monoubiquitylation promotes mitochondrial p53 translocation. *EMBO J*. 26:923-934.

Marchenko, N.D., A. Zaika, and U.M. Moll. 2000. Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling. *J Biol Chem*. 275:16202-16212.

Marquez-Valadez, B., R. Lugo-Huitron, V. Valdivia-Cerda, L.R. Miranda-Ramirez, V. Perez-De La Cruz, O. Gonzalez-Cuahutencos, I. Rivero-Cruz, R. Mata, A. Santamaria, and J. Pedraza-Chaverri. 2009. The natural xanthone alpha-mangostin reduces oxidative damage in rat brain tissue. *Nutr Neurosci*. 12:35-42.

Martinez, J.D. 2010. Restoring p53 tumor suppressor activity as an anticancer therapeutic strategy. *Future Oncol*. 6:1857-1862.

Martins, C.P., L. Brown-Swigart, and G.I. Evan. 2006. Modeling the therapeutic efficacy of p53 restoration in tumors. *Cell*. 127:1323-1334.

Maslon, M.M., and T.R. Hupp. 2010. Drug discovery and mutant p53. *Trends Cell Biol*. 20:542-555.

Matsuura, A., M. Tsukada, Y. Wada, and Y. Ohsumi. 1997. Apg1p, a novel protein kinase required for the autophagic process in Saccharomyces cerevisiae. *Gene*. 192:245-250.

Meulmeester, E., R. Frenk, R. Stad, P. de Graaf, J.C. Marine, K.H. Vousden, and A.G. Jochemsen. 2003. Critical role for a central part of Mdm2 in the ubiquitylation of p53. *Mol Cell Biol*. 23:4929-4938.

Mihara, M., S. Erster, A. Zaika, O. Petrenko, T. Chittenden, P. Pancoska, and U.M. Moll. 2003. p53 has a direct apoptogenic role at the mitochondria. *Mol Cell*. 11:577-590.

Mills, A.A., B. Zheng, X.J. Wang, H. Vogel, D.R. Roop, and A. Bradley. 1999. p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature*. 398:708-713.

Miyashita, T., and J.C. Reed. 1995. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell*. 80:293-299.

Mizushima, N., B. Levine, A.M. Cuervo, and D.J. Klionsky. 2008. Autophagy fights disease through cellular self-digestion. *Nature*. 451:1069-1075.

Momand, J., A. Villegas, and V.A. Belyi. 2011. The evolution of MDM2 family genes. *Gene*. 486:23-30.

Momand, J., G.P. Zambetti, D.C. Olson, D. George, and A.J. Levine. 1992. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell*. 69:1237-1245.

Monasterio, A., M.C. Urdaci, I.V. Pinchuk, N. Lopez-Moratalla, and J.J. Martinez-Irujo. 2004. Flavonoids induce apoptosis in human leukemia U937 cells through caspase-and caspase-calpain-dependent pathways. *Nutr Cancer*. 50:90-100.

Monks, A., D. Scudiero, P. Skehan, R. Shoemaker, K. Paull, D. Vistica, C. Hose, J. Langley, P. Cronise, A. Vaigro-Wolff, and et al. 1991. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J Natl Cancer Inst*. 83:757-766.

Morgan, D.O. 1995. Principles of CDK regulation. *Nature*. 374:131-134.

Nakagawa, T., M. Takahashi, T. Ozaki, K. Watanabe Ki, S. Todo, H. Mizuguchi, T. Hayakawa, and A. Nakagawara. 2002. Autoinhibitory regulation of p73 by Delta Np73 to modulate cell survival and death through a p73-specific target element within the Delta Np73 promoter. *Mol Cell Biol*. 22:2575-2585.

Nakano, K., and K.H. Vousden. 2001. PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell*. 7:683-694.

Neves, M.P., S. Cravo, R.T. Lima, M.H. Vasconcelos, M.S. Nascimento, A.M. Silva, M. Pinto, H. Cidade, and A.G. Correa. 2012a. Solid-phase synthesis of 2'-hydroxychalcones. Effects on cell growth inhibition, cell cycle and apoptosis of human tumor cell lines. *Bioorg Med Chem*. 20:25-33.

Neves, M.P., R.T. Lima, K. Choosang, P. Pakkong, M. de Sao Jose Nascimento, M.H. Vasconcelos, M. Pinto, A.M. Silva, and H. Cidade. 2012b. Synthesis of a natural chalcone and its prenyl analogs--evaluation of tumor cell growth-inhibitory activities, and effects on cell cycle and apoptosis. *Chem Biodivers*. 9:1133-1143.

Newmeyer, D.D., and S. Ferguson-Miller. 2003. Mitochondria: releasing power for life and unleashing the machineries of death. *Cell*. 112:481-490.

Nigro, J.M., R. Sikorski, S.I. Reed, and B. Vogelstein. 1992. Human p53 and CDC2Hs genes combine to inhibit the proliferation of Saccharomyces cerevisiae. *Mol Cell Biol*. 12:1357-1365.

Noda, T., and Y. Ohsumi. 1998. Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. *J Biol Chem*. 273:3963-3966.

Nurse, P. 2000. A long twentieth century of the cell cycle and beyond. *Cell*. 100:71-78.

Oda, E., R. Ohki, H. Murasawa, J. Nemoto, T. Shibue, T. Yamashita, T. Tokino, T. Taniguchi, and N. Tanaka. 2000a. Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science*. 288:1053-1058.

Oda, K., H. Arakawa, T. Tanaka, K. Matsuda, C. Tanikawa, T. Mori, H. Nishimori, K. Tamai, T. Tokino, Y. Nakamura, and Y. Taya. 2000b. p53AIP1, a potential mediator of p53-dependent apoptosis, and its regulation by Ser-46-phosphorylated p53. *Cell*. 102:849-862.

Ohki, R., J. Nemoto, H. Murasawa, E. Oda, J. Inazawa, N. Tanaka, and T. Taniguchi. 2000. Reprimo, a new candidate mediator of the p53-mediated cell cycle arrest at the G2 phase. *J Biol Chem*. 275:22627-22630.

Olsson, A., C. Manzl, A. Strasser, and A. Villunger. 2007. How important are post-translational modifications in p53 for selectivity in target-gene transcription and tumour suppression? *Cell Death Differ*. 14:1561-1575.

Ongkeko, W.M., X.Q. Wang, W.Y. Siu, A.W. Lau, K. Yamashita, A.L. Harris, L.S. Cox, and R.Y. Poon. 1999. MDM2 and MDMX bind and stabilize the p53-related protein p73. *Curr Biol*. 9:829-832.

Owen-Schaub, L.B., W. Zhang, J.C. Cusack, L.S. Angelo, S.M. Santee, T. Fujiwara, J.A. Roth, A.B. Deisseroth, W.W. Zhang, E. Kruzel, and et al. 1995. Wild-type human p53 and a temperature-sensitive mutant induce Fas/APO-1 expression. *Mol Cell Biol*. 15:3032-3040.

Park, B.J., S.J. Lee, J.I. Kim, S.J. Lee, C.H. Lee, S.G. Chang, J.H. Park, and S.G. Chi. 2000. Frequent alteration of p63 expression in human primary bladder carcinomas. *Cancer Res*. 60:3370-3374.

Pedraza-Chaverri, J., N. Cardenas-Rodriguez, M. Orozco-Ibarra, and J.M. Perez-Rojas. 2008. Medicinal properties of mangosteen (Garcinia mangostana). *Food Chem Toxicol*. 46:3227-3239.

Pei, D., Y. Zhang, and J. Zheng. 2012. Regulation of p53: a collaboration between Mdm2 and Mdmx. *Oncotarget*. 3:228-235.

Pereira, C., I. Coutinho, J. Soares, C. Bessa, M. Leao, and L. Saraiva. 2012a. New insights into cancer-related proteins provided by the yeast model. *FEBS J*. 279:697-712.

Pereira, C., M. Leao, J. Soares, C. Bessa, and L. Saraiva. 2012b. New therapeutic strategies for cancer and neurodegeneration emerging from yeast cell-based systems. *Curr Pharm Des*. 18:4223-4235.

Perez, C.A., J. Ott, D.J. Mays, and J.A. Pietenpol. 2007. p63 consensus DNA-binding site: identification, analysis and application into a p63MH algorithm. *Oncogene*. 26:7363-7370.

Petre-Lazar, B., G. Livera, S.G. Moreno, E. Trautmann, C. Duquenne, V. Hanoux, R. Habert, and H. Coffigny. 2007. The role of p63 in germ cell apoptosis in the developing testis. *J Cell Physiol*. 210:87-98.

Phillips, A., A. Teunisse, S. Lam, K. Lodder, M. Darley, M. Emaduddin, A. Wolf, J. Richter, J. de Lange, M. Verlaan-de Vries, K. Lenos, A. Bohnke, F. Bartel, J.P. Blaydes, and A.G. Jochemsen. 2010. HDMX-L is expressed from a functional p53-responsive promoter in the first intron of the HDMX gene and participates in an autoregulatory feedback loop to control p53 activity. *J Biol Chem*. 285:29111-29127.

Popowicz, G.M., A. Czarna, U. Rothweiler, A. Szwagierczak, M. Krajewski, L. Weber, and T.A. Holak. 2007. Molecular basis for the inhibition of p53 by Mdmx. *Cell Cycle*. 6:2386-2392.

Reed, D., Y. Shen, A.A. Shelat, L.A. Arnold, A.M. Ferreira, F. Zhu, N. Mills, D.C. Smithson, C.A. Regni, D. Bashford, S.A. Cicero, B.A. Schulman, A.G. Jochemsen, R.K. Guy, and M.A. Dyer. 2010. Identification and characterization of the first small molecule inhibitor of MDMX. *J Biol Chem*. 285:10786-10796.

Reinhardt, H.C., and B. Schumacher. 2012. The p53 network: cellular and systemic DNA damage responses in aging and cancer. *Trends Genet.* 28:128-136.

Ries, S., C. Biederer, D. Woods, O. Shifman, S. Shirasawa, T. Sasazuki, M. McMahon, M. Oren, and F. McCormick. 2000. Opposing effects of Ras on p53: transcriptional activation of mdm2 and induction of p19ARF. *Cell*. 103:321-330.

Rippin, T.M., V.J. Bykov, S.M. Freund, G. Selivanova, K.G. Wiman, and A.R. Fersht. 2002. Characterization of the p53-rescue drug CP-31398 in vitro and in living cells. *Oncogene*. 21:2119-2129.

Rong, J.J., R. Hu, Q. Qi, H.Y. Gu, Q. Zhao, J. Wang, R. Mu, Q.D. You, and Q.L. Guo. 2009. Gambogic acid down-regulates MDM2 oncogene and induces p21(Waf1/CIP1) expression independent of p53. *Cancer Lett*. 284:102-112.

Rutkowski, R., K. Hofmann, and A. Gartner. 2010. Phylogeny and function of the invertebrate p53 superfamily. *Cold Spring Harb Perspect Biol*. 2:a001131.

Ryan, K.M. 2011. p53 and autophagy in cancer: guardian of the genome meets guardian of the proteome. *Eur J Cancer*. 47:44-50.

Sagot, I., B. Pinson, B. Salin, and B. Daignan-Fornier. 2006. Actin bodies in yeast quiescent cells: an immediately available actin reserve? *Mol Biol Cell*. 17:4645-4655.

Saraiva, L., R.D. Silva, G. Pereira, J. Goncalves, and M. Corte-Real. 2006. Specific modulation of apoptosis and Bcl-xL phosphorylation in yeast by distinct mammalian protein kinase C isoforms. *J Cell Sci*. 119:3171-3181.

Sax, J.K., P. Fei, M.E. Murphy, E. Bernhard, S.J. Korsmeyer, and W.S. El-Deiry. 2002. BID regulation by p53 contributes to chemosensitivity. *Nat Cell Biol*. 4:842-849.

Sayan, A.E., J.P. Roperch, B.S. Sayan, M. Rossi, M.J. Pinkoski, R.A. Knight, A.E. Willis, and G. Melino. 2007. Generation of DeltaTAp73 proteins by translation from a putative internal ribosome entry site. *Ann N Y Acad Sci*. 1095:315-324.

Scharer, E., and R. Iggo. 1992. Mammalian p53 can function as a transcription factor in yeast. *Nucleic Acids Res*. 20:1539-1545.

Secchiero, P., E. Rimondi, M.G. di Iasio, R. Voltan, A. Gonelli, and G. Zauli. 2012. Activation of the p53 pathway induces alpha-smooth muscle actin expression in both myeloid leukemic cells and normal macrophages. *J Cell Physiol*. 227:1829-1837.

Seo, Y.W., J.N. Shin, K.H. Ko, J.H. Cha, J.Y. Park, B.R. Lee, C.W. Yun, Y.M. Kim, D.W. Seol, D.W. Kim, X.M. Yin, and T.H. Kim. 2003. The molecular mechanism of Noxa-induced mitochondrial dysfunction in p53-mediated cell death. *J Biol Chem*. 278:48292-48299.

Serber, Z., H.C. Lai, A. Yang, H.D. Ou, M.S. Sigal, A.E. Kelly, B.D. Darimont, P.H. Duijf, H. Van Bokhoven, F. McKeon, and V. Dotsch. 2002. A C-terminal inhibitory domain controls the activity of p63 by an intramolecular mechanism. *Mol Cell Biol*. 22:8601-8611.

Shadfan, M., V. Lopez-Pajares, and Z.M. Yuan. 2012. MDM2 and MDMX: Alone and together in regulation of p53. *Transl Cancer Res*. 1:88-89.

Shangary, S., and S. Wang. 2009. small molecule inhibitors of the MDM2-p53 protein-protein interaction to reactivate p53 function: a novel approach for cancer therapy. *Annu Rev Pharmacol Toxicol*. 49:223-241.

Shibata, M.A., M. Iinuma, J. Morimoto, H. Kurose, K. Akamatsu, Y. Okuno, Y. Akao, and Y. Otsuki. 2011. alpha-Mangostin extracted from the pericarp of the mangosteen (Garcinia mangostana Linn) reduces tumor growth and lymph node metastasis in an immunocompetent xenograft model of metastatic mammary cancer carrying a p53 mutation. *BMC Med*. 9:69.

Smardova, J., J. Smarda, and J. Koptikova. 2005. Functional analysis of p53 tumor suppressor in yeast. *Differentiation*. 73:261-277.

Smits, V.A., R. Klompmaker, T. Vallenius, G. Rijksen, T.P. Makela, and R.H. Medema. 2000. p21 inhibits Thr161 phosphorylation of Cdc2 to enforce the G2 DNA damage checkpoint. *J Biol Chem*. 275:30638-30643.

Sniezek, J.C., K.E. Matheny, M.D. Westfall, and J.A. Pietenpol. 2004. Dominant negative p63 isoform expression in head and neck squamous cell carcinoma. *Laryngoscope*. 114:2063-2072.

Stevens, C., and N.B. La Thangue. 2003. E2F and cell cycle control: a double-edged sword. *Arch Biochem Biophys*. 412:157-169.

Stiewe, T., C.C. Theseling, and B.M. Putzer. 2002. Transactivation-deficient Delta TA-p73 inhibits p53 by direct competition for DNA binding: implications for tumorigenesis. *J Biol Chem*. 277:14177-14185.

Sugars, K.L., V. Budhram-Mahadeo, G. Packham, and D.S. Latchman. 2001. A minimal Bcl-x promoter is activated by Brn-3a and repressed by p53. *Nucleic Acids Res*. 29:4530-4540.

Sui, X., L. Jin, X. Huang, S. Geng, C. He, and X. Hu. 2011. p53 signaling and autophagy in cancer: a revolutionary strategy could be developed for cancer treatment. *Autophagy*. 7:565-571.

Takimoto, R., and W.S. El-Deiry. 2000. Wild-type p53 transactivates the KILLER/DR5 gene through an intronic sequence-specific DNA-binding site. *Oncogene*. 19:1735-1743.

Tasdemir, E., M. Chiara Maiuri, E. Morselli, A. Criollo, M. D'Amelio, M. Djavaheri-Mergny, F. Cecconi, N. Tavernarakis, and G. Kroemer. 2008. A dual role of p53 in the control of autophagy. *Autophagy*. 4:810-814.

Taylor, W.R., A.H. Schonthal, J. Galante, and G.R. Stark. 2001. p130/E2F4 binds to and represses the cdc2 promoter in response to p53. *J Biol Chem*. 276:1998-2006.

Thanos, C.D., and J.U. Bowie. 1999. p53 Family members p63 and p73 are SAM domain-containing proteins. *Protein Sci*. 8:1708-1710.

Tomasini, R., K. Tsuchihara, M. Wilhelm, M. Fujitani, A. Rufini, C.C. Cheung, F. Khan, A. Itie-Youten, A. Wakeham, M.S. Tsao, J.L. Iovanna, J. Squire, I. Jurisica, D. Kaplan, G. Melino, A. Jurisicova, and T.W. Mak. 2008. TAp73 knockout shows genomic instability with infertility and tumor suppressor functions. *Genes Dev*. 22:2677-2691.

Tomkova, K., W. El-Rifai, A. Vilgelm, M.C. Kelly, T.C. Wang, and A.I. Zaika. 2006. The gastrin gene promoter is regulated by p73 isoforms in tumor cells. *Oncogene*. 25:6032-6036.

Uldrijan, S., W.J. Pannekoek, and K.H. Vousden. 2007. An essential function of the extreme C-terminus of MDM2 can be provided by MDMX. *EMBO J*. 26:102-112.

Urist, M.J., C.J. Di Como, M.L. Lu, E. Charytonowicz, D. Verbel, C.P. Crum, T.A. Ince, F.D. McKeon, and C. Cordon-Cardo. 2002. Loss of p63 expression is associated with tumor progression in bladder cancer. *Am J Pathol*. 161:1199-1206.

Utrera, R., L. Collavin, D. Lazarevic, D. Delia, and C. Schneider. 1998. A novel p53-inducible gene coding for a microtubule-localized protein with G2-phase-specific expression. *EMBO J*. 17:5015-5025.

Valentin-Vega, Y.A., J.A. Barboza, G.P. Chau, A.K. El-Naggar, and G. Lozano. 2007. High levels of the p53 inhibitor MDM4 in head and neck squamous carcinomas. *Hum Pathol*. 38:1553-1562.

van Bokhoven, H., and H.G. Brunner. 2002. Splitting p63. *Am J Hum Genet*. 71:1-13.

Vassilev, L.T. 2004. small molecule antagonists of p53-MDM2 binding: research tools and potential therapeutics. *Cell Cycle*. 3:419-421.

Vassilev, L.T., B.T. Vu, B. Graves, D. Carvajal, F. Podlaski, Z. Filipovic, N. Kong, U. Kammlott, C. Lukacs, C. Klein, N. Fotouhi, and E.A. Liu. 2004. In vivo activation of the p53 pathway by small molecule antagonists of MDM2. *Science*. 303:844-848.

Ventura, A., D.G. Kirsch, M.E. McLaughlin, D.A. Tuveson, J. Grimm, L. Lintault, J. Newman, E.E. Reczek, R. Weissleder, and T. Jacks. 2007. Restoration of p53 function leads to tumour regression in vivo. *Nature*. 445:661-665.

Vichai, V., and K. Kirtikara. 2006. Sulforhodamine B colorimetric assay for cytotoxicity screening. *Nat Protoc*. 1:1112-1116.

Vikhanskaya, F., W.H. Toh, I. Dulloo, Q. Wu, L. Boominathan, H.H. Ng, K.H. Vousden, and K. Sabapathy. 2007. p73 supports cellular growth through c-Jun-dependent AP-1 transactivation. *Nat Cell Biol*. 9:698-705.

Vilgelm, A., W. El-Rifai, and A. Zaika. 2008. Therapeutic prospects for p73 and p63: rising from the shadow of p53. *Drug Resist Updat*. 11:152-163.

Wade, M., Y.C. Li, and G.M. Wahl. 2013. MDM2, MDMX and p53 in oncogenesis and cancer therapy. *Nat Rev Cancer*. 13:83-96.

Wade, M., and G.M. Wahl. 2009. Targeting Mdm2 and Mdmx in cancer therapy: better living through medicinal chemistry? *Mol Cancer Res : MCR*. 7:1-11.

Wade, M., Y.V. Wang, and G.M. Wahl. 2010. The p53 orchestra: Mdm2 and Mdmx set the tone. *Trends Cell Biol*. 20:299-309.

Wager, M., J. Guilhot, J.L. Blanc, S. Ferrand, S. Milin, B. Bataille, F. Lapierre, S. Denis, T. Chantereau, C.J. Larsen, and L. Karayan-Tapon. 2006. Prognostic value of increase in transcript levels of Tp73 DeltaEx2-3 isoforms in low-grade glioma patients. *Br J Cancer*. 95:1062-1069.

Wang, W., R. Takimoto, F. Rastinejad, and W.S. El-Deiry. 2003. Stabilization of p53 by CP-31398 inhibits ubiquitination without altering phosphorylation at serine 15 or 20 or MDM2 binding. *Mol Cell Biol*. 23:2171-2181.

Wang, X., T. Arooz, W.Y. Siu, C.H.S. Chiu, A. Lau, K. Yamashita, and R.Y.C. Poon. 2001. MDM2 and MDMX can interact differently with ARF and members of the p53 family. *FEBS Lett*. 490:202-208.

Weber, A., U. Bellmann, F. Bootz, C. Wittekind, and A. Tannapfel. 2002. Expression of p53 and its homologues in primary and recurrent squamous cell carcinomas of the head and neck. *Int J Cancer*. 99:22-28.

Wei, C.L., Q. Wu, V.B. Vega, K.P. Chiu, P. Ng, T. Zhang, A. Shahab, H.C. Yong, Y. Fu, Z. Weng, J. Liu, X.D. Zhao, J.L. Chew, Y.L. Lee, V.A. Kuznetsov, W.K. Sung, L.D. Miller, B. Lim, E.T. Liu, Q. Yu, H.H. Ng, and Y. Ruan. 2006. A global map of p53 transcription-factor binding sites in the human genome. *Cell*. 124:207-219.

Wei, J., E. Zaika, and A. Zaika. 2012. p53 Family: Role of Protein Isoforms in Human Cancer. *J Nucleic Acids*. 2012:687359.

Weinberg, R.A. 1995. The retinoblastoma protein and cell cycle control. *Cell*. 81:323-330.

Wilhelm, M.T., A. Rufini, M.K. Wetzel, K. Tsuchihara, S. Inoue, R. Tomasini, A. Itie-Youten, A. Wakeham, M. Arsenian-Henriksson, G. Melino, D.R. Kaplan, F.D. Miller, and T.W. Mak. 2010. Isoform-specific p73 knockout mice reveal a novel role for delta Np73 in the DNA damage response pathway. *Genes Dev*. 24:549-560.

Wu, G., S. Nomoto, M.O. Hoque, T. Dracheva, M. Osada, C.C. Lee, S.M. Dong, Z. Guo, N. Benoit, Y. Cohen, P. Rechthand, J. Califano, C.S. Moon, E. Ratovitski, J. Jen, D. Sidransky, and B. Trink. 2003. DeltaNp63alpha and TAp63alpha regulate transcription of genes with distinct biological functions in cancer and development. *Cancer Res*. 63:2351-2357.

Wu, X., J.H. Bayle, D. Olson, and A.J. Levine. 1993. The p53-mdm-2 autoregulatory feedback loop. *Genes Dev*. 7:1126-1132.

Xue, W., L. Zender, C. Miething, R.A. Dickins, E. Hernando, V. Krizhanovsky, C. Cordon-Cardo, and S.W. Lowe. 2007. Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature*. 445:656-660.

Yang, A., M. Kaghad, Y. Wang, E. Gillett, M.D. Fleming, V. Dotsch, N.C. Andrews, D. Caput, and F. McKeon. 1998. p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol Cell*. 2:305-316.

Yang, A., N. Walker, R. Bronson, M. Kaghad, M. Oosterwegel, J. Bonnin, C. Vagner, H. Bonnet, P. Dikkes, A. Sharpe, F. McKeon, and D. Caput. 2000. p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. *Nature*. 404:99-103.

Yang, Z., and D.J. Klionsky. 2010. Mammalian autophagy: core molecular machinery and signaling regulation. *Curr Opin Cell Biol*. 22:124-131.

Yorimitsu, T., S. Zaman, J.R. Broach, and D.J. Klionsky. 2007. Protein kinase A and Sch9 cooperatively regulate induction of autophagy in Saccharomyces cerevisiae. *Mol Biol Cell*. 18:4180-4189.

Yousef, A.F., G.W. Xu, M. Mendez, C.J. Brandl, and J.S. Mymryk. 2008. Coactivator requirements for p53-dependent transcription in the yeast Saccharomyces cerevisiae. *Int J Cancer*. 122:942-946.

Yu, J., L. Zhang, P.M. Hwang, K.W. Kinzler, and B. Vogelstein. 2001. PUMA induces the rapid apoptosis of colorectal cancer cells. *Mol Cell*. 7:673-682.

Yuan, Z.M., H. Shioya, T. Ishiko, X. Sun, J. Gu, Y.Y. Huang, H. Lu, S. Kharbanda, R. Weichselbaum, and D. Kufe. 1999. p73 is regulated by tyrosine kinase c-Abl in the apoptotic response to DNA damage. *Nature*. 399:814-817.

Yun, J., H.D. Chae, H.E. Choy, J. Chung, H.S. Yoo, M.H. Han, and D.Y. Shin. 1999. p53 negatively regulates cdc2 transcription via the CCAAT-binding NF-Y transcription factor. *J Biol Chem*. 274:29677-29682.

Zaika, A.I., N. Slade, S.H. Erster, C. Sansome, T.W. Joseph, M. Pearl, E. Chalas, and U.M. Moll. 2002. DeltaNp73, a dominant-negative inhibitor of wild-type p53 and TAp73, is up-regulated in human tumors. *J Exp Med*. 196:765-780.

Zauberman, A., D. Flusberg, Y. Haupt, Y. Barak, and M. Oren. 1995. A functional p53-responsive intronic promoter is contained within the human mdm2 gene. *Nucleic Acids Res*. 23:2584-2592.

Zdzalik, M., K. Pustelny, S. Kedracka-Krok, K. Huben, A. Pecak, B. Wladyka, S. Jankowski, A. Dubin, J. Potempa, and G. Dubin. 2010. Interaction of regulators Mdm2 and Mdmx with transcription factors p53, p63 and p73. *Cell Cycle*. 9:4584-4591.

Zeng, X., L. Chen, C.A. Jost, R. Maya, D. Keller, X. Wang, W.G. Kaelin, Jr., M. Oren, J. Chen, and H. Lu. 1999. MDM2 suppresses p73 function without promoting p73 degradation. *Mol Cell Biol*. 19:3257-3266.

Zetterberg, A., O. Larsson, and K.G. Wiman. 1995. What is the restriction point? *Curr Opin Cell Biol*. 7:835-842.

Zha, J., S. Weiler, K.J. Oh, M.C. Wei, and S.J. Korsmeyer. 2000. Posttranslational N-myristoylation of BID as a molecular switch for targeting mitochondria and apoptosis. *Science*. 290:1761-1765.

Zhan, Q., M.J. Antinore, X.W. Wang, F. Carrier, M.L. Smith, C.C. Harris, and A.J. Fornace, Jr. 1999. Association with Cdc2 and inhibition of Cdc2/Cyclin B1 kinase activity by the p53-regulated protein Gadd45. *Oncogene*. 18:2892-2900.

Zhao, J., Q. Qi, Y. Yang, H.Y. Gu, N. Lu, W. Liu, W. Wang, L. Qiang, L.B. Zhang, Q.D. You, and Q.L. Guo. 2008. Inhibition of alpha(4) integrin mediated adhesion was involved in the reduction of B16-F10 melanoma cells lung colonization in C57BL/6 mice treated with gambogic acid. *Eur J Pharmacol*. 589:127-131.

Zhao, Y., D. Bernard, and S. Wang. 2013. Small Molecule Inhibitors of MDM2-p53 and MDMX-p53 Interactions as New Cancer Therapeutics. *BioDiscovery*. 8:4.

Zhu, J., and X. Chen. 2000. MCG10, a novel p53 target gene that encodes a KH domain RNA-binding protein, is capable of inducing apoptosis and cell cycle arrest in G(2)-M. *Mol Cell Biol*. 20:5602-5618.