“Yeast as a model for the study of human p53”

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1. INTRODUCTION

1.1 S. cerevisiae AS A MODEL ORGANISM

The unicellular yeast S. cerevisiae is one of the most intensively used model organism for studying the principles of microbiology, characterizing biochemical pathways, and understanding the biology of more complex eukaryotic organisms. In fact, many pathways are conserved from yeast to humans, including DNA replication, recombination, and repair; RNA transcription and translation; intracellular trafficking; enzymatic activities of general metabolism, mitochondrial biogenesis, apoptosis and aging. The reasons that make yeast particularly suitable for these studies include its easy genetic manipulation, short generation time, the ability to be maintained in a haploid or diploid state, ease of mutant isolation, a well-defined genetic
system, and a highly versatile DNA transformation system that allow for either the addition of new genes or their deletion.

For all these reasons *S. cerevisiae* has developed as a very useful model organism. In fact, many proteins important in human biology were first discovered by studying their homologs in yeast.

Growth in yeast is synchronized with the growth of the bud, which reaches the size of the mature cell by the time it separates from the parent cell. In rapidly growing yeast cultures, all the cells can be seen to have buds, since bud formation occupies the whole cell cycle. Both mother and daughter cells can initiate bud formation before cell separation has occurred. In yeast cultures growing more slowly, cells lacking buds can be seen, and bud formation only occupies a part of the cell cycle. The cell cycle in yeast normally consists of the following stages – G1, S, G2, and M – which are the normal stages of mitosis.

*S. cerevisiae* was the first eukaryotic genome that was completely sequenced. The genome sequence was released in the public
domain on April 24, 1996. Since then, regular updates have been maintained at the Saccharomyces Genome Database (SGD). This database is a highly annotated and cross-referenced databases for yeast researchers. Another important \textit{S. cerevisiae} database is maintained by the Munich Information Center for Protein Sequences (MIPS). The genome is composed of about 12,156,677 base pairs and 6,275 genes, compactly organized on 16 chromosomes. Only about 5,800 of these are believed to be true functional genes. Yeast is estimated to share about 23\% of its genome with that of humans (Williams N, 1996).

The availability of the \textit{S. cerevisiae} genome sequence and the complete set of deletion mutants has further enhanced the
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power of *S. cerevisiae* as a model for understanding the regulation of eukaryotic cells.
Figure 1.1.1 Picture showing the structure of budding yeast
1.2 APOPTOSIS AND AGING

Apoptosis is a highly regulated cellular suicide program crucial for metazoan. However, disfunction of apoptosis also leads to several diseases (Madeo et al., 2004). Indeed, misregulation of apoptosis can result in human diseases such as neurodegenerative disorders, spread of viral infections, AIDS and cancer (Herker et al., 2004). During the past years, yeast has been successfully established as a model to study mechanisms of apoptosis regulation (Herker et al., 2004).

Typical markers of apoptosis are DNA fragmentation, phosphatidyserine externalization and chromatin condensation. These markers, as shown in figure 1.2.2, are conserved in yeast.

Further, a caspase-like protease (Yca1p) mediates oxygen-stress-dependent apoptosis in yeast.

In mammals, there are two pathways of apoptosis that leading to caspase-dependent apoptosis: the intrinsic apoptotic
pathway and the extrinsic apoptotic pathway. The intrinsic pathway is characterized by mitochondrial outer membrane permeabilization (MOMP) that is a crucial step in the intrinsic apoptotic pathway in mammalian cells (Mazzoni and Falcone, 2008), while the extrinsic pathway begins outside the cell through the activation of specific pro-apoptotic receptors on the cell surface. These are activated by specific molecules known as pro-apoptotic ligands. These ligands include Apo2L/TRAIL and CD95L/FasL and bind their cognate receptors DR4/DR5/Fas, respectively. Unlike the intrinsic pathway, the extrinsic pathway triggers apoptosis independently of the p53 protein.

Two complementary and partly overlapping models of aging have been investigated in yeast: the replicative life span model and the chronological life span model.

Chronological life span is defined as the length of time cells survive after they are driven into a non-dividing state by depletion of nutrients from the medium, while replicative life span
is determined by counting the number of times cells divide in the presence of nutrients before they senescence and die.

Both chronologically and replicatively aged yeast cells dye exhibit typical markers of apoptosis, accumulate oxygen radicals, and show caspase activation (Herker et al., 2004).

Apoptosis in yeast confers a selective advantage for this unicellular organism, and it has been demonstrated that old yeast cells release substances into the medium that stimulate survival of the clone (altruistic suicide) (Herker et al., 2004).

In recent years, several homologues of classical apoptotic regulators have been identified and characterized, i.e. caspase (YCA1) (REF), AIF1 (REF), EndoG (endonuclease G, NUC1) (REF) and Omi (NMA111)(REF), suggesting that the basic machinery of apoptosis is indeed present and functional also in yeast.
Figure 1.2.1 DAPI staining shows nuclear fragmentation, TUNEL assay shows fragmentation of DNA, the exposition of the phosphatidylinerine was observed through annexin V and electron micrographs show condensation of chromatin. This picture shows the importance of the conserved features between yeast and mammalian. n nucleus, m mitochondria v vacuole.
1.2.1 THE CASPASE YCA1

Caspases are members of a family, or structurally related group, known as cysteine proteases. The name caspase derives from cysteine-dependent aspartate specific protease: catalysis is driven by a critical conserved cysteine side chain of the enzyme, and by a stringent specificity for cleaving protein substrates containing aspartic acid. The activation of caspases has been recognized as one of the key processes linked to apoptosis in mammalian cells in which several caspases, grouped into two classes, have been described (Mazzoni and Falcone, 2008).

The analysis of the S. cerevisiae genome did not reveal the presence of caspases, but by sequence comparison ORF YOR197 was identified coding for a caspase-like protein, fitting into the type I category of metacaspases, and it was named MCA1 (Uren et al., 2000; Szallies et al., 2002).
Following studies demonstrated that, following an apoptotic stimulus, the product of MCA1 was processed by the proteolytic removal of a 14 kDa peptide leading, as in mammalian caspases, to the activation of the metacaspase.

Moreover, in the same study, it has been demonstrated that the conserved Cys297 residue is necessary for the proteolytic cleavage and is important for the fully activity of the caspase (Madeo et al., 2002).

Lysates from yeast over-expressing the MCA1 gene could efficiently cleave IETD-AMC peptide, a caspase substrates typical of initiator caspases (i.e. caspase 8), while no activity was found toward DEVDAMC, a characteristic substrate for effector caspases (i.e. caspase 3) suggesting that MCA1 could operate as the initiator caspase 8. The over-expression of MCA1 rendered cells more susceptible to cell death following an apoptotic stimulus, being cell death completely abolished in the presence of the caspase inhibitor zVAD-fmk. Conversely, yeast mutants lacking the MCA1 gene
survived to hydrogen peroxide treatment and to aging better than wild type cells (Madeo et al., 2002).

From these results, Yor197w was proposed as a bona fide caspase gene in S. cerevisiae and it was renamed YCA1 (Yeast CAspase) (Madeo et al., 2002).

The apoptotic events observed in S. cerevisiae, due to external stimuli as well as internal signals, were often dependent on the activity of the yeast metacaspase Yca1p while, in other cases, cell death occurred also in the absence of this activity.

Disruption of the apoptotic machinery by deletion of YCA1 increases the survival in long term cultures but it is disadvantageous for the population as, in direct competition assays, the wild type cells outlast the disruptants (Herker et al., 2004). An aged yca1 - null mutant strain is no longer able to regrow when nutrients become available after a period of starvation, leading to a population with a high percentage of damaged and old cells. This indicates a physiological role of apoptosis in yeast essential to
operate a selection for the fitter cells in aging-related cell death.

(Buttner et al., 2006).
1.2.2 THE ORTHOLOGUE OF THE GENE AIF IN YEAST, aif1

Apoptosis - inducing factor (AIF) in mammalian cells is a flavoprotein with oxido-reductase activity localized in the mitochondrial intermembrane space (Susin et al., 1999; Miramar et al., 2001). Upon apoptosis induction, AIF translocates to the nucleus, where it leads to chromatin condensation and DNA degradation. AIF has been suggested to control caspase-independent pathway of apoptosis, important for neurodegeneration and normal development (Susin et al., 1999; Cregan et al., 2002).

An orthologue of AIF has been described in yeast cells. Yeast Aif1p shows the same localization and exhibits similar death executing pathways as mammalian AIF. Further Aif1p is dependent on cyclophilin A (CypA) and partially on caspase action (Wissing et al., 2004).
1.2.3 THE SERIN PROTEASE Nma 111p (Omi)

In the yeast *Saccharomyces cerevisiae* Nma111p functions as a nuclear serine protease that is necessary for apoptosis under cellular stress conditions, such as elevate temperature or treatment of cells with hydrogen peroxide to induce cell death.

The role of nuclear protein import in the function of Nma111p in apoptosis has been examined.

Nma111p contains two small clusters of basic residues towards its N-terminus, both of which are necessary for efficient translocation into the nucleus. Nma111p does not shuttle between the nucleus and cytoplasm during either normal growth conditions or under environmental stresses that induce apoptosis.

The N-terminal half of Nma111p is sufficient to provide the apoptosis - inducing activity of the protein, and the nuclear localization signal (NLS) sequences and catalytic serine 235 are both necessary for this function. Now there is evidence that
intranuclear Nma111p activity is necessary for apoptosis in yeast (Belanger et al., 2009).

Nma111p is a nuclear protein that interacts with nuclear-pore complexes (NPCs) (Fahrenkrog et al., 2004). Both Yca1p, the yeast caspase, and Bir1p, the only identified substrate for Nma111p, are also nuclear proteins (Walter et al., 2006). Therefore, some of the processes that occur in the cytoplasm during mammalian apoptosis seem to occur in the nucleus during yeast cell death. The importance of the nucleus for the yeast apoptotic programme is further supported by the notion that Aif1p and Nuc1p move from mitochondria to the nucleus upon induction of apoptosis (Buttner et al., 2007; Wissing et al., 2004).
1.2.4 THE YEAST HOMOLOGOUS OF EndoG,  

Nuc1p

Endonuclease G (EndoG) is located in mitochondria yet translocates into the nucleus of apoptotic cells during human degenerative diseases. Nonetheless, a direct involvement of EndoG in cell death execution remains equivocal, and the mechanism for mitochondrial-nuclear translocation is not known.

Has been shown that the yeast homologous of EndoG (Nuc1p) can efficiently trigger apoptotic cell death when excluded from mitochondria. Nuc1p induces apoptosis in yeast independently of metacaspase or of apoptosis inducing factor AIF1. Instead, the permeability transition pore, karyopherin Kap123p, and histone H2B interact with Nuc1p and are required for cell death upon Nuc1p overexpression, suggesting a pathway in which mitochondrial pore opening, nuclear import, and chromatin association are successively involved in EndoG-mediated death. Deletion of NUC1 diminishes apoptotic death when mitochondrial
respiration is increased but enhances necrotic death when oxidative phosphorylation is repressed, pointing to dual – lethal and vital – roles of EndoG (Buttner et al., 2007).

EndoG has been described as a mitochondrial nuclease that digests both DNA and RNA (Ruiz-Carrillo and Renaud, 1987; Schafer et al., 2004). Upon apoptosis induction, translocation of mammalian EndoG to the nucleus coincides with large-scale DNA fragmentation (Li et al., 2001; Parrish et al., 2001). Recently, nuclear translocation of EndoG has been associated with progression of several degenerative disorders, such as cerebral ischemia (Lee et al., 2005) and muscle atrophy (Leeuwen-burgh et al., 2005).

Similar to other mitochondrially located cell-death regulators like cytochrome c or apoptosis inducing factor (AIF) (Cheng et al., 2006; Vahsen et al., 2004), EndoG may have a genuine vital function, besides its proapoptotic function. In fact, EndoG was suggested to play a role in cell proliferation and replication (Huang et al., 2006)
Mitochondria are highly dynamic organelles that continuously fuse and divide. In many organisms, including worms and mammals, mitochondrial division promotes the release of cytochrome c to trigger apoptosis. Furthermore, mitochondrial dynamics is thought to counteract aging and constitute an organellar quality control mechanism.
1.3 THE TUMOUR SUPPRESSOR p53

1.3.1 STRUCTURE OF p53

The human p53 gene was shown to span about 20 kb of DNA and to be localized to the short arm of chromosome 17 (17p13). This gene is composed of 11 exons, the first of which is non-coding and localized 9-10 kb away from exons 2 through 11 (Benchimol et al., 1985; Isobe et al., 1986; Oren, 1985).

The p53 has been conserved during evolution (Soussi et al., 1987). Through cross-species comparison of amino acid sequences, the p53 proteins showed the existence of five highly conserved regions within the amino acid residues 13-23, 117-142, 171-181, 234-250 and 270-286 (Soussi et al., 1990; Soussi and May, 1996). These regions, termed domains I-V, were expected to be crucial for the p53 functions.
The human p53 protein is a transcription factor, consists of 393 amino acids and contains four major functional domains: the activation domain (AD, residues 1-45), a proline rich domain, the DNA binding domain (DBD, residues 100-300) and the tetramerization domain (TD, 324-356).

Whitin the N-terminus are the transactivation domain and a proline-rich region, which is required for apoptotic function. Whitin the N-terminus are the interaction sites of p53 with components of the transcriptional machinery as well as ubiquitin ligase Mdm2. In the central domain harboring the sequence specific DBD domain occur most of the tumor associated mutations. This central region also contains binding sites for interaction with members of the Bcl2 protein family. The C-terminal region contains the oligomerization domain as well as nuclear localization and export signals. Several sites within the N-terminal region have been shown to be phosphorylated and the C-terminal region contains numerous sites of modification which influence stability, localization and activity of p53.
Figure 1.3.1 Diagram showing the domain structure of the p53 protein (Yee and Vousden, 2005)
1.3.2 ROLE OF p53

p53 may be considered the best known of all tumor suppressors. The loss or inhibition of p53 activity and functions prevents cellular senescence and apoptosis, thus favoring cancer development and progression. p53 is indeed mutated in 50% human cancers.

In normal cells, p53 levels are maintained low by the activity of the E3 ubiquitin ligase MDM2. Many types of stress signals, (i.e. DNA damage, oncogene activation, hypoxia-anoxia) increase the levels of p53 by inducing its transcription and/or by stabilizing the protein.

p53 is a transcription factor that directly activates the expression of both cell cycle and apoptosis regulating genes (Vousden and Lu, 2002). Over 4,000 putative p53-binding sites were identified in several gene promoters. Whether all of these will prove to be bona fide target genes of p53 is not known. Although far from complete, these examples illustrate that many of
the genes that are induced by p53 can be divided into groups that might mediate a specific p53 function, such as inhibition of cell growth, DNA repair, activation of apoptosis or regulation of angiogenesis (Vousden and Lu, 2002).

The tumour suppressor function of p53 depends principally on its ability to prevent cellular proliferation in response to stress stimuli that are encountered during tumourigenic progression. Activation of p53 leads to cell cycle arrest and apoptosis, and can play a role in the induction of differentiation and cellular senescence (Almog and Rotter, 1997; Lundberg et al., 2000). Wild-type p53 has been shown to inhibit angiogenesis in tumour, by activating or repressing genes that regulate new blood vessels formation (Dameron et al., 1994; Nishimori et al., 1997; Bouvet et al., 1998). p53 can also play a direct role in the repair of DNA damage, both through nucleotide excision repair and base excision repair (Wani et al., 1999; Offer et al., 2001).
Function of cell cycle arrest of p53

The cell cycle arrest function of p53 correlates well with its ability to function as a transcription factor (Crook et al., 1994; Pietenpol et al., 1994). One of cell cycle regulating p53 target genes is p21<sub>Waf/Cip1</sub> (El-Deiry et al., 1993). p21<sub>Waf/Cip1</sub> is a cyclin-dependent kinase inhibitor that can activate both G1 and G2 cell cycle arrest similar to those seen in response to p53 induction (Agarwal et al., 1995; Bates et al., 1998). Importantly, p21<sub>Waf/Cip1</sub>-deficient cells are defects in both G1 and G2 arrest, DNA synthesis and mitosis. Another target of p53 that contributes to the p53-induced G2 arrest is 14-3-3 σ (Hermeking et al., 1997). The 14-3-3 family proteins play a role in signal transduction and cell cycle control (Musling and Xing, 2000). Although 14-3-3 σ-deficient cells may transiently arrest in G2 phase after DNA damage, they are unable to maintain the cell cycle arrest status. Further potential mediators of the G2 arrest include GADD45 and Reprimo (Ohki et al., 2000).
Recent data implicate p53 as part of a checkpoint that senses microtubule disruption, and that prevents the outgrowth of aneuploid variants that emerge after exposure of cells to microtubule inhibitors.

A key link was established when Brian Reid and colleagues demonstrated that in rodent cells microtubule disruption in combination with p53 loss allows downstream events (DNA replication) to occur prior to the successful completion of upstream events (mitosis) (Cross et al., 1995). The consequence of this loss of coordination is an increase in the number of cells with greater than 4N DNA content. Thus, loss of p53 leads to DNA reduplication after exposure to agents that inhibit mitotic division, such as nocodazole and colcemid. These data led to the proposal that p53 functions in a mitotic spindle checkpoint in which it monitors and prevents exit from mitosis under conditions that hinder cell division (Cross et al., 1995).

Furthermore, p53 contributes either to repair or cell death by stimulating expression of either pro- or antioxidant genes.
Figure 1.3.2  p53 drives different responses under conditions of low stress (where cell survival and repair is supported) and high stress (where the damaged cell is eliminated through death or senescence) (Gottlieb and Vousden, 2010).
p53 pro-apoptotic function

In most cell types, the apoptotic response reflects the cumulative action of several p53-induced signals. The ability to activate apoptosis through different apoptotic pathways may be relevant for the tumour-suppressor activity of p53 (Vousden and Lu, 2002).

The concept that apoptosis is a cumulative response to many signals, including p53, indicates that loss of any of these might enhance survival by reducing the apoptotic burden to below the threshold that is necessary for the execution of death (Vousden and Lu, 2002).

Recent studies have suggested that the straightforward role of p53 as a transcription factor that eliminates developing tumor cells by inducing the expression of apoptotic target genes is only part of a much more complicated story. There is now a firm body of evidence supporting a transcriptionally independent activity that involve a direct role of p53 in mitochondria, thus favoring the
release of cytochrome C and the activation of the Apaf-1/caspase-9 apoptotic cascade (Yee and Vousden, 2005).

A number of early studies suggest that p53 may have an apoptotic function that is completely separate from the regulation of gene expression. An explanation of this activity has been provided by the recent observations that, following stress, a proportion of p53 appears to function outside the nucleus, in the cytoplasm or at the mitochondria, and that p53 can be found in association with several members of the Bcl2 family of proteins. The Bcl2 family proteins are key components of the intrinsic apoptotic pathway and fall into three main groups; the proapoptotic effectors proteins (Bax and Bak), which directly perturb mitochondrial membrane potential, the BH-3 only proteins that directly or indirectly drive the activation of Bax and Bak, and the anti-apoptotic proteins that sequester the pro-apoptotic family members and hold them inactive (such as Bcl2, BclxL and Mc11). The BH-3 only proteins are further divided into two groups; the “activators” like Bim and Bid that directly bind Bax and Bak to
activate them and the “enablers” like Bad and Bik that bind the anti-apoptotic family members to release the activators. Unexpectedly, p53 may function in a manner analogous to the BH3-only proteins, with evidence to support two broad, but not mutually exclusive, models. In the first, p53 acts as an “enabler” BH3 domain protein, interacting with the anti-apoptotic proteins and presumably releasing pro-apoptotic BH3 domain proteins to drive apoptosis. The second model is based on the observation that p53 may function as an “activator” BH3-only proteins, by directly activating the apoptotic functions of Bax or Bak (Yee and Vousden, 2005). Indeed, p53 protein itself can localize to the mitochondria presenting a potential additional transcription-independent way of mediating apoptosis (Marchenko et al., 2000).

Recently, other pro-apoptotic members of the BCL2-family named NOXA (Oda et al., 2000) and PUMA (Nakano and Vousden, 2001; Yu et al., 2001) have been identified as p53 targets. These proteins, as well as another p53 target gene product, p53AIP1 (Oda et al., 2000), localize to the mitochondria and
promote loss of the mitochondrial membrane potential and cytochrome c release (Bossy-Wetzel and Green, 1999).

Perturbation of mitochondrial integrity may also be mediated by several genes encoding for redox-controlling enzymes, which were identified as p53-induced genes (PIGs). It has been proposed that reactive oxygen species (ROS) produced by these PIGs cause damage to mitochondria, which in turns initiate apoptosis.

p53 has been also implicated in the regulation of the extrinsic death receptor-induced pathway of apoptosis. The expression of at least two death receptors, FAS/FASL and DR5/KILLER, is up-regulated by p53 (Owen-Schaub et al., 1995; Wu et al., 1997). The activation of FAS and DR5 death-receptors by their ligands FASL and TRAIL respectively, results in their trimerization and recruitment of intracellular adapter molecules which initiate the caspase-8/10 cascade and apoptosis (Ashkenazi and Dixit, 1998). The DR5 promoter is a direct target of p53 (Takimoto and El-Deiry, 2000), while cell surface expression of
FAS is enhanced by p53 that promotes its trafficking from Golgi to the plasma membrane (Bennett et al., 1998).

The loss of survival signalling can also augment p53-mediated apoptosis (Gottlieb et al., 1994; Abrahamson et al., 1995; Canman et al., 1995; Lin and Benchimol, 1995; Prisco et al., 1997) and p53 may both negatively regulate the growth factor (i.e. insulin growth factor, IGF) signalling pathway (Buckbinder et al., 1995) and inhibit integrin-associated survival signalling thus further sensitizing cells to apoptosis (Bachelder et al., 1999). The NF-κB transcription factor has lately been shown to play an important role in p53-mediated apoptosis (Ryan et al., 2000).

In conclusion, several cellular responses to p53 activation have been described, and the choice of response depends on factors such as cell type, cell environment and other oncogenic alterations that are sustained by the cell. In general, however, the effects of p53 activation is to inhibit cell growth, either through cell cycle arrest or induction of apoptosis, thereby preventing tumor development (Vousden and Lu, 2002).
Indeed, under some circumstances, when the damage is reversible, p53 contributes to the repair of genotoxic damage, blocking cell cycle, potentially allowing for the release of the rehabilitated cell back into proliferating pool. On the other hand, rather in most cases, when the cell damage is extensive, induction of p53 leads to an irreversible inhibition of cell growth, most decisively by activating apoptosis.
Figure 1.3.3  p53 induces apoptosis by activating target genes such as PIGs, Bax, Noxa; PUMA, p53AIP1, FAS PIDD and IGF-BP3. p53-induced apoptosis involves change in mitochondrial membrane potential ($\Delta Y$), cytochrome C release and caspase activation (Bálint and Vousden, 2001).
1.3.3 REGULATION OF FUNCTIONS OF p53

Since p53 is a powerful inhibitor of cell proliferation, control of its activity is essential during normal growth and development. Regulation of p53 has been described at the level of transcription, translation, conformational changes, and various covalent and non-covalent modifications. At present, it is clear that one of the key mechanisms by which p53 functions are regulated, relies on the control of protein stability. MDM2 plays a pivotal role in regulating p53 ubiquitination and degradation and multiple forms of stress stimuli activate p53 by countering MDM2-mediated degradation of p53 (Ryan et al., 2000).

Two related proteins – MDM2 and MDMX (MDM4) – have been described as crucial in regulating p53 levels. MDM2, which participates in an autoregulatory loop with p53, contains a RING finger domain and, similarly to many RING finger proteins has been shown to function as a E3 - ubiquitin ligase that targets p53 for degradation. MDM2 targets both itself and p53 for
ubiquitination, and mutations in its RING finger domain lead to the stabilization of both proteins (Honda et al., 1997). The activation and stabilization of p53 is generally associated with inhibition of the function of MDM2, and defects in the pathways that curb MDM2 activity are common in tumours that retain wild-type p53 (Vousden and Lu, 2002). Interestingly, the transcriptional co-activator p300/CBP that functions in acetylating and activating p53, also participates in the degradation of p53 by MDM2, possibly by acting as a platform to allow efficient p53/MDM2 interaction.

MDM2 has been shown to inhibit p53 activity in several ways: by binding to the transactivation domain of p53, by targeting p53 for ubiquitination, by inhibiting acetylation of p53 and by shuttling p53 to cytoplasm. The importance of MDM2 in the regulation of p53 activity is illustrated by the observation that MDM2 deficiency causes early embryonic lethality in mice, an event that may be rescued in a p53 - null background. Conversely, amplification of MDM2 is associated with the development of
tumours that retain wild-type p53 suggesting that overexpression of MDM2 prevents the normal p53-mediated response to oncogenic stress (Ryan et al., 2000).

Mechanisms regulating p53 function by MDM2

Binding of MDM2 to p53 and its ubiquitin-dependent degradation

MDM2 interacts with the N-terminal region of p53, which also contains the major acidic transcriptional activation domain (Momand et al., 1992). This binding of MDM2 to p53 can inhibit p53's transcriptional activity by impairing the association of p53 with transcriptional coactivators such as p300/CBP.

The binding of MDM2 with p53 plays an additional important role in controlling p53 function by contributing to the regulation of p53 protein stability (Kubbutat et al., 1997). In most unstressed tissues p53 is expressed at very low levels are
maintained in large part by the constant removal of newly synthesized p53 through ubiquitin-dependent degradation by the proteasome. Ubiquitin modification of proteins involves a multienzyme cascade, with substrate specificity determined by a large group of ubiquitin protein ligase (E3). One recently described group of E3 ligases are RING-finger proteins, which are thought to serve as a bridge to allow transfer of ubiquitin from the E2 ubiquitin-conjugating enzyme to the substrate (Joazeiro and Weissman, 2000). MDM2 is a RING-finger protein that functions as a ubiquitin ligase for both p53 and itself (Honda et al., 1997; Midgley et al., 2000).

Mutations within the RING-finger domain of MDM2 inhibit the ubiquitination of p53 and MDM2 itself resulting in the stabilization of both proteins. Interestingly, the ability of MDM2 to target p53 for degradation is also regulated by other factors. A role for p300/CBP has been described in supporting efficient degradation of p53, probably by facilitating the interaction between p53 and MDM2 in cells, although this activity depends on an
interaction between these proteins different from those involving the p53 transactivation domain.

- **Nuclear export of p53 by MDM2**

  Although ubiquitination of p53 by MDM2 has the main function to target the p53 protein to the proteasome, this activity may also allow the export of p53 from the nucleus to the cytoplasm, where degradation of p53 takes place.

- **Other mechanisms regulating p53 function**

  - **Modifications of p53**

    - **Phosphorylation**

      Phosphorylation sites in p53 are clustered within the N- and C-termini and include potential targets for numerous cellular kinases (Meek, 1998). Phosphorylation within to the N-terminal MDM2 binding domain in p53 is mostly involved in the regulation of p53 stability, by interfering with the ability to bind to MDM2.
Several stress-response kinases such as ATM, ATR, Chk1, and Chk2 have been shown to phosphorylate this region of p53, and inhibition or loss of these kinases renders cells deficient in mounting a p53 response to stress signals. Both ATM and ATR appear to have a multiples roles in allowing activation of p53 in response to DNA damage. These kinases directly phosphorylate p53 on serine 15 and 37, thereby enhancing the transcriptional activity of p53 (Sakaguchi et al., 1998; Dumaz and Meek, 1999), ATM also activate Chk2, a kinase that phosphorylate p53 directly within the MDM2 binding domain, thus reducing binding to MDM2 (Hirao et al., 2000). ATM activation also induces dephosphorylation of the C-terminus of p53, and ATM can also directly phosphorylate MDM2 (Khosravi et al., 1999), although the consequences of this activity are less understood. Many other kinases have been shown to be capable of phosphorylating the N-terminus of p53, including DNA-PK, JNK, p38, CK1 and CAK (Ljungman, 2000).
Many proteins that shuttle between the cytoplasm and nucleus are regulated by phosphorylation mediated by CK or Cdc2/cyclin B at sites near their NLS (Nuclear Localization Signal) and NES ((Nuclear Export Signal) domains (Vandromme et al., 1996). While phosphorylation of these proteins by CK is associated with nuclear localization, phosphorylation by Cdc2/cyclin B is associated with cytoplasmic localization. The C terminal domain of p53 has both Cdc2/cyclin B and CK phosphorylation sites. Phosphorylation of ser392 by CK has been shown to stimulate tetramerization of p53 (Sakaguchi et al., 1997). Since tetramerization of p53 has been suggested to hide the NES of p53 and thus block nuclear export (Stommel et al., 1999)), phosphorylation of p53 by CK may stimulate nuclear accumulation of p53 by blocking p53’s export. In contrast, phosphorylation induced by Cdc2/cyclin B is associated with cytoplasmic localization of many different types of proteins. It has been shown that the tetramerization stimulating effect of ser392 phosphorylation by CK II can be blocked by phosphorylation of
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ser315 by Cdc2/cyclin B (Sakaguchi et al., 1997). Thus, it is possible that the nucleocytoplasmic shuttling of p53 could be regulated, in part, by these two kinases where CK may stimulate nuclear import and Cdc2/cyclin B may stimulate nuclear export (Ljungman, 2000).

- Acetylation

p300/CBP has been shown to enhance p53's transcriptional activity by interacting with the N-terminus of p53 and acetylating lysine 382 in the C-terminus of the p53 protein (Sakaguchi et al., 1998). The acetylation of this site activates p53's binding function, thus reinforcing the role of p300/CBP as a key coactivator for p53 transcriptional activity. Interestingly, phosphorylation of p53 within the N-terminus by kinase like ATM or ATR enhances p300/CBP binding and C-terminal acetylation, revealing a complex pathway in each multiple codependent modifications of p53 may be necessary for full activation. The inhibition of acetylation by either histone deacetylases (Fogal et al., 2000) or MDM2 (Kobet et al., 2000) results in a reduction in p53's transcriptional activity.
• Sumoylation and other modifications of p53

SUMO is a small ubiquitin-related protein that is conjugated to target proteins through enzyme cascades similar to those responsible for ubiquitination. p53 is modified by SUMO at one of the lysine residues that is also ubiquitinated, and although SUMO modification does not regulate p53 stability, this modification enhances p53’s transcriptional activity (Gostissa et al., 1999). Interestingly this modification of p53 may also be regulated by phosphorylation, again illustrating the complex codependence of regulatory modifications of p53. Several other modifications of p53 have been described to affect both protein stability and functions, including glycosilation and ribosylation by PARP (Vaziri et al., 1997; Kumari et al., 1998), which might affect both protein stability and protein function.
• Interaction of p53 with other proteins

Many p53-interacting proteins that might play a role in the stabilization and activation of p53 have been described (Prives et al., 1999). Interaction of p53 with Ref-1 (Jayaraman et al., 1997) and HMG-1 (Jayaraman et al., 1998) leads to the activation of p53, possibly without requirement for covalent modification of the p53 protein. Numerous other proteins such as WT1, BRCA1, WRN, E2F1, and pRB, have been shown to augment p53 function, either by stabilizing the p53 proteins or by activating p53’s transcriptional activity (Sionov and Haupt, 1999).

• The ARF protein

Another mechanism to stabilize p53 in response to stress involves the protein ARF. The alternative reading frame product of the INK4A locus, called p14ARF (mouse p19ARF) binds directly to MDM2, inhibiting p53 ubiquitination by MDM2 (Bates et al., 1998; Kamijo et al., 1998). In some systems, ARF also leads to the relocalization of MDM2 from the nucleoplasm to the nucleolus, through nucleolar localization signals present in both ARF and
MDM2 (Lohrum et al., 2001). As a consequence, p53 is efficiently stabilized and activated.

The importance of the ARF/p53 pathways is illustrated in mice, where deletion of ARF results in tumour development (Kamijo et al., 1997). Although mutations of ARF have been rarely found in human tumours, the loss of ARF expression by either methylation of the ARF promoter (Esteller, 2000)

- The role of MDMX

Another regulator of p53 stability is the protein MDMX. MDMX, a protein related to MDM2, also possesses a p53-binding domain and a RING-finger domain. Binding of MDMX inhibits p53 transactivation domain. Binding of MDMX inhibits p53 transactivation function (Shvarts et al., 1996) through a mechanism independent of p53 for degradation. It is possible that MDMX does not show ubiquitin ligase activity, and hetero-oligomerization of MDMX with MDM2 through their RING finger domain results in the stabilization of MDM2 (Sharp et al., 1999; Tanimura et al.,
1999) but protects p53 by MDM2-mediated ubiquitination and degradation (Jackson and Berberich, 2000).

Figure 1.3.4 Cartoon depicting the p53 protein, showing the major functional domains, sites of interactions with some other proteins and regions of covalent modifications (Woods and Vousden, 2001).
1.3.4 LOCALIZATION AND REGULATION OF

LOCALIZATION OF p53

In the past few years it has become clear that in addition to modulation of protein stability, p53 function is also regulated by its subcellular localization. Since one of the key functions of p53 is the regulation of transcription, localization of p53 in the nucleus plays an important role for the p53 response. Active transport of p53 towards the nucleus by dynein and microtubule network has been described, and several nuclear localization signals in the C-terminus of p53 can contribute to nuclear import. Once entered into the nucleus, regulatory mechanisms exist to control the export of p53 back out to the cytoplasm.

p53 nuclear accumulation in response to certain stress stimuli, such as γ-irradiation, is cell cycle-dependent (Komarova et al., 1997). On the contrary, other stresses, such as hydrogen peroxide and heat shock, induce p53 nuclear translocation and
accumulation independently of cell cycle phase (Sugano et al., 1995).

The microtubule network shows an inherent polarity from the dynamic “plus” end (usually at the cell periphery) to the relatively stable “minus” end (at the centrosome, usually near, or even linked to, the nucleus) (Vousden and Woude, 2000).

The study of Giannakakou and colleagues shows that transport of p53 along the microtubules towards the nucleus occurs only in response to stress, and that disruption of the microtubule network or interference with dynein function impairs localization of p53 to the nucleus, thus inhibiting p53 activation (Vousden and Woude, 2000).

Although, the accumulation of p53 into getting p53 into the nucleus is obviously important in allowing p53 to function, dampening p53 activity in normally dividing cells seems to require transport out of the nucleus (Vousden and Woude, 2000).

Besides direct ubiquitination of p53, MDM2 also plays a role in regulating the subcellular localization of p53, driving
degradation of p53 through the proteasome. Several lines of evidence indicate that this degradation of p53 occurs in the cytoplasm, although both MDM2 and p53 are nuclear proteins (Vousden and Woude, 2000). MDM2's ubiquitin ligase activity contributes to the efficient nuclear export of p53 (Boyd et al., 2000; Geyer et al., 2000), which depends on the nuclear export sequence (NES) identified in the C-terminus of p53 (Stommel et al., 1999). It is possible that the ubiquitination of p53 reveals the NES, possibly by driving p53 into a monomeric form (Stommel et al., 1999), allowing access to the nuclear export machinery (Bálint and Vousden, 2001).

Several models of how p53 is exported from the nucleus have been put forward. MDM2 contains nuclear-export signals in p53 has complicated this model, and there is some evidence that the export of p53 continues unabated even in the absence of MDM2. Now a further wrinkle on the system has been uncovered by two studies that have shown that the ubiquitin-ligase activity of
MDM2 is necessary for p53 export, but export of MDM2 itself is not required (Boyd et al., 2000; Geyer et al., 2000).

Together, the studies indicate that, in the absence of stress signals, p53 activity may be regulated both by suppressing localization to the nucleus and by enhancing transport out of the nucleus. In response to the appropriate signals, nuclear import of p53 is activated, whereas export is inhibited, so maximizing the accumulation of p53 in the nucleus. In tumours that retain wild-type p53 (roughly 50%), defects in either of these pathways impinge on the ability to activate the p53 response, several tumour-associated defects have been described that lead to a failure to block MDM2 function in response to stress signals, and so result in an inability to stop the nuclear export and degradation of p53 (Vousden and Woude, 2000).

Although previous studies have shown that cytoplasmic localization of p53 in tumour cells may be the result of a hyperactive nuclear-export system, the study by Giannakakou and colleagues raises the possibility that cytoplasmic retention may
also result from defects in the ability of p53 to associate with microtubules in response to stress (Vousden and Woude, 2000).

The importance of microtubules in p53 function may have important repercussions in tumour therapy, as microtubule-disrupting drugs like taxol are commonly used for chemotherapy (Vousden and Woude, 2000).

**Figure 1.3.5** Domain structure of p53 and MDM2 proteins. NLS, nuclear localization signal; NES, nuclear export signal; NoLs, nucleolar localization signal (Bálint and Vousden, 2001).
**Figura 1.3.6** Model for regulation of the subcellular localization of p53. Association with microtubules directs p53 towards the nucleus, using the minus-end-directed molecular motor dynein, where import receptors recognize localization signals in the C-terminus of p53. Export from the nucleus requires nuclear-export sequences in p53 and MDM2 activity, possibly through ubiquitination of p53. Defects in the import pathway, or failure to inhibit the export pathway, could compromise the ability to activate p53 and contribute to tumour development. Ub, ubiquitin. (*Vousden and Woude, 2000*).
1.3.5 MUTATIONS OF p53

From the data available, it would seem that only 5% of TP53 mutations are found in the regulatory domains (amino terminus, amino acids 1-99; carboxyl terminus, amino acids 301-393), whereas 95% of the mutations occur in the central region of TP53, which is responsible for sequence-specific DNA binding (amino acids 100-300) (Vousden and Lu, 2002).

Moreover, mutations within the TD (tetramerization domain) have been recently reported and associated with human tumors. The TD of p53 is essential for efficient p53 transcriptional activity and affects both the affinity and conformation of the interaction with DNA (Muscolini et al., 2009).

Tumour-associated mutations in TP53 are predominantly point mutation (93.6%) that result in single amino-acid substitutions - a mutational spectrum that is quite different from that seen in other tumour-suppressor genes, in which large
deletions or frameshift mutations tend to result in the complete loss of protein expression (Vousden and Lu, 2002).

The result of mutational inactivation of TP53 by single-amino-acid substitutions is that many tumour cells retain the ability to express the mutant p53 protein. These proteins are often more stable than wild-type p53, and are present at very high levels in the tumour cell. One explanation of this selection of such mutations is that the mutant p53 proteins can act as dominant-negative inhibitors of wild-type p53, which functions as a tetramer. The observation that many tumours that harbour TP53 point mutations also show loss of heterozygosity – effectively eliminating the wild-type allele – indicates that the efficiency of dominant-negative inhibition might not be complete, and almost certainly depends on the nature of the initial point mutation (Vousden and Lu, 2002).

Tumour therapy that is based on the reactivation of p53 is therefore an attractive goal, and might be achieved by reactivating mutant p53, introducing exogenous wild-type p53 or activating
endogenous p53 in those tumours that retain wild-type p53 (Vousden and Lu, 2002).

Furthermore, accumulating evidence that the apoptotic activity of p53 is regulated separately from its other functions indicate that it might be possible to selectively induce p53-mediated apoptosis for cancer therapy (Vousden and Lu, 2002).

The abnormal proliferation, loss of normal cellular environment and stress that accompanies malignant progression all enhance the apoptotic sensivity of the cancer cells by either increasing death signalling or hampering survival signals.

The suggestion that tumour cells show a greater propensity to die in response to p53 than their normal counterparts has triggered much excitement in the use of p53 as a tumour-specific therapy (Vousden and Lu, 2002).

The group of Muscolini et al. has recently identified mutation of Lys351 to Asn (K351N) as a new cancer-associated mutant of p53 in a cisplatin-resistant clone derived from the A2780 ovarian carcinoma cell-line (A2780 CIS). This mutation was
generated by the substitution AAG/AAT in heterozygosis. Although K351N mutation did not affect p53 expression, it was associated to the acquisition of resistance to apoptosis in A2780CIS, particularly to defects in both CDDP-induced Bax expression and associated mitochondrial membrane depolarization, cytochrome C release and activation of caspase (Muscolini et al., 2009).

Muscolini et al. found that K351N substitution did not affect the overall half-life of p53 protein but significantly reduced its ability to form tetramers, when compared to p53wt. Tetrameric p53 is most effective at binding and transactivating p53 response elements, and mutations which prevent tetramerization compromise DNA binding, diminish transactivation, and lead to tumorigenesis (Friedman et al., 1993; Hainaut et al., 1994; Halazonetis and Kandil, 1993; Hupp and Lane, 1994; Ishioka et al., 1995; Lomax et al., 1998; Pietenpol et al., 1994; Tarunina et al., 1996; Varley et al., 1996). As a consequence, p53K351N, exhibited a reduced ability to transactivate specific target gene (Es.
Bax, p53AIP1 and Δnp73) when overexpressed in A2780 or Jurkat cells. They also found that K351N substitution affected subcellular localization of p53 by impairing its export from nucleus induced by CDDP. These data identify K351N as a new cancer-associated mutant in the TD of p53 with reduced tumor suppressor activity and involved in the induction of resistance to drug-induced apoptosis (Muscolini et al., 2009).
AIM OF THE THESIS

The p53 tumor suppressor gene is a major barrier against cancer, preventing tumor development and promotes apoptosis induced by chemotherapy. P53 is able to regulate apoptosis both through its transcriptional activity and by the induction of the outer mitochondrial membrane (OMM) permeabilization promoting the release of cytochrome c.

In the yeast *S. cerevisiae*, the machinery of the basic apoptotic process seems to be conserved as it presents many of the cytological markers of apoptosis such as chromatin condensation, DNA fragmentation and the release of cytochrome c from mitochondria to the cytoplasm. It was recently reported that the expression of p53 in *S. cerevisiae* is able to activate the apoptotic processes, making this system a good model to analyze the basic mechanisms of apoptosis induced by p53.

It was recently identified a novel mutation in p53, in which lysine 351 is replaced by an asparagine (K351N) in a cisplatin-
resistant ovarian carcinoma cell line (A2780 CIS). The K351N mutation is associated with the acquisition of resistance to apoptosis induced by cis-platinum in this cell line.

The K351N mutation significantly reduces the thermodynamic stability of p53 tetramers, the transcriptional activity of p53 and affects the export from the nucleus to the cytosol induced by cisplatinum treatment. The characterization of this mutant could thus help to envisage the development of new cancer drugs that can suppress this phenotype in tumors.

We cloned the two human genes, p53 and its mutated form p53K351N, under the control of Gal1-Gal10 vector and we transformed them in both wild type and S. cerevisiae strains mutated in genes involved in the apoptotic response. We then analyzed the effect of the p53 expression during an apoptotic stimulus ($H_2O_2$) and during aging.
2. RESULTS AND DISCUSSION

2.1 EXPRESSION OF p53wt AND p53K351N IN YEAST CELLS

To understand the involvement of p53 the and its mutated form p53K351N in the phenomena of apoptosis and aging, we have cloned these gene in the yeast pESC-HIS vector under the control of Gal1-10 promoter. Gene expression is repressed in the presence of glucose and induced by the presence of galactose in the media. These vectors, were transformed in Saccharomyces cerevisiae wild type (BY4741, see materials and methods) and mutated strains in genes involved in the yeast apoptotic pathways and mitochondrial morphology. In particular, we addressed our attention to the following genes, also described in the introduction sections 1.1.3-1.1.8:
• **YCA1**: The *YCA1* gene (YOR197w) encodes a metacaspase that exhibits proteolytic activity homologous to mammalian caspases (*Madeo F. et al.*, 2002).

• **AIF1**: The yeast homologue AIF1 (YNR074c) encodes a protein of 41.3 kDa, and shows 41% identity with human AIF.

• **NUC1**: *NUC1* encodes for a yeast homologue of mammalian EndoG, a mitochondrial nuclease that digests DNA and RNA. Nuc1 has a role in both mitochondrial recombination, apoptosis, and in the maintenance of polyploidy.

• **NMA111**: The protein Nma111, encoded by the YNL123W ORF in yeast, is homologous to HtrA2(Omi) in mammalian and, like all HtrA owns PDZ domains at the C-terminal (*Pallene and Wren, 1997; Harris and Lim, 2001; Clausen et al., 2002*). They mediate protein-protein interactions involved in signal transduction events. *Δnma111* mutants show a lifespan extension while the over expression of Nma111 promotes cell death (*Fahrenkrog et al.*, 2004).
All these strains were also transformed, as a control, with the empty plasmid (pESCHIS) and clones were selected on minimal medium lacking histidine at 28 ° C.

The expression of p53 was verified by western blot analysis (Figure 2.1.1). Protein extracts were prepared from cells grown in minimal medium containing glucose (SD, promoter Gal repressed), or after an incubation for about twenty hours in minimal medium containing galactose (SGal) which induce Gal1-10 promoter and induces the expression of p53 proteins.

Protein extracts were subjected to SDS-PAGE, transferred to nitrocellulose membrane and hybridized with an antibody directed against the p53.

As can be seen in figure 2.1.1, both p53 and p53K351N were present only when cells are grown in the presence of galactose, and not in glucose, suggesting that p53 cloned genes were functional and produce proteins recognized by the p53 antibody.
**Figure 2.1.1** Western blot analysis of protein extracts from yeast strains containing pESC-HIS-p53 (p53wt), pESC-HIS-p53K351N (p53K351N) and the control vector p-ESC-HIS. Cells grown in minimal medium containing glucose (SD) were shifted for 20h in minimal medium containing galactose (SG). Ponceau red staining of membranes is also shown for quantization of transferred proteins.
2.2 p53wt AND p53K351N INHIBIT THE GROWTH OF YEAST CELLS

To explore the effect of p53wt and p53K351N on yeast cell growth, we have performed a dilution spot assay on cells plated in glucose, galactose, caffeine and glycerol. As shown in figure 2.1.4, in the presence of glucose (SD), a condition that does not allow the protein expression, there was no difference between strains expressing the two forms of p53 and those expressing the empty plasmid. On the contrary, in the presence of galactose (SGal) growth of strains expressing both wt p53 and the K351N mutated form was completely inhibited.
Figure 2.2.1. Dilution spot assay of the indicated strains on plates containing 2% glucose (SD), 2% galactose (SGal), 3% glycerol (SY), 0.1% caffeine. Plates were recorded after 3 days of incubation at 28°C or, where indicated, at 37°C.

The same experiment carried out at a temperature of 37 °C, gave the same results, demonstrating that the protein inhibited cell growth independently of growth temperature.

In the presence of glycerol, a respiratory carbon source, the promoter (GAL1-10) is not suppressed, but results in a basal transcription of genes located downstream. This low expression of p53 genes resulted in the arrest of cell growth, suggesting that few molecules of p53 are sufficient to block the cell cycle.
The presence of 0.10% caffeine inhibited more than 50% cell growth in all tested conditions, so we can conclude that it did not induce apoptosis in a p53 dependent manner.

We have performed the same experiment also with mutant strains. As can be seen in figure 2.1.3, the expression of both forms of p53 blocked the cell growth also when expressed in the \textit{yca1} mutant strain. In fact, compared to growth on SD, which is complete up to the fifth dilution, growth on galactose (sGAL) at 28°C was completely inhibited. This indicated that the expression of protein blocks cell growth, despite the lack of the \textit{YCA1} gene, which is involved in the apoptotic response. The growing conditions are similar at 37°C, except when it is expressed p53K351N, which determined the cell growth up to the first dilution. This could be due to the fact that the mutated protein defects in forming tetrameres increases with temperature and, in absence of \textit{yca1}, high levels of functional p53 protein are required to block cell cycle.
This can be also confirmed by the observation that cell growth is only partially inhibited in the presence of glycerol indicating that, at least in yca1 mutant strains, the basal expression of p53 proteins is not sufficient for complete inhibition of cell growth and over-expression is required.
**Figure 2.2.2.** Dilution spot assay of the indicated strains on plates containing 2% glucose (SD), 2% galactose (SGal), 3% glycerol (SY), 0.1% caffeine. Plates were recorded after 3 days of incubation at 28°C or, where indicated, at 37°C.

The presence of caffeine, in conjunction with the expression of p53, did not completely inhibit cell growth, possibly related to the lack of the gene *YCA1*. 
The partial resistance to the cell growth inhibition following p53 expression was also observed in the *aif1*, *nucl* and *nma111* (Figure 2.1.3).
Figure 2.2.3. Dilution spot assay of the indicated strains on plates containing 2% glucose (SD), 2% galactose (SGal), 3% glycerol (SY), 0.1% caffeine. Plates were recorded after 3 days of incubated at 28°C or, where indicated, at 37°C.

The lack of growth in glycerol (SY) for aif1, nuc1 and nma111 is due to the mitochondrial defects shown by these mutant strains.

To explore the contribution of p53wt and p53K351N in accumulation of reactive oxygen species (ROS), we have used the fluorescence DHR123 labelling assay, which identify cells containing ROS. These assays showed that cells transformed with the plasmids containing p53wt and p53K351N have an accumulation of intracellular ROS higher respect to cells transformed with the empty plasmid pESC-HIS. In figure 2.2.4 are shown a sample of cells ROS positive (left panel) and the
quantification in percentage (right panel). About 35-40% of cells expressing both forms of p53 were ROS positive, 3-4 fold more than the same strain expressing the control plasmid p-ESC.

Figure 2.2.4 Cells containing p-ESC, p53wt and p53K351N were precultivated in SD and then shifted for 20h in minimal medium containing galactose to allow the expression of p53 genes. DHR123 was added to the cultures at the concentration of 5µg/ml and, after 3 hours at 28°C, cells were observed at the fluorescence microscope (left panel). Quantification of ROS positive cell was made in three independent experiments. In the right panel is shown the average with SD.
2.3 p53wt AND p53K351N INDUCE CELL DEATH WHEN EXPRESSED IN YEAST CELLS

In order to study the effect of the expression of p53 and p53K351N on cell viability and apoptosis, we compared the percentage of viability, measured by clonogenicity, after twenty hours from the expression of both protein forms in galactose without (Figure 2.3.1) or in the presence of the apoptotic inducer hydrogen peroxide (3mM H$_2$O$_2$ Figure 2.3.2).

The over-expression of both forms of p53 proteins resulted, in the wild type strain, in a reduction of about 50% viability. The reduction of viability was about 40% in yca1 and nma111 mutants. In the case of aif1 and nuc1 mutants, the loss of viability was quite low, suggesting that these strains are resistant to p53-induced cell death.

Aif1 and Nuc1/EndoG are two mitochondrial proteins that are released from this organelle upon an apoptotic stimulus. The fact the aif1 and nuc1 mutants were more resistant, compared to
the wild type, to p53 expression, suggests that Aif1 and Nuc1 proteins are involved in p53-promoted cell death. On the contrary, p53-cell death is only partially dependent from active caspase Yca1 or Nma111.

These results indicate that most of the p53 toxicity in yeast is mediated by the association of p53 to the mitochondria. There isn’t any evident difference between cell death induced by p53WT and p53K351N, except for the aif1 and nuc1 strains, that seem, especially nuc1, extremely resistant to the mutated form. As p53K351N shows a reduced capability to tetramerize and to move from the nucleus to cytoplasm, this mutated form would still capable to induce cell death only in the presence of an active Nuc1-EndoG protein.
Figure 2.3.1. Survival determined by clonogenicity of the indicated strains overexpressing p53, p53K351 and vector control (p-ESC) yeast cells after 20 hr induction on galactose. Average and standard deviation, obtained from three independent experiments, are reported.
We then analyze cell viability in the presence of p53 proteins upon an apoptotic stimulus such as hydrogen peroxide treatment. The comparison of cell viability after H$_2$O$_2$ treatment (Figure 2.3.2) has first pointed out differences between mutant and wild-type strains containing the plasmid control. In fact, all the mutated strains have a higher resistance to hydrogen peroxide, according to their role in mediating the apoptotic process. The expression of p53 leads to increased sensitivity to apoptotic stimuli, with the exception of nuc1 mutant in which is absent the mammalian EndoG homologous nucleases.

In fact, cell viability of nuc1 mutant that expresses both forms of p53 was similar to that shown by the control strain and is very high compared with that of all other analyzed strains over expressing the two forms of p53.

This result suggests that, at least in the yeast S. cerevisiae, cell death induced by an apoptotic insult in the presence of p53 is mediated through the action of Nuc1/EndoG.
**Figure 2.3.2.** Survival determined by clonogenicity of the indicated strains overexpressing p53, p53K351 and vector control (p-ESC) yeast cells after 20 hr induction on galactose + 4 h in 3mM H2O2. Average and standard deviation, obtained from three independent experiments, are reported.
2.4 EFFECTS OF p53wt AND p53K351N ON YEAST CHRONOLOGICAL LIFESPAN

Long term cultivation causes an aging processes in the whole yeast culture, called chronological aging (Fabrizio and Longo, 2003), which leads to physiological-induced apoptosis in yeast (Herker et al., 2004). Therefore, we investigated the effect of p53wt and p53K351N in chronological aging in wild type and mutant strains in genes involved in apoptosis. We observed that the wild type strains BY4741 expressing p53wt and p53K351N, as already discussed above, at the first day show a viability loss of about 50%. Cell death kinetics was then very similar for both p53 and p53K351N expressing strains (Figure 2.4.1).
Figure 2.4.1. Chronological aging determined by clonogenicity of BY4741 strains overexpressing p53, p53K351 and vector control (p-ESC). Average and standard deviation, obtained from three independent experiments, are reported.
In the absence of the gene YCA1 (Figure 2.4.2) or NMA11 (Figure 2.4.3) cell death kinetics were slower compared to the wild type. Anyway, also in these strains at the first day of p53 expression viability was approximately 50%.
**Figure 2.4.2.** Chronological aging determined by clonogenicity of *ycal* strains overexpressing p53, p53K351 and vector control (p-ESC). Average and standard deviation, obtained from three independent experiments, are reported.
Figure 2.4.3. Chronological aging determined by clonogenicity of *nma111* strains overexpressing p53, p53K351 and vector control (p-ESC). Average and standard deviation, obtained from three independent experiments, are reported.

The chronological aging of *aifl* and *nucl* mutant strains expressing the p53 proteins, showed that cells expressing the mutated form p53K351N had a cell death kinetics very similar to
that of the same strain expressing the control plasmid p-ESC (figure 2.4.4 and 2.4.5).

These results are in agreement with those described in the above paragraph and point to Aif1 and Nuc1 as the principles mediator of cell death upon p53 expression.
Figure 2.4.4. Chronological aging determined by clonogenicity of nuc1 strains overexpressing p53, p53K351 and vector control (p-ESC). Average and standard deviation, obtained from three independent experiments, are reported.
Figure 2.4.5. Chronological aging determined by clonogenicity of nuc1 strains overexpressing p53, p53K351 and vector control (p-ESC). Average and standard deviation, obtained from three independent experiments, are reported.
2.5 STUDY OF MITOCHONDRIAL MORPHOLOGY UPON p53 AND p53K351N EXPRESSION

It has been recently reported that the functional transcription-independent p53 apoptotic functions and p53-induced mitochondria fragmentation are conserved in yeast (Abdelmoula-Souissi et al, 2011; Coutinho et al, 2011).

The recombinant strains BY4741/pESC (negative control), BY4741/p53WT (expressing wt p53) and BY4741/p53K351N were transformed with a plasmid encoding the GFP targeted to the mitochondria allowing visualization of the mitochondrial morphology (Westermann and Neupert 2000). Cells were cultivated in SD and shifted in SGal for 20h to allow the expression of p53. As illustrated in Figure 2.5.1, panel A, cells expressing p53 presented fragmented mitochondria, whereas in those expressing the p53K351N the percentage of aberrant mitochondria was much lower. The panel B in figure 2.5.1 represents the quantification of glucose (SD) or galactose (SGal)
grown cells presenting fragmented mitochondria. It is evident that p53K351N mutated protein was not able to accumulate to mitochondria and promote its fragmentation (Muscolini et al., 2011 and Dott. Palermo personal communication).
Picture 2.5.1 K351N mutation affects p53-induced fragmentation of mitochondria in S. cerevisiae. The BY4741 S. cerevisiae strain cells containing pEsc-His, p53WT, and p53K351N, were all transformed with mito-GFP. Transformants were grown in glucose (SD), and then shifted for 21 h in minimal medium containing galactose (SGal). The mitochondria morphology (A) was observed by analyzing the GFP with an Axioskop2 fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with a digital camera (micro-charge-coupled device). The quantification of cell population showing fragmented mitochondria is reported in B. Data represent the mean and SD of three independent experiments.
3. CONCLUSIONS

The p53 phosphoprotein is probably the best known of all tumor suppressors. It is well established that loss or inhibition of p53 prevents cellular senescence and apoptosis. p53 is either lost or mutated in about half of all human cancers.

Muscolini et al. found that K351N substitution did not affect the overall half-life of p53 protein but significantly reduced its ability to form tetramers, when compared to p53wt. They also found that K351N substitution affected subcellular localization of p53 by impairing its export from nucleus induced by CDDP. These data identify K351N as a new cancer-associated mutant in the TD of p53 with reduced tumor suppressor activity and involved in the induction of resistance to drug-induced apoptosis (Muscolini et al., 2009).
The characterization of this mutant could thus help to envisage the development of new cancer drugs that can suppress this phenotype in tumors.

In the yeast *S. cerevisiae*, the machinery of the basic apoptotic process seems to be conserved as it presents many of the cytological markers of apoptosis such as chromatin condensation, DNA fragmentation and the release of cytochrome c from mitochondria to the cytoplasm. It was recently reported that the expression of p53 in *S. cerevisiae* is able to activate the apoptotic process, making this system a good model to analyze the basic mechanisms of apoptosis induced by p53.

The data obtained in this thesis, have highlighted that the over-expression of wild-type p53 and p53K351N proteins in yeast blocks cell cycle and reduces viability.

We noticed the loss of viability of about half of the cell population, suggesting that the over expression of p53 may play a role in removing old or damaged cells of the cell population.
This effect, pointed out in wild yeast strains, was attenuated in strains mutated in genes encoding component of the yeast apoptotic pathway. In fact, a complete block of cell cycle in yca1 mutant can be achieved only when p53 are over-expressed at 37°C only the p53 wild type is able to inhibit cell growth. The strains that showed the higher resistance to p53 expression were aif1 and nuc1, indicating that Aif1 and Nuc1 (EndoG) are the major proteins mediating p53-induced cell death in yeast. The p53 expression rendered cells more susceptible to cells death induced by H$_2$O$_2$, an apoptotic inducer, except for the nuc1 mutant that showed very little difference in cell viability of strains expressing both forms of p53 and the control plasmid, pointing to Nuc1 as an important protein mediating cell death induced by H$_2$O$_2$ in the presence of p53.

The aif1 and nuc1 mutants, in particularly the latter one, were also highly resistant to the mutated form p53K351N, measures both after 20h of gene expression induction and during chronological aging. Upon p53 over-expression we also observed
an increase in mitochondrial fragmentation, a phenomenon greatly reduced for the mutated K351N form. We can speculate that, in yeast cells, the expression of p53, beside its transcriptional effect, cause mitochondrial fragmentation that release the proapoptotic factors Aif1 and Nuc1. In the absence of these proteins this effect is attenuated and, when mitochondrial fragmentation does not occur (K351N) cell death is not completely induced.

On the contrary, the yeast caspase Yca1 and the Nma111 (Omi homologue), seem marginally involved in mediating p53 toxicity.

In the future we plan to explore more p53 functions through the use of multiple mutants involved in mitochondrial morphology and post-translational modifications linked to the p53 cytoplasmic function.

The genetic tractability of budding yeast, its ease of manipulation and the wealth of functional genomics tools available will be very useful to isolate proteins involved in this particular function of p53, a protein so important in human disease.
4. MATERIALS AND METHODS

*Transformation of yeast cells with lithium acetate*

Exponentially growing cells were collected at the concentration of $2 \times 10^7$ cells / ml, collected by centrifugation, washed once with sterile water and then washed with 1 ml of 100 mM lithium acetate.

After centrifugation, cells were incubated with a reaction mixture composed of:

- 240 µl PEG (50% w/v)
- 36 µl 1.0 M Liac
- 50 l SS-DNA (2.0 mg/ml)
- plasmid DNA (0.1-1.0 g)
- 34-µl sterile dd H$_2$O

After incubation at 28 °C for 30 min, cells were heat shocked at 42 °C for 30 min., centrifuged, resuspended in 1 ml of sterile H$_2$O and plated on selective medium.
Cell viability determination

Cells were grown at 28 °C in SD medium (yeast nitrogen base) containing auxotrophic requirements as needed. 5 µl of cell suspensions containing approximately 6·10⁶ cells / ml were poured on a thin layer of YPD agar on a microscope slide. A cover slip was placed over the samples and, after 24 h, viable and unviable cells were scored on the basis of their ability to form microcolonies.

H₂O₂ sensitivity determination

Exponentially growing cells were treated with 0.8, 1.2 and 3 mM H₂O₂ for 4h at 28°C. After treatments, cell viability was measured as described above.

Fluorescence microscopy

The presence of ROS was detected with DHR (Sigma Aldrich Co. D1054), as described previously (Madeo et al,1999).
Free 30-OH ends were detected by TUNEL on cells grown for 9 days, as described (Madeo et al., 1997).

*Phenotypes determination by microtiter analysis*

Cells were grown at 28 °C in SD medium additioned with auxotrophic requirements. 5 µl of 10-fold dilutions were the spotted on solid SD medium supplemented, as specified in the text, with 2% galactose (S-Gal), 3% glycerol (SY) 0,10% caffeine (SD+caffeine) or temperature at 37° C. Growth of colonies was observed after incubation of plates for 3 days at 28 °C.

*Protein extracts, SDS-PAGE and western blot analysis*

Exponential and stationary phase cells

Cell extracts were prepared from culture growing at 28 °C in shaked flasks containing SD medium with the addition of auxotrophic requirements. Exponential cells were collected at 0.2 OD600 density while stationary cells were grown for two additional days.
Samples were centrifuged at 4 °C and washed with water. Cells were resuspended with 0.2 ml of 2M NaOH, 5% β-mercaptoethanol and incubated in ice for 10 min. After the addition of 40 ml of 50% trichloro acetic acid, samples were incubated in ice for further 10 min. After centrifugation, cell pellets were dried at room temperature for 5 min and resuspended in lysis buffer (50mM Tris-HCl, pH 6.8, 10% β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol).

Proteins in the extracts were separated by standard SDS-polyacrylamide gel electrophoresis procedures.

Detection of proteins by western blot analysis

After SDS-polyacrylamide gel electrophoresis fractionation, proteins were transferred following standard protocols onto commercial membrane. The protein p53 was detected by hybridization with the antibody goat anti-mouse SC126 and goat anti-mouse.
Signals were revealed with Super Signal West Pico chemiluminescent substrate HRP (Pierce).
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RINGRAZIAMENTI

Nel completare questo mio percorso di studi desidero ringraziare la mia relatrice, Cristina Mazzoni, che mi ha seguito scrupolosamente nel corso del dottorato di ricerca, tutte le persone che ho incontrato in questi tre anni, Vanessa Palermo, Eleonora Mangiapelo, Francesco Giorgi, Federica Straforini, Luisa Pieri, Patrizia Luise, Michele Saliola, Michela Pitorri, tutti coloro che sono passati per il lab 25, tutti i “residenti” del piano rialzato, e tutti gli altri dottorandi che stanno raggiungendo con me questo traguardo.

Ringrazio il Prof. Claudio Falcone e tutti gli altri docenti e ricercatori che sono stati presenti nel corso delle mie giornate lavorative in questi tre anni, e quelli che mi hanno invece guidato verso la laurea.

Desidero ringraziare anche i miei vecchi compagni di studi, che hanno proseguito con me, anche se in modi diversi, i loro percorsi formativi.
Cristina Piloto

Un ringraziamento particolare va al dott. Carlo Anzilotti e a tutte le persone che insieme a me stanno lavorando per conquistarsi un'identità sempre più bella e solida, e che permetta di raggiungere obiettivi concreti.

Ringrazio tutte le persone che mi stanno vicino, e che credono in me e nelle mie capacità.