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**Effect of aberrant ribosome biogenesis on
wild-type and mutant p53 in cancer cells**

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**Effect of aberrant ribosome biogenesis on wild-type and
mutant p53 in cancer cells**

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ABSTRACT

The tumor suppressor p53 plays a central role in the protection against DNA damage and other forms of stress, primarily by inducing cell cycle arrest or apoptosis. Missense mutation of p53, which is one of the most frequent genetic alterations detected in human cancers, inactivates these growth regulatory functions; in addition, mutant p53 often acquires tumor-promoting activities (gain-of-function). A complete and thorough understanding of the signaling circuitry that regulates wild-type and mutant p53 functions is therefore a primary objective for basic cancer research, since it may lead to development of important tools for diagnosis and therapy of tumors. One crucial component of such knowledge is the molecular mechanism leading to protein activation.

Deregulated ribosome biogenesis is commonly observed in cancers as a result of increased biosynthetic demand due to uncontrolled cell proliferation. Cells actively monitor the fidelity of ribosome biogenesis, activating cellular checkpoints when this process is altered. Indeed, perturbations to many aspects of ribosome biogenesis generate a “nucleolar stress” that triggers a p53 response. Evidence in animal models indicates that the ribosomal-p53 checkpoint is indeed important for tumor suppression *in vivo*.

In the recent past, we identified the nucleolar protein GTPBP4 as a novel p53 interactor, and established a functional link between the two proteins by demonstrating that GTPBP4 depletion promotes p53 accumulation and activation.

In this Thesis, I demonstrate that GTPBP4 depletion affects 28S rRNA processing, generating a “nucleolar stress” responsible for p53 stabilization. The molecular mechanism responsible for such accumulation relies on the binding of ribosomal proteins to Mdm2. In parallel, I report that the same molecular mechanism leading to wild-type p53 stabilization upon ribosomal stress, can also promote mutant p53 accumulation in cancer cells. Thus, deregulated ribosome biogenesis may contribute to the stability of mutant p53 in tumors.

These results collectively suggest that upon alterations in ribosome biogenesis, the TP53 status may be critical to drive the nucleolar surveillance pathway towards a tumor suppressive or oncogenic outcome.

FOREWORD

During my PhD I worked on two different projects. Although both concern the analysis of the effects of deregulated ribosome biogenesis in cancer progression, they are markedly distinct; for this reason I describe them as separate parts in this Thesis.

The first project is focused on the molecular mechanism leading to p53 accumulation upon depletion of the nucleolar protein GTPBP4. This project derived from a study that was made in our laboratory and that was already published (Lunardi et al., 2010). This work is described in PART 1.

The second project stemmed from some observations made during the previous project, and is focused on the study of a molecular link between deregulated ribosome biogenesis and mutant p53. This work is described in PART 2.

PAPERS NOT DIRECTLY RELEVANT TO THE THESIS

During my PhD I was also involved in a collaboration to study the impact of some Parkinson's disease associated proteins on ribosome biogenesis and rRNA maturation. This collaboration led to the following publications:

Parkinson's disease DJ-1 L166P alters rRNA biogenesis by exclusion of TTRAP from the nucleolus and sequestration into cytoplasmic aggregates via TRAF6.

Vilotti S, Codrich M, **Dal Ferro M**, Pinto M, Ferrer I, Collavin L, Gustincich S, Zucchelli S. PLoS One. 2012.

The PML nuclear bodies-associated protein TTRAP regulates ribosome biogenesis in nucleolar cavities upon proteasome inhibition.

Vilotti S, Biagioli M, Foti R, **Dal Ferro M**, Lavina ZS, Collavin L, Del Sal G, Zucchelli S, Gustincich S. Cell Death Differ. 2012.

INTRODUCTION

Cancer is a complex disease involving dynamic changes in the genome that allow cells to bypass those regulatory circuits which modulate normal cell proliferation and homeostasis. Besides many individual differences, all human tumors share common features: self-sustained proliferative abilities, insensitivity to growth inhibitors and apoptosis, limitless replicative potential, sustained angiogenesis, and acquired ability to invade and metastasize (Hanahan and Weinberg, 2000). Besides these six features, human tumors may take advantage from an inflammatory microenvironment (Mantovani, 2009), and from reprogramming cellular metabolism to sustain deregulated cell proliferation (Hanahan and Weinberg, 2011).

From a molecular point of view, oncogenic transformation is a multistep process characterized by a gradual alteration of normal cells into cancerous cells and requires alterations in various cellular processes including both activation of oncogenic pathways and inhibition of tumor suppressor pathways, therefore promoting growth and survival of cancer cells (Hanahan and Weinberg, 2000).

The p53 pathway

Among the tumor suppressor pathways frequently deregulated in cancer, that of p53 is one of the most important. The hub of this pathway is the protein p53, a transcription factor that was discovered in 1979, as a protein associated with simian virus 40 (SV40) large T-antigen (Kress et al., 1979; Lane and Crawford, 1979; Linzer and Levine, 1979). More than 30 years of research unveiled the complexity and the importance of the p53 pathway in tumor suppression. p53 is indeed at the center of a signaling network: many upstream pathways that work as a sensor for a great variety of potentially oncogenic stresses, converge to p53, which in turn translates stress signals into different cellular outcomes (Kruse and Gu, 2009). For this crucial role, p53 has been defined the “guardian of the genome” (Lane, 1992).

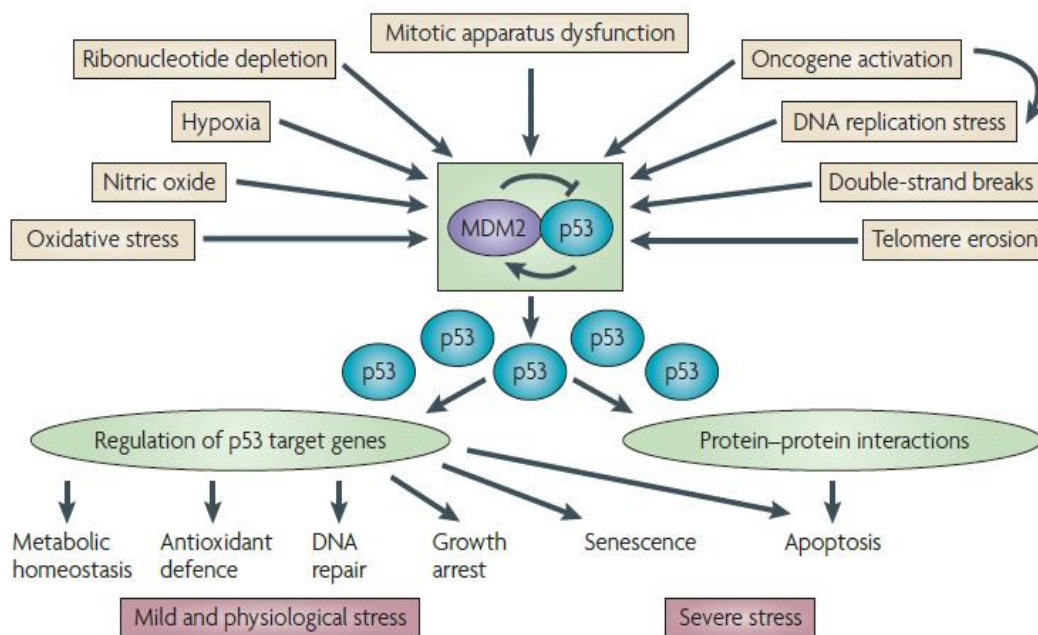


FIGURE 1. Simplified scheme of the p53 pathway. The p53–MDM2 feedback loop is the ‘heart’ of the p53 pathway. Under normal conditions, it maintains constantly low steady-state p53 levels and activity. Various stress signals, related in many ways to carcinogenesis, impinge on this central loop to release p53 from MDM2-mediated inhibition. This increases p53 protein levels and activity, inducing various phenotypic changes. The downstream effects of p53 are largely due to its ability to transactivate and repress various subsets of target genes; however, at least in the case of apoptosis, protein–protein interactions also have an important role. The nature of the phenotypic response to p53 activation is, at least partially, proportionate to the amplitude, duration and nature of the activating signal. Severe stress induces more extreme, usually irreversible, responses, namely apoptosis and senescence, whereas milder stress leads to a transient growth arrest coupled with an attempt to deal with the cause of stress and repair the damage caused by it. Recent evidence indicates that p53 also has an important role in enabling the cell to adjust its metabolism in response to mild normal physiological fluctuations, including those in glucose and other nutrient levels, oxygen availability and reactive oxygen species levels. (Levine and Oren, 2009).

The core of this complex pathway is the regulatory mechanism that controls p53 protein levels. Under physiological conditions p53 is maintained at low levels by continuous ubiquitination by its major E3 ubiquitin ligase Mdm2 and subsequent degradation by 26S proteasome (Haupt et al., 1997; Kubbutat et al., 1997). Many stress signals can impinge on this regulatory axis, leading to p53 stabilization, accumulation and activation. These events are regulated by a defined combination of post-translational modifications within p53 and by protein-protein interactions. Once activated, p53 acts mainly as a transcription factor able to promote the expression of a set of genes whose expression mediates different cellular outcomes such as cell cycle arrest, senescence or apoptosis (Junttila and Evan, 2009).

The importance of the p53 pathway in tumor suppression is highlighted by the fact that the p53 gene is mutated in more than 50% of human cancers (Brosh and Rotter, 2009), while in the remaining cancers, mutations affecting upstream or downstream components of the p53 regulatory network are frequently found, thus perturbing the outcome of p53 pathway functionality (Vousden and Lane, 2007; Vousden and Prives, 2009). Moreover, patients affected by the Li-Fraumeni syndrome, in which one mutant allele of p53 is germ-line inherited, show increased chance of developing early-onset cancers (Malkin et al., 1990). Finally, mice homozygous for the null allele of p53 have normal embryonic development but are prone to the spontaneous development of a variety of neoplasms by 6 months of age (Donehower et al., 1992).

The p53 protein

p53 is a 393 aa protein with evolutionary conserved functional domains: an N-terminal transactivation domain (TAD), a prolin-rich domain (PR), a central DNA-binding domain (DBD), an oligomerization domain (OD) and a C-terminal regulatory domain.

The N-terminal TAD is crucial for p53 transcriptional activity since it mediates the interaction with components of the basal transcriptional machinery. Moreover is finely regulated both by post-translational modifications and by interaction with p53 partners such as p300/CBP, Mdm2 and Pin1 (Mantovani et al., 2004; Mantovani et al., 2007; Marine and Lozano, 2010).

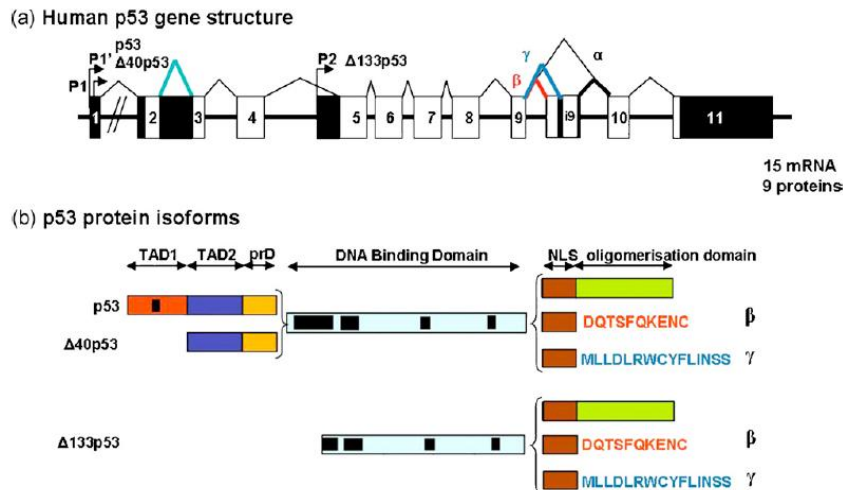


FIGURE 2. Schematic representation of human p53. (A) Structure of the human p53 gene: alternative splicing (α , β , γ) and alternative promoters (P1, P1' and P2) are indicated. (B) p53 protein isoforms: p53, p53 β and p53 γ proteins encoded from P1 or P1' promoters contain the conserved N-terminal domain of transactivation (TA). $\Delta 133p53$ isoforms encoded from promoter P2 are amino-truncated proteins deleted of the entire TA domain and deleted of part of the DNA-binding domain. Translation is initiated at ATG-133. $\Delta 40p53$ protein isoforms encoded from P1 or P1' promoters are amino-truncated proteins due to alternative splicing of exon 2 and/or alternative initiation of translation at ATG-40). $\Delta 40p53$ protein isoform have lost the conserved N-terminal domain of transactivation, but still contain part of the transactivation domain. $\Delta p53$ protein isoform is due to noncanonical alternative splicing between the exon 7 and 9. $\Delta p53$ has lost 66 residues including the highly conserved domain V of the DNA-binding domain. The isoforms $\Delta p53\beta$, $\Delta p53\gamma$, $\Delta 40\Delta p53\beta$, $\Delta 40\Delta p53\gamma$, $\Delta 133\Delta p53$, $\Delta 133\Delta p53\beta$ and $\Delta 133\Delta p53\gamma$ should theoretically be generated (Bourdon, 2007).

The PR domain (aa 61-94) derives its name from the presence of five repeats of the amino acid motif PXXP (P=prolin; X=any amino acid). The PXXP motifs may influence both p53 function, by indirectly affecting p53 acetylation by p300 (Dornan et al., 2003) and stability, since the prolyl isomerase Pin1, upon Thr81 phosphorylation, binds to Thr81-Pro82 site and induces a conformational change leading to reduced Mdm2 binding and increasing p53 stability (Zacchi et al., 2002).

The central region of the protein contains the sequence-specific DNA binding domain that is essential for the p53 activity as a transcriptional factor. The canonical p53-responsive element (RE) contains two copies of the 10 base pair motif 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3' separated by 0-13 base pairs (el-Deiry et al., 1992). Besides DNA, the DBD is bound also by p53 cofactors that play important roles in defining p53 functions, such as Hzf and ASPP proteins (Ahn et al., 2009; Das et al., 2007) that positively affect p53 activities and other proteins that negatively regulate p53, such as Mdm2. Mdm2 indeed has been shown to interact also with DBD

(Shimizu et al., 2002) and this interaction strengthens the binding between the two proteins (Dawson et al., 2003).

The oligomerization domain (OD) is required for the formation of a high affinity DNA binding and transcriptional competent p53 tetramer (Shieh et al., 1999).

The C-terminal (CT, aa 364-393) domain of p53, in particular the last 30 amino acid is a regulatory domain for p53 activity, indeed, upon stress signaling, it undergoes a great number of post translational modifications at almost every residue that are essential for p53 activity (Kruse and Gu, 2009).

Interestingly, additional p53 isoforms have been identified, but their biological functions and expression are still largely unknown. An alternative internal promoter coupled to alternative initiation and splicing sites allow for nine distinct isoforms. The alternative splicing of the intron 9 produces three isoforms different in their C-terminus: p53 (or p53 α), p53 β and p53 γ , with p53 β and p53 γ lacking the oligomerization domain. The use of an alternative initiation of translation or an alternative splicing of intron 2 may encode for Δ 40p53, which lacks part of the TAD, while, an alternative promoter in intron 4 (P2) leads to the expression of an amino-terminally truncated p53 protein (Δ 133p53) (Machado-Silva et al., 2010).

Mechanism involved in the regulation of p53

Modulation of p53 stability

In normal physiological conditions, p53 levels are kept low by ubiquitin-dependent proteasomal degradation (Vogelstein et al., 2000). Upon stress conditions post-translational modifications and interaction with different partners promote p53 stabilization and activation, thereby leading to a fully active protein (Kruse and Gu, 2009).

p53 inhibition occurs mainly through the E3 ubiquitin ligase Mdm2. Mdm2 regulates p53 in two ways by direct binding at the N-terminal of the protein, masking the TAD to the basal transcriptional machinery, and by promoting p53 C-terminal lysine ubiquitination (Clegg et al., 2008). Mdm2 promotes both mono- and poly-ubiquitination of p53; when p53 is mono-ubiquitinated, it is exported into the cytoplasm and translocates at the mitochondria (Marchenko et al., 2007). Conversely, p53 poly-ubiquitination induces its degradation by the 26S proteasome (Lee and Gu,

2010). p53, in turn, promotes the expression of Mdm2, thus generating a negative feedback loop controlling its own levels (Stommel and Wahl, 2005). The other important regulator of the Mdm2-p53 axis is MdmX (Shvarts et al., 1996). Mdm2 and MdmX are structurally related proteins, however, despite its sequence homology with Mdm2 and the presence of a RING domain, MdmX does not have intrinsic E3-ligase activity for p53. By binding Mdm2, MdmX can instead promote its activity towards p53 (Linares et al., 2003) and inhibits p53-induced transcription by masking its transactivation domain (Oliner et al., 1993). Mdm2 and Mdmx are thus non redundant p53 inhibitors, and this is further highlighted by the fact that both are overexpressed in several cancers acting as oncogenes (Bond et al., 2004; Danovi et al., 2004).

Many proteins can modulate Mdm2-p53 interaction. Upon oncogenic signaling, the tumor suppressor ARF (p14 alternative reading frame) increases p53 stability down-modulating Mdm2 ubiquitin ligase activity by sequestering it to the nucleolus (Brooks and Gu, 2004). Notably, p53 itself down-regulates ARF expression (Robertson and Jones, 1998). Similarly, p53 stabilization and activation is affected by other pathways that crosstalk with the Mdm2-p53 axis. For example the AKT kinase phosphorylates Mdm2 inducing its translocation into the nucleus, where it down-regulates p53. Conversely, the tumor suppressor PTEN inhibits AKT, thus promoting p53 levels (Mayo and Donner, 2002). The positive feed-back loop is realized by p53, that promotes PTEN expression at a transcriptional level (Stambolic et al., 2001).

Upon stress, p53 activity rapidly increases, and the required step is the abrogation of Mdm2-mediated degradation. This is achieved by the action of different kinases, such as ATM, Chk1 and Chk2, that upon stress signaling lead to the coordinated phosphorylation of p53, Mdm2 and MdmX, thus releasing p53 from negative regulation (Efeyan and Serrano, 2007). Mdm2 and MdmX phosphorylation, enhance their degradation by both auto-ubiquitination and by reduced association with the de-ubiquitinating enzyme HAUSP, which in turn, can promote also p53 de-ubiquitination (Brooks et al., 2007).

Furthermore, the cytoplasmic ubiquitin protease USP10 plays an important role in modulating p53 localization and stability. Indeed, USP10 specifically de-ubiquitinates p53, thereby reversing Mdm2-induced p53 nuclear export and degradation (Yuan et al., 2010).

In addition to Mdm2, a number of other p53 E3-ubiquitin ligases have been described in the last decade. Among them Pirh2 and COP1, are p53-induced genes that participate in an auto regulatory negative feedback loop similar to that of Mdm2 (Dornan et al., 2004; Leng et al., 2003).

p53 post-translational modifications

p53 is subjected to a great variety of post-translational modifications (PTMs) that influence its stability and transcriptional activity. Most of these modifications occur both at the N- and C-terminal of the protein. In general, the N-terminal phosphorylations affect p53 stability and allow the C-terminal acetylations that, in turn, promote p53 transcriptional activity. More than 36 residues within p53 have been reported to be modified. Besides phosphorylation and acetylation, p53 is mono- and poly-ubiquitinated, sumoylated, neddylated, and methylated (Kruse and Gu, 2009).

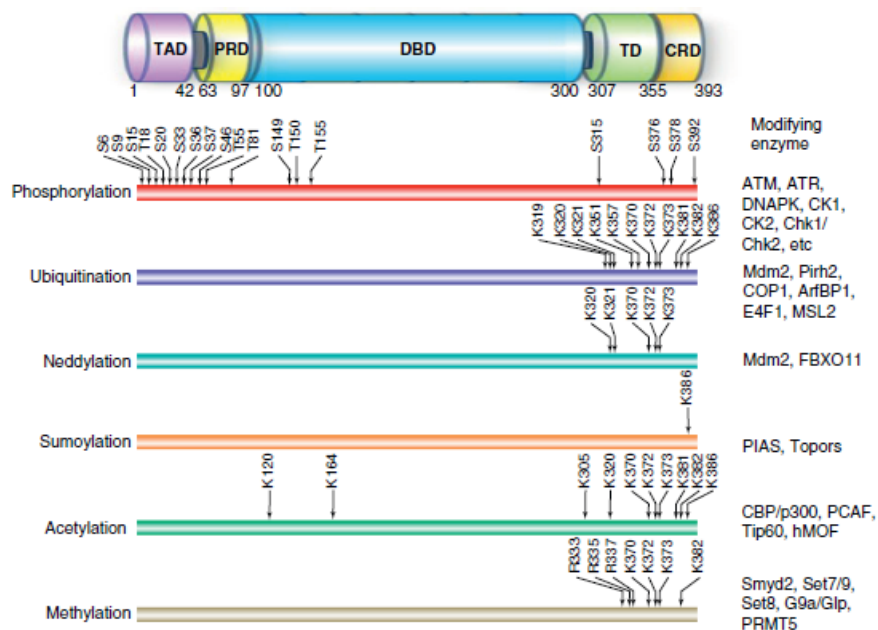


Figure 3. Overview of p53 domain structure and post-translational modifications. The major sites for p53 phosphorylation, ubiquitination, neddylation, sumoylation, acetylation and methylation are plotted. The enzymes responsible for each type of modification are shown on the right. Abbreviations: TAD, transactivation domain; PRD, proline rich domain; DBD, DNA-binding domain; TD, tetramerization domain; CRD, C-terminal regulatory domain (Dai and Gu, 2010).

Phosphorylation. Phosphorylation is the best characterized p53 post-translational modification. Many kinases, including ATM, ATR, Chk1, Chk2, CK1, CK2, JNK,

Erk, HIPK2, and others, have been reported to phosphorylate p53 after cellular stress (Kruse and Gu, 2009; Vousden and Prives, 2009). Interestingly, a single site can be phosphorylated by different kinases and vice versa a single kinase can phosphorylate different sites. This redundancy might be explained as a failsafe mechanism of protection or by the fact that a distinctive pattern of phosphorylation is required for further modifications, thus allowing a fully and specific activation (Kruse and Gu, 2009). There are, however, residues that are constitutively phosphorylated in unstressed conditions and are dephosphorylated following stress (e.g. T55) (Li et al., 2007).

Acetylation. Acetylation is a powerful mechanism to promote p53 stability and transcriptional activity. Indeed C-terminal lysines acetylation excludes ubiquitination on the same sites and prevents the formation of Mdm2/MdmX inhibitory complexes on target gene promoters; moreover, it allows recruitment of cofactors essential for p53 transcriptional activity (Dai and Gu, 2010).

Histone Acetyl transferases (HATs), such as p300/CBP and PCAF (p300/CBP-associated factor), are responsible for p53 acetylation in response to DNA damaging agents, such as UV- and γ -irradiation (Carter and Vousden, 2009). Conversely, the equilibrium in p53 acetylation is maintained by Histone Deacetylases (HDACs), such as HDAC1 and SIRT1 (Luo et al., 2001; Luo et al., 2000).

Regulation by co-factors

There are proteins that affect p53 activity by selective interaction with the tumor suppressor, indeed, more than 150 p53-interacting proteins have been described so far (source: BioGrid and Mint databases). Many of them, influence specifically p53 transcriptional activity, and thereby its functions, acting as co-activators or co-repressors. For example, ASPP (Apoptosis stimulating proteins of p53) family members ASPP1 and ASPP2 enhance the proapoptotic function by selectively promoting the binding of p53 to proapoptotic genes like BAX, PUMA and PIG3 (Samuels-Lev et al., 2001), conversely, iASPP (inhibitory member of the ASPP family) prevents transcriptional activity of p53 bound to pro-apoptotic promoters (Bergamaschi et al., 2003).

Some p53 interactors possess histone modifying activity, suggesting that targeted chromatin remodeling might be important for p53 transcriptional activity. Indeed,

p53-dependent changes in histone acetylation have been observed at the promoter of many p53 target genes (Kaeser and Iggo, 2002; Shan et al., 2003).

p53 post-translational modifications may selectively guide co-regulator recruitment, thus providing a means for a fine tune p53 modulation in response to different stresses.

p53 biological functions

Upstream events activating the p53 pathway

A wide range of stress signals, that could affect genome integrity and cell proliferation, promote the activation of the p53 pathway.

Historically, DNA damage was the first type of stress found to activate the p53 pathway (Lane, 1992). DNA damage can originate from exogenous stimuli, such as UV, ionizing radiations and from chemical mutagenic compounds, or from normal cellular processes such as metabolism and replication. For example, DNA damage can be induced by by-products of the oxidative metabolism such as reactive oxygen species (ROS), premature termination of replication fork progression (Branzei and Foiani, 2005, 2010), and by telomere erosion consequent to continuous replication (d'Adda di Fagagna et al., 2003). Remarkably, specifically in precancerous lesions and not in normal tissue, the DNA damage response pathway is permanently activated, suggesting that cellular transformation generates DNA damage, and cells activate a barrier to counteract cancer progression (Bartkova et al., 2005).

DNA damage activates different signaling pathways that converge on p53 promoting a complex combination of p53 post-translational modifications necessary to promote its stabilization and its transcriptional activity (Kruse and Gu, 2009). The two DNA damage sensor kinases, ATR and ATM, and their downstream kinases Chk1 and Chk2, are indeed able to phosphorylate p53 at different sites, promoting its stabilization and activation (Hammond et al., 2002; Hirao et al., 2000; Pabla et al., 2008; Perona et al., 2008).

Other kinases that have been reported to phosphorylate and activate p53 in response to different stress signals are for example JNK, HIPK2 and p38 (Kruse and Gu, 2009).

Oncogenic signaling activates p53 not only through the induction of the DNA damage cascade, but also through the transcriptional activation of p14^{ARF} (de Stanchina et al., 1998; Palmero et al., 1998).

Other stimuli have been reported to induce and activate the p53 pathway: among them altered ribosome biogenesis (that will be discussed below), heat and cold shock (Ohnishi et al., 1998a; Ohnishi et al., 1998b), presence of denaturated proteins, depletion of ribonucleotide-phosphate pool in the cell (Khan et al., 2000), spindle damage leading to defective chromosomal segregation (Lanni and Jacks, 1998), nitric oxide production associated with infections and inflammation (Hofseth et al., 2003), and hypoxia (Liu et al., 2007).

Outcomes of the activation of the p53 pathway

The activation of the p53 pathway by stress signals promotes different cellular outcomes, such as the induction of reversible cell cycle arrest, apoptosis, senescence and others, depending on the cell type, the nature, duration and intensity of the stimuli, and finally on the protein partners that affect its stability and activity.

Once activated, p53 acts mainly as a sequence-specific transcription factor to induce the expression of a large set of genes. Moreover, p53 has been also shown to promote cellular outcomes in a transcription-independent way, through direct interaction with other proteins.

The activation of the p53 pathway can induce cell cycle arrest mostly promoting the expression of three target genes: p21, 14-3-3 σ and GADD45 (Vousden and Lane, 2007; Vousden and Lu, 2002). The expression of the cyclin-dependent kinase inhibitor p21, results in cell cycle arrest at G1 phase due to inhibition of cyclinE/CDK2, cyclinA/CDK2 and cyclinD/CDK4 (Harper et al., 1993), while 14-3-3 σ and GADD45 expression is responsible for p53-induced G2 arrest (Hermeking et al., 1997; Kastan et al., 1992). 14-3-3 σ prevents nuclear import of cyclin B1 and CDC2 (Chan et al., 1999) whereas GADD45 destabilizes CDC2/cyclinB complexes, and these two processes cooperate to prevent initiation of mitosis (Jin et al., 2002).

Cell cycle arrest allows the activation of pathways that promote the correction of the damage. Indeed p53 has been reported to directly promote DNA repair through the modulation of nucleotide excision repair (NER), base excision repair (BER) and

double strand break (DSB) repair (Albrechtsen et al., 1999; Bertrand et al., 2004; Paquet et al., 2004). p53 is also involved in inhibiting homologous recombination (HR) in response to replication fork stalling (Janz and Wiesmuller, 2002) and it prevents the accumulation of DSBs at stalled-replication forks (Kumari et al., 2004).

The elimination of damaged cells that might develop into malignant counterpart can be achieved through the induction of apoptosis (Clarke et al., 1993; Debbas and White, 1993). p53 promotes apoptosis through the transcriptional modulation of genes involved in both the extrinsic and intrinsic pathway. p53 promotes the expression of genes coding for death receptors and associated proteins such as Fas, DR4 and KILLER/DR5, PERP and Pidd, thus activating the extrinsic apoptotic pathway (Balint and Vousden, 2001). Other p53 regulated genes such as Bax, Puma and Noxa are involved in the release of the cytochrome c from the mitochondria into the cytoplasm thus activating the intrinsic apoptotic pathway (Miyashita and Reed, 1995; Nakano and Vousden, 2001) (Yu et al., 2001). p53 can promote apoptosis also in a transcription independent manner, in response to genotoxic, hypoxic, and oxidative stress a fraction of p53 translocates to the mitochondria (Marchenko et al., 2000) where it interacts with Bcl-2, Bcl-xL, and Bak promoting the release of cytochrome c (Leu et al., 2004; Mihara et al., 2003).

Another mechanism by which the p53 pathway counteracts neoplastic transformation is through the induction of senescence. Senescence is characterized by irreversible cell cycle arrest and morphological changes in the cell triggered by physiological conditions, such as telomeres shortening (replicative senescence), or by endogenous and exogenous stimuli such as oncogene activation, loss of tumor suppressor genes, oxidative stress and others (Itahana et al., 2004; Zuckerman et al., 2009). The p53 and p16/INK4a-RB pathways are mainly responsible for the execution of the proliferative arrest that characterizes senescence (Campisi, 2005). The main executor in mediating p53-induced senescence is its transcriptional target p21 (Serrano et al., 1997) which is transcribed as a result of the activation of the DNA damage response pathway (Saretzki, 2010). Several lines of evidence suggest that *in vivo* the induction of cellular senescence program is the main barrier that p53 opposes to cellular transformation (Xue et al., 2007).

In recent years, the p53 pathway has been demonstrated to be involved in the regulation of other cellular processes such as cell metabolism (Vousden and Ryan, 2009), and cell polarity (Cicalese et al., 2009).

Mutant p53

Mutations in the TP53 gene are very frequently found in human tumors, even exceeding 50% of cases depending on tumor type (COSMIC database <http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/>).

Unlike other tumor suppressor genes, whose expression is usually abrogated by deletions or truncating mutations, in the case of TP53, more than 70% of the mutations are missense, consequently leading to the expression of a full length protein, which differs from the wild-type counterpart in a single amino acid substitution.

Mutations in the TP53 gene are not randomly dispersed along the whole sequence, but are generally found in exons 4-9, which encode for the DNA-binding domain of the protein (Fig. 4). Interestingly, about 30% of all p53 mutations fall within 6 “hotspot” codons (Hollstein et al., 1991; Petitjean et al., 2007). TP53 mutations can be grouped into two main categories accordingly to their effect on the thermodynamic stability of the p53 protein (Bullock and Fersht, 2001). Mutations affecting residues directly involved in DNA binding, such as R248Q and R273H, are referred to as “DNA-contact” mutations. Conversely, mutations that cause local, such as R249S and G245S, or global, such as R175H and R282W, structural distortions are referred to as “conformational” mutations.

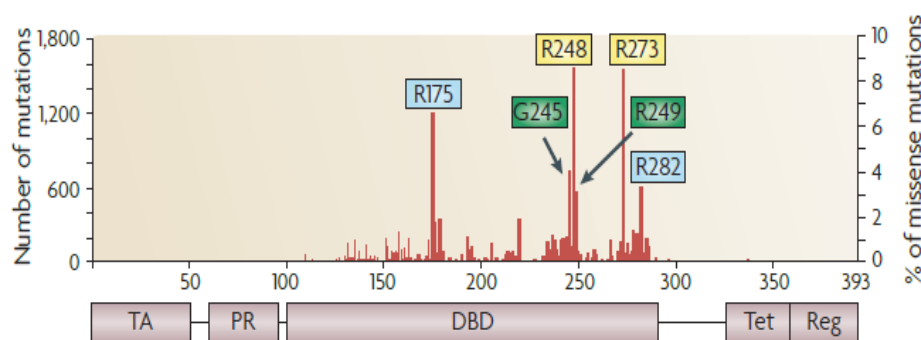


FIGURE 4. The distribution of reported missense mutations along the p53 sequence. The six most common hotspot mutations are highlighted in yellow for DNA-contact mutations, green for locally distorted mutants and blue for globally denatured mutants. The domain architecture of p53 is aligned below. TA, transactivation domain; PR, proline-rich domain; DBD, DNA binding domain; Tet, tetramerization domain; Reg, carboxy-terminal regulatory domain. Data derived from the *IARC TP53 Database*.

The frequency of point mutations suggests that mutation of p53 might provide to cancer cells a selective advantage with respect to simple loss of the protein.

In fact, p53 mutations are involved in the process of tumorigenesis by three non-mutually exclusive mechanisms (Brosh and Rotter, 2009).

First, mutations abolish the tumor suppressive functions of p53, mainly reducing or abrogating the binding of p53 to its consensus DNA sequence, thereby preventing the transcriptional activation of p53 target genes (Kato et al., 2003). Wild type p53 loss of functions (LOF), besides missense mutations of the TP53 gene, can be achieved also by gene deletion and truncations, splicing and nonsense mutations.

Second, most missense mutations produce a full length protein capable of inhibiting, to varying degrees, the function of the wild type protein encoded by the second allele. This dominant negative (DN) effect is achieved by the oligomerization of mutant with wild type p53. The result is a heterotetramer defective in sequence-specific DNA binding (Milner and Medcalf, 1991). Although this concept has been proved in various models, heterozygous p53mut/wt tumors are rarely detected, indicating this condition is not stable during cell transformation.

Finally, mutant p53 acquires new functions, termed gain of function properties (GOF), not present in the wild type protein, that actively contribute to various aspect of tumor progression.

Mechanism of the Gain of function of mutant p53

There are multiple proposed mechanism that account for different p53 gain of function activities. These include both transcriptional and non-transcriptional mechanisms (fig. 5).

Mutant p53 has been reported to interact with p53 family members p63 and p73 (Di Como et al., 1999; Gaiddon et al., 2001) and to inhibit transactivation of their target genes (Marin et al., 2000; Strano et al., 2002). The binding to p63 and p73 has been linked to the ability of mutant p53 to promote chemoresistance, migration, invasion and metastasis (Adorno et al., 2009; Muller et al., 2009; Sampath et al., 2001).

Despite mut-p53 proteins are unable to recognize wt-p53 consensus (Kato et al., 2003), mutant p53 has been shown to transactivate genes involved in different aspects of tumorigenesis (Brosh and Rotter, 2009; Girardini et al., 2011). A proposed mechanism is through the interaction with other sequence specific transcription

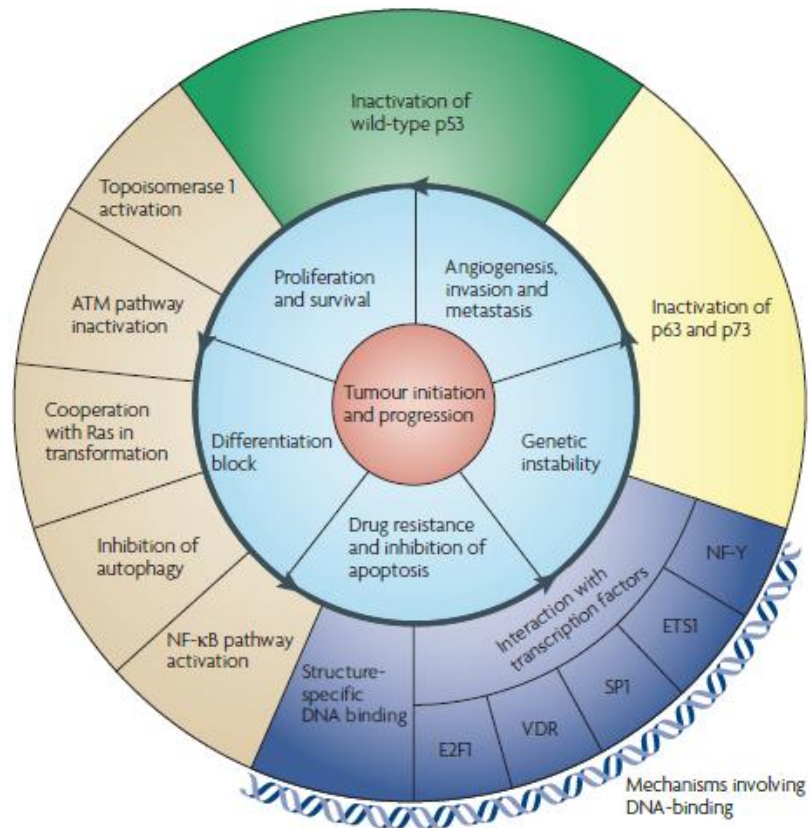


Figure 5. Selected oncogenic properties of mutant p53 and their underlying mechanisms. The inner circle (shaded blue) represents oncogenic phenotypes associated with the activities of mutant p53 proteins. The outer circle depicts key mechanistic properties of p53 mutants that underlie the phenotypes listed in the inner circle. Each of the phenotypic effects can be attributed to almost each of the mechanistic properties; hence the inner blue circle can be freely rotated (Brosh and Rotter, 2009).

factors, specific co-activators, histone-acetyltransferases or de-acetylases augmenting or repressing the expression of genes that are targeted directly by partner factors (Oren and Rotter, 2010). Besides p63 and p73, the first two transcription factors shown to interact with mutant p53 were SP1 and ETS1 (Chicas et al., 2000); (Sampath et al., 2001). The transcriptional complex modulated by mutant p53 and these transcription factors promotes pro-survival signals.

In addition several p53 mutants have been reported to bind specific DNA structure with high affinity (Gohler et al., 2005), therefore suggesting that mutant p53 specificity towards certain regulatory elements could be mediated by preferential binding to structural DNA motif rather than consensus sequences.

Interactions between mutant p53 and other partners not directly linked to transcription have been reported to play a role in mut-p53 GOF. For example, it was found that mut-p53 binding to the upstream component MRe11, can disrupt ATM-mediated

cellular responses to double stranded DNA breaks (Song et al., 2007; Song and Xu, 2007) while the interaction with topoisomerase I, increasing aberrant homologous DNA recombination and mutagenic DNA rearrangement, contributes to genomic instability (Restle et al., 2008).

Regulation of mutant p53 stability

In contrast to the plethora of information available for wild type p53, the regulation of mutant p53 stability and activity, and the status of its post-translational modifications, are much less characterized (Brosh and Rotter, 2009).

In opposition to the wild type counterpart, mutant p53 is often found at high levels in tumors (Bartek et al., 1991); in fact, immunohistochemical detection of p53 in tissue sections is commonly used as a surrogate for detecting TP53 missense mutation and can provide prognostic and predictive informations (Soussi and Beroud, 2001). Moreover, high mutant p53 protein levels are thought to be responsible for most of its oncogenic functions, since its depletion reduces the aggressive features associated to GOF (Muller and Vousden, 2013) and conversely, its precocious stabilization in knock-in mice is associated with enhanced aggressiveness (Suh et al., 2011; Terzian et al., 2008).

Interestingly, in mutant p53 knock-in mice models, no stabilization of the protein is observed in normal tissue, but mut-p53 is found accumulated exclusively in tumors (Lang et al., 2004; Olive et al., 2004). Similarly, in patients with Li-Fraumeni syndrome, mutant p53 does not accumulate in normal tissues, but only in tumors.

Interestingly, when mutant p53 knock-in mice were crossed into a Mdm2-null background, the mut-p53 protein was stabilized in nearly all normal tissues (Terzian et al., 2008), suggesting that the same mechanisms regulating the wild type protein are responsible for maintaining mutant p53 at low levels in normal (but not tumor) tissues.

A clue to the nature of mutant p53 stabilization may be provided by the observation that in primary cells derived from mutant p53 knock in mice, the unstable protein can be stabilized by genotoxic stress very much like wt-p53 (Lang et al., 2004), however, mutant p53 is incapable of transactivating Mdm2 (Oren and Rotter, 2010), thus breaking the regulatory feedback loop.

These results strongly suggest that p53 mutations alone are insufficient for mutant p53 accumulation, but additional events occurring in tumors are required to release mut-p53 from Mdm2-mediated degradation. For example, since tumor cells experience chronic DNA damage and other alterations, it is possible that the resulting signaling events, normally aimed at activating the endogenous wt-p53 and preventing cancer cell proliferation, could also promote mut-p53 protein stability, resulting in cancer promotion.

Interestingly, Mdm2 isoforms A, B, and C, are frequently found overexpressed in high grade tumors indicating a role of such isoforms in tumorigenesis (Bartel et al., 2002). A common feature of these isoforms is the lack of p53 binding domain, while they retain the ability to bind full length Mdm2 (Fridman et al., 2003). A recent work established a role of Mdm2-B in promoting mutant p53 accumulation and GOF, through the inhibition of Mdm2-mediated mutant p53 degradation (Zheng et al., 2013).

Other ubiquitin ligases that target wild type p53 regulate also mutant p53 stability, such as C-terminus of HSP70-interacting protein (CHIP) (Esser et al., 2005; Lukashchuk and Vousden, 2007). CHIP- and Mdm2-mediated ubiquitination of mutant p53 is counteracted by the chaperone HSP90, which binds mutant p53 increasing its stability (Esser et al., 2005; Li et al., 2011). Notably, HSP90 is found overexpressed in tumors (Kamal et al., 2003).

Mutant p53 oncogenic properties

Mutant p53 plays a fundamental role in cellular transformation as demonstrated in both human and mouse models: introduction of mut-p53 forms can, indeed, transform p53-null cells and endow them with an increased ability to form colonies in soft agar and tumors in mice (Dittmer et al., 1993). Conversely, down-regulation of endogenous mutant p53 renders cancer cells less tumorigenic (Bossi et al., 2008).

Mutant p53 forms seem to take part in almost every aspect of cancer development, including most of the Hanahan and Wienberg hallmarks (Hanahan and Weinberg, 2011).

In particular several studies investigated the role of mutant p53 in promoting migration and invasion of tumor cells by controlling pathways that are involved in the regulation of these phenotypes.

Indeed, mutant p53 was found to enhance the activation of EGFR/integrin signaling and modulating TGF β pathway thus facilitating cell migration and invasion (Adorno et al., 2009; Dhar et al., 2008; Muller et al., 2009). This is achieved by two non-mutually exclusive mechanisms of inhibition of p63 anti-metastatic functions.

Furthermore, mutant p53 can induce EMT in several cancer models, including prostate, lung, and breast cancer, thus allowing invasiveness: mutant p53 supports EMT transition modulating Slug or Twist activity (Kogan-Sakin et al., 2011; Wang et al., 2009), or by suppressing the anti-invasive gene CCN-5/WISP2 (Dhar et al., 2008). In addition, it has also been demonstrated a role of mutant p53 in coordinating three-dimensional growth: mutant p53 depletion in aggressive breast cancer-derived cells reverts malignant-appearing cells into more benign cells, which form acinous-like structures (Freed-Pastor et al., 2012).

Ribosome biogenesis

Ribosome biogenesis is a very important and complex process in the life cycle of a cell: it requires the coordinated action of all three RNA polymerases to transcribe four types of different rRNAs, approximately 79 ribosomal proteins, and hundreds of ribosome assembly factors. In yeast, it has been estimated, that ribosome biosynthesis accounts for up to 60% of total transcriptional activity and the synthesis of mRNA encoding ribosomal proteins accounts for 50% of all RNA Polymerase II initiation events (Warner, 1999).

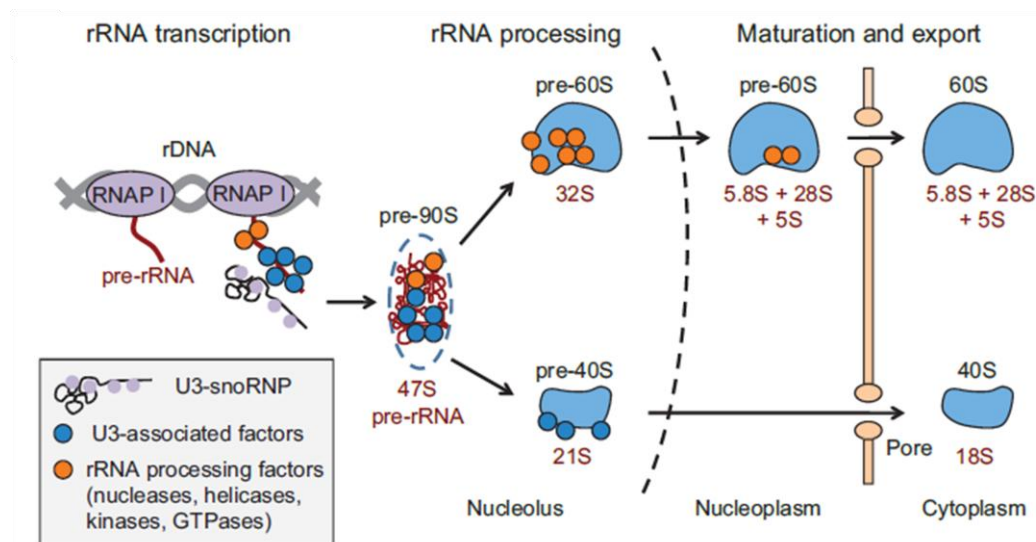


Figure 6. Mammalian ribosome biogenesis. Ribosome biogenesis consists of rRNA synthesis, rRNA processing and ribosome maturation. RNA Pol I transcribes a 14 kb-long, poly- cistronic 47S rRNA precursor that contains 18S, 5.8S, and 28S rRNAs. RNA Pol II transcription facilitates the synthesis of 79 ribosomal L- and S-proteins, a large cohort of small nucleolar (sno)RNAs, and > 200 mRNAs encoding non-ribosomal nucleolar factors, including kinases, helicases, nucleases, and GTPases. In addition, 5S rRNA is produced by RNA Pol III. The orchestrated activity of all three RNA polymerases ensures the correct transcription, processing, and modification of rRNA and its assembly into 40S and 60S ribosomal subunits that compose functional 80S ribosomes in the cytoplasm (Eichler and Craig , 1994 ; Boisvert et al. , 2007).

Eukaryotic ribosomes (80S) are composed of two distinct subunits, small (40S) and large (60S), respectively. The small 40S subunit contains a single 18S rRNA and 33 ribosomal proteins (Rabl et al., 2011), while the larger 60S subunit contains 3 different rRNAs: 5S, 5.8S and 28S, and 46 ribosomal proteins (Klinge et al., 2011).

The nucleolus is the cellular compartment where most of ribosome biogenesis takes place. In the nucleolus RNA polymerase I (Pol I) transcribes a single 47S rRNA precursor (pre-rRNA) (Roeder and Rutter, 1970), which is then modified and cleaved

to obtain mature 18S, 5.8S and 28S rRNA. 5S rRNA is transcribed independently in the nucleus by RNA polymerase III, after processing the mature 5S rRNA is imported in the nucleolus as a ribonucleoprotein complex (Lee and Nazar, 2003).

rRNA maturation process requires chemical modifications, cleavage steps, assembly to ribosomal proteins and transport through different cellular compartments to obtain mature ribosomes (fig. 6).

The first step of this highly organized pathway is the covalent modification of pre-rRNA: in mammals more than 200 nucleotides are modified in methylated sugars, pseudouridines and methylated bases (Maden, 1990); these modifications are carried out by small nucleolar ribonucleoparticles (snoRNPs) most of which are guided to the site of RNA modification by base pairing with small nucleolar RNAs (snoRNAs) (Lafontaine and Tollervey, 1998). These modifications are important for translation performance and fidelity, and binding of ligands to ribosomes, such as tRNA (Mullineux and Lafontaine, 2012). In addition, some snoRNAs (e.g. U3, U8, U17, U22 in mammals) are involved in pre-rRNA processing.

In the pre-rRNA transcript, mature rRNAs are separated by two internal transcribed spacers, ITS1 and ITS2, and are flanked by two external transcribed spacers, the 5' ETS and 3' ETS. The removal of external and internal spacer sequences proceeds through an ordered series of endo- and exonucleolytic cleavages at about 10 sites in the pre-rRNA, with alternative processing pathways (Fig. 7).

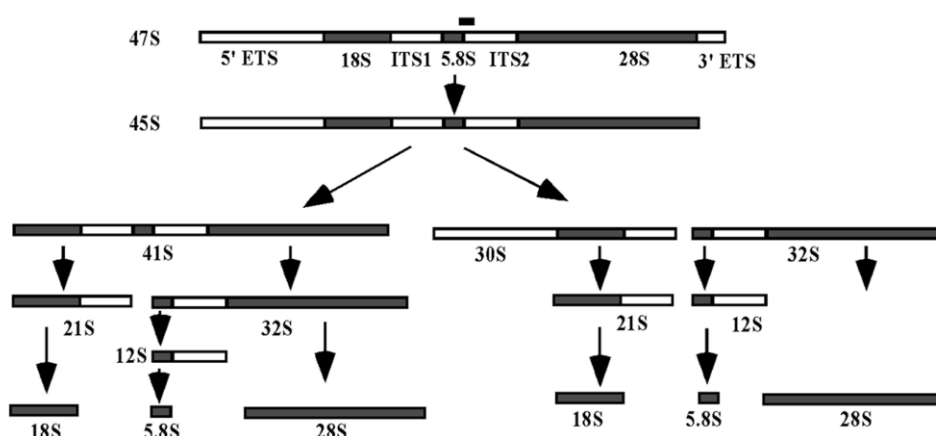


Figure 7. Pre-rRNA processing pathway in mammals. ETS and ITS are external and internal transcribed spacers, respectively (Liang and Crooke, 2011).

The primary transcript, the 47S pre-rRNA, is initially cleaved at both ends of the molecule, generating the 45S precursor. This intermediate rRNA is further processed by at least two alternative pathways. These pathways differ mainly in kinetics and order of cleavage, and can vary according to species, cell type, physiological and developmental stages, and disease state.

In the first pathway for example, the 41S pre-rRNA is generated after the complete removal of 5'-ETS from the 45S pre-rRNA, and subsequently cleaved at the ITS1. This late cleavage generates the 32S pre-rRNA and the 18S pre-rRNA, which is further modified to generate mature 18S rRNA. 32S pre-rRNA undergoes other additional cleavages, mainly in the ITS2, to finally generate mature 5.8 and 28SrRNAs (Mullineux and Lafontaine, 2012).

More than 300 proteins have been described to be involved and assist ribosome biogenesis directly or indirectly. In particular, hundreds of factors assist and drive pre-rRNA during its maturation process. A recent screening identified 286 proteins required for rRNA processing in HeLa cells, many of them with yeast homologues, highlighting the evolutionary conservation of this key process across eukaryotes (Tafforeau et al., 2013). Most of these proteins associate with 47S pre-rRNA to generate 90S ribonucleoparticle (RNP).

Pre-rRNA particles processing begins with the cleavage of the large 90S RNP complex within the ITS1 to generate pre-40S and pre-60S particles.

After ITS1 cleavage in the nucleus, pre-40S particles are rapidly exported to the cytoplasm where last maturation steps generate mature 40S ribosomal subunits. The nuclear export of pre-40S requires Crm1 (Thomas and Kutay, 2003) and ribosomal protein Rps15p through a direct or indirect interaction with the nuclear transport machinery (Leger-Silvestre et al., 2004). In the cytoplasm the kinase hRio2 is required for the conversion of pre-40S in mature 40S particle (Rouquette et al., 2005). Pre-60S particle maturation is instead more complex, and requires many intermediate steps to generate mature 5.8S and 28S rRNA. In the nucleolus about 50 non-ribosomal proteins associate to the early pre-60S ribosomal particle: among them Helicases, rRNA processing and modifier factors, and GTPases that promote a series of RNA processing reactions (Fatica and Tollervey, 2002). During rRNA maturation, pre-60S ribosomal subunits move from the nucleolus to the nucleoplasm with major changes in protein composition. Nuclear export requires the dissociation

of pre-rRNA particle from most of the GTPases, and the association with the NES-containing adapter protein Nmd3 and Mtr2, and with the export receptor XpoI/Crm1/exportin1.

In the cytoplasm, most of the non-ribosomal proteins are released and pre-60S rRNAs particles undergo the final structural rearrangement that will convert them into functional ribosomes competent for translation (Nissan et al., 2002).

Ribosome biogenesis and cancer

Though the discovery that the nucleolus is the site for ribosome biogenesis can be dated to the 1960s, nucleoli have attracted the attention of pathologists as early as the late 19th century.

In 1896 the Italian pathologist Giuseppe Pianese first observed and described that malignant cells have hyperthrophic and irregularly shaped nucleoli. Since then a lot of studies have been conducted to understand whether nucleolar parameters could be used for diagnostic purposes or to determine the clinical output of the disease, and to clarify whether nucleolar alterations could be the consequence of cellular transformation, or the leading cause of neoplastic transformation.

It is now accepted that enlarged nucleoli in actively proliferating cells are the result of increased ribosome biogenesis, indeed it has been demonstrated that all mammalian cells stimulated to proliferate have nucleolar hypertrophy as a result of the higher biosynthetic demand (Derenzini and Ploton, 1991). This observation allowed mainly two considerations: since nucleolar size is strongly correlated with cell proliferation this parameter is not useful for diagnostic purposes in tumors. On the other hand since proliferation is the major cause in determining the growth rate of a tumor mass and one of the most important prognostic factors in oncology, nucleolar size turned to be a parameter to predict the clinical outcome in many forms of cancer (Derenzini et al., 2009).

For many years the increased ribosome biogenesis observed in cancer cells has been considered as a mere consequence of increased biosynthetic demand due to uncontrolled proliferation. However many works clearly demonstrate that ribosome biogenesis is highly controlled and many components of the protein synthesis machinery are deregulated or mutated in different tumors (Ruggero and Pandolfi,

2003). Indeed, important oncogenes and tumor suppressor genes can influence and modulate ribosome biogenesis (fig. 8).

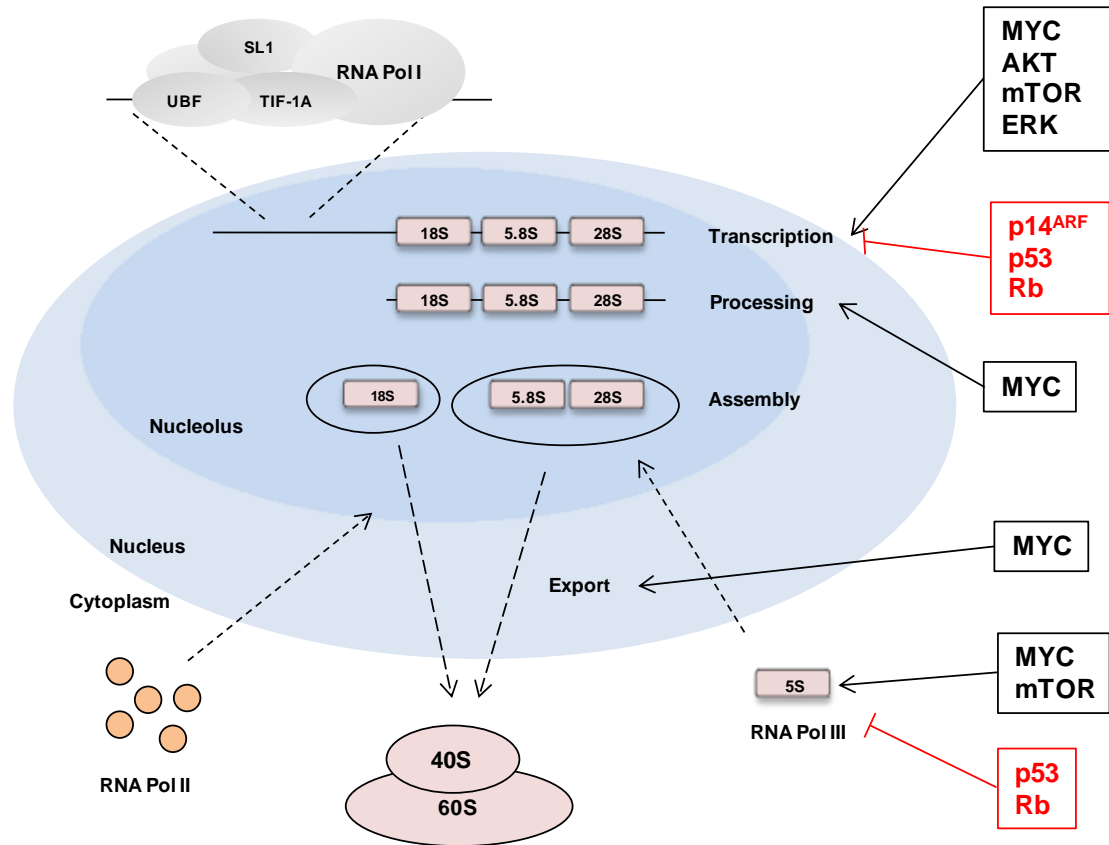


FIG 8. Oncogenes and tumor suppressor genes control of ribosome biogenesis.

Ribosome biogenesis consists of rRNA synthesis, rRNA processing and ribosome maturation. RNAPI transcribes poly-cistronic 47S rRNA precursor that contains 18S, 5.8S, and 28S rRNAs. RNAPII transcribes ribosomal L- and S-proteins, small nucleolar (sno)RNAs, and non-ribosomal nucleolar factors. 5S rRNA is produced by RNAPIII. The orchestrated activity of all three RNA polymerases ensures the correct transcription, processing, nuclear export, and assembly into 40S and 60S ribosomal subunits that compose functional 80S ribosomes in the cytoplasm. The tumor suppressor p53 controls Pol I transcriptional activity preventing the interaction between UBF and SL-1. Moreover, it inhibits Pol III transcription by binding to the transcription factor TFIIB. The Rb family also plays a role in the inhibition of rRNA synthesis and Pol III transcription, binding to UBF and TFIIB, respectively. p14ARF interacts with the Pol I transcription termination factor TTF-I, preventing its localization to the nucleolus and inhibiting rRNA synthesis. Oncogenes, by contrast, have the opposite effect and increase ribosome biogenesis. The principal enhancers of ribosome biogenesis are Myc and ERK1/2. Myc associates with SL-1, increasing Pol I transcription, and is also found associated with 5S rRNA activating Pol III transcription, whereas ERK phosphorylates and activates UBF, TIF1A, and TFIIB.

Oncogenes and tumor suppressors regulating ribosome biogenesis

Myc. Myc is a key transcription factor that directly modulates genes involved in ribosome biogenesis, protein synthesis and proliferation (Schmidt, 2004), and is

frequently overexpressed in some tumor types such as Burkitt's lymphomas, neuroblastomas and colon carcinomas (Lin and Medeiros, 2013).

Myc controls multiple steps in ribosome biogenesis, from the transcription of rRNA, ribosomal proteins and accessory factors, to the nuclear export of mature ribosomal subunits.

Myc has been demonstrated to promote 18S, 28S, and 5.8S rRNAs transcription at different levels. Myc binds rDNA loci and activates the transcription of rRNA through direct binding with cofactors that are required for the recruitment of RNA pol I to rDNA promoters (Arabi et al., 2005), and it activates a chromatin remodeling program that opens chromatin structure near rDNA loci (Shiue et al., 2009). In addition, Myc enhances the expression and recruitment of essential RNA pol I cofactors: UBF (upstream binding transcription factor) and SL1 (selectivity factor 1) (Grandori et al., 2005).

Myc is also involved in the transcription of 5S rRNA outside the nucleolus by a direct interaction with TFIII3B (Gomez-Roman et al., 2003).

This key transcription factor regulates the expression of many ribosomal proteins of both large and small subunit (Kim et al., 2000) and it coordinates the transcription of genes encoding factors required for rRNA processing such as NOP56, BOP1, PES1, FBL, DKC1, NCL and NPM1 (Schlosser et al., 2003).

Finally, Myc has been observed to regulate the machinery responsible for proper ribosomal translocation by promoting the expression of IPO7 and XPO1, two proteins regulating import and export, respectively of RPs and ribosomal subunits (Golomb et al., 2012).

PI3K/AKT. The PI3-kinase (PI3K)/AKT signaling pathway plays a central role in modulating cell survival, proliferation and motility. This statement is supported by the evidence that in transformation, malignant cells develop different systems to activate this pathway (Vivanco and Sawyers, 2002).

Following IGF-1 treatment, the PI3K/AKT pathway is able to stimulate rRNA gene expression by modulating the activity of RNA pol I transcriptional machinery. This is achieved by increasing pol I transcription factor SL1 occupancy in rDNA promoter region (James and Zomerdijk, 2004).

mTOR. The mammalian target of rapamycin (mTOR) is a kinase that regulates cellular functions coordinating anabolic and catabolic processes in response to growth

factors and nutrients (Laplante and Sabatini, 2013). mTOR regulates ribosome biogenesis at different levels by promoting both rRNA transcription and processing.

In response to nutrients, mTOR is able to promote rRNA transcription both stimulating the binding between the essential transcription factor TIF-1A and the initiation competent form of RNA pol I (Mayer et al., 2004), and promoting the interaction of UBF with SL1 thereby promoting Pol I activity (Hannan et al., 2003).

mTOR is also able to stimulate the synthesis of 5S rRNA by RNA pol III facilitating the association of the transcription factors TFIIB and TFIIC with the 5S gene (Mayer and Grummt, 2006).

Ras/Raf/ERK. The Ras-dependent mitogen-activated protein kinase (MAPKs) cascades play a key role in coordinating extracellular signals to a transcriptional program promoting cellular growth and malignant transformation. The importance of this pathway in cellular transformation is highlighted by the fact that it is frequently altered in human tumors, and mutations in Ras are the most common mutations in colon, lung, and pancreatic carcinomas (Fernandez-Medarde and Santos, 2011).

The Ras pathway has been demonstrated to stimulate ribosome biogenesis mainly through its main downstream molecules MEKs and ERKs (James and Zomerdijk, 2004; Wang and Chen, 1998), which bind and phosphorylate TIF-1A and UBF, thus promoting pol I rRNA transcription, and TFIIB, thus promoting 5S rRNA synthesis (Felton-Edkins et al., 2003; Zhao et al., 2003).

A crosstalk between Ras and Myc has been demonstrated: in fact, by activating ERKs, Ras can promote Myc stabilization by direct phosphorylation (Zhu et al., 2008), and counteracting the inhibitory activity of GSK3 β through the activation of PI3K-Akt pathway (Lee et al., 2008).

RB. Rb, the product of the retinoblastoma-associated gene RB1, has a major role in the regulation of cell cycle progression through its capability to arrest cells in G1 phase and to prevent uncontrolled cell proliferation (Chau and Wang, 2003). The variety of human cancers in which the retinoblastoma protein Rb is inactivated reflects its importance as tumor suppressor, indeed pRb is frequently mutated or expressed at low levels in several tumors such as retinoblastoma, osteosarcoma, lung, prostate, bladder and breast carcinomas (Piva, 2014).

Besides its role in inhibiting cell cycle progression, Rb has been demonstrated to regulate ribosome biogenesis through its interaction with UBF and TFIIB, thereby

inhibiting RNA pol I and pol III activity, respectively (Cavanaugh et al., 1995; White et al., 1996). Remarkably, during cell cycle progression, phosphorylated Rb actively induces rRNA transcription, so Rb knock-out mice display reduced pol III activity (White et al., 1996).

INK4A-ARF. The INK4A-ARF locus encodes for the tumor suppressors p16^{INK4A} and p14^{ARF} that activate the growth suppressor functions of Rb and p53, respectively. Giving its role in modulating two important tumor suppressive pathways, deletions of the INK4A-ARF locus is a frequent event in tumors (Ozenne et al., 2010).

p14^{ARF} is able to inhibit ribosome biogenesis by direct interaction with Nucleophosmin (NPM). NPM is a chaperone able to control nucleoplasm nucleolar shuttling of the RNA polymerase I transcription termination factor, TTF-I. ARF binds and inhibit NPM functions promoting its ubiquitination and subsequent proteosomal degradation (Lessard et al., 2010).

p53. In addition to its well established role in promoting cell cycle arrest and apoptosis, the tumor suppressor p53 has been reported to inhibit ribosome biogenesis at different stages.

p53 over-expression in HeLa cells results in a strong reduction in RNA pol I activity by interfering with the formation of the initiation complex between SL-1, UBF, and pol I (Zhai and Comai, 2000). Moreover p53 inhibits 5S rRNA transcription by RNA pol III through binding to the transcription factor TFIIB.

Recently, p53, once activated has been observed to directly negatively regulate expression of U1 and U6 snoRNAs by both RNA pol I and pol III (Krastev et al., 2011).

Ribosome biogenesis and p53

For many years the function of the nucleolus has been thought to be limited to the production of the ribosomal subunits. However, recent studies have challenged this view by showing that among the 4500 proteins associated with the nucleolus only 30% are directly involved in ribosome biogenesis (Ahmad et al., 2009). These studies strongly supported the concept of a “multifunctional nucleolus” proposed by Thoru Pederson in 1998, demonstrating that besides ribosome biogenesis, the nucleolus is involved in controlling other cellular functions (Pederson, 1998). Some of these

additional functions link the nucleolus to the regulation of cell cycle progression, induction of apoptosis and others (Boisvert et al., 2007).

The proposed mechanism by which the nucleolus can integrate different signals to trigger diverse cellular outcomes is by modulating the activity of regulatory proteins essential for these processes. Proteomic studies have indeed revealed that the protein content of the nucleolus is highly dynamic and modified upon stress conditions, thus allowing a crosstalk between altered nucleolar functions and cell responses (Boulon et al., 2010).

The tumor suppressor p53 is deeply linked to the nucleolar status. A landmark study by Rubbi and Milner even revealed that stabilization and activation of the p53 pathway by radiation-induced nuclear DNA damage occurs only when irradiation also affects nucleolar integrity (Rubbi and Milner, 2003), thus indicating the nucleolus as a central hub in controlling p53 functions.

In line with this observation, the nucleolus has been proposed to work as a stress sensor responsible both for maintaining low p53 levels under physiological conditions, and to promote p53 accumulation as soon as nucleolar functions are impaired (Rubbi and Milner, 2003).

Mechanism of p53 activation upon ribosomal stress

The key downstream event in promoting p53 accumulation after ribosomal stress is probably the inactivation of the p53-inhibitor Mdm2 by direct binding of ribosomal proteins (RPs). This surveillance mechanism has been defined as the “RPs-Mdm2-p53 stress response pathway” (Deisenroth and Zhang, 2010). In principle, conditions that disrupt ribosome biogenesis at different levels are postulated to create a pool of unused ribosomal proteins that are free to interact with other molecules, in this specific case Mdm2, thus altering their functions. Indeed in recent years, many ribosomal proteins, including RPL5, RPL11, RPL23, RPL26, RPS3, RPS7, RPS14, RPS25, RPS26, RPS27/L, have been reported to bind and inhibit Mdm2 functions mostly *in vitro* and in tissue culture (Shcherbik and Pestov, 2010).

Mechanistically, since most of these ribosomal proteins bind the central region of Mdm2, they are thought to create a conformational block that prevents the transfer of ubiquitin from the C-terminal RING finger domain to the N-terminal p53 binding site.

In support of this thesis, it was demonstrated that RPs-bound Mdm2 retains the ability to interact with p53, but loses its ubiquitin ligase activity (Dai and Lu, 2004; Jin et al., 2004).

A recent work by the group of Yanping Zang addressed the *in vivo* role of RPs-Mdm2 interactions. The starting observation was that many cancer-associated Mdm2 mutations that target the central zinc finger domain, including point mutations in conserved zinc-coordinating cysteines, disrupt Mdm2 binding to RPL5 and RPL11, thus compromising the ability of p53 to be accumulated in response to ribosomal but not genotoxic stress (Lindstrom et al., 2007).

They generated mice carrying such a cancer associated mutation (Mdm2^{C305F}) in the Mdm2 zinc finger and analyzed the effect of uncoupling the RP-Mdm2-p53 axis in a model of c-MYC induced tumorigenesis: they demonstrated that in cells derived from Mdm2^{C305F} knock-in mice (expressing a functional Mdm2 that cannot bind RPL5 and RPL11) p53 responds normally to DNA damage, but is not stabilized by nucleolar stress. These mice displayed strongly increased Myc-induced lymphomagenesis (Macias et al., 2010).

This work established the central role of RPL5 and RPL11, among the many other ribosomal proteins, in promoting p53 activation upon aberrant ribosome biogenesis. Therefore, the RP-MDM2-p53 pathway can be considered tumor suppressive, restraining proliferation of cells with defective or uncontrolled ribosome biogenesis (de Las Heras-Rubio et al., 2013).

Disruption of rRNA synthesis activates p53

Perturbation at any step in the process of ribosome biogenesis, such as rRNA transcription, early and late processing, and ribosomal proteins imbalance, has been demonstrated to contribute to nucleolar stress, leading to accumulation and activation of the p53 tumor suppressor protein (fig. 9).

Inhibition of *de novo* rRNA synthesis is an established mechanism for inducing ribosomal stress and to activate RP-MDM2-p53 pathway.

A commonly used approach to inhibit rRNA production is treatment with drugs that inhibit the activity of RNA Pol I, or deplete the available pool of nucleotides for new rRNA synthesis.

The most widely used compounds for this purpose are Actinomycin D (ActD) and 5-Fluorouracil (5-FU). ActD is an antineoplastic compound that at low concentrations (<10nM) specifically disrupts ribosome biogenesis by intercalating into the GC-rich regions of rDNA to inhibit Pol I-mediated transcription of nascent rRNA (Perry and Kelley, 1968, 1970; Sobell et al., 1971). Most of the studies demonstrating the RP-MDM2-p53 axis use this agent to generate ribosomal stress and activate p53.

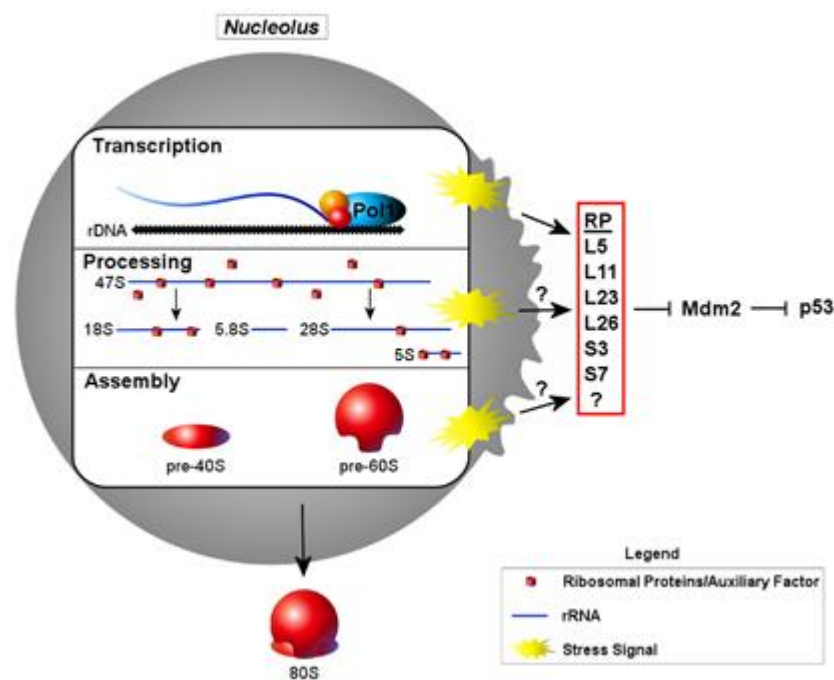


Figure 9. Perturbations to ribosome biogenesis trigger a p53 response. Dysfunction at discrete phases of rRNA transcription, processing, and assembly during ribosome biogenesis are known to lead to p53-mediated responses. Select RPs are known to transduce nucleolar stress signals to p53, particularly in response to attenuation of rRNA transcription. (Deisenroth and Zhang, 2010)

5-FU is another commonly used chemotherapeutic compound that alters ribosome biogenesis, activating the RP-Mdm2-p53 pathway (Sun et al., 2007). 5-FU, as uracil analogue, is incorporated into nascent rRNA and completely blocks rRNA synthesis (Longley et al., 2003).

Accumulation and activation of p53 by inhibition of rRNA transcription has been also demonstrated by altering the expression of key regulatory elements. For example, siRNA depletion of components of RNA Pol I machinery, such as transcription factor TIF-1A, has been demonstrated to trigger loss of nucleolar structure, reduced rRNA transcription, and activation of the p53 pathway (Yuan et al., 2005).

Disruption of rRNA processing activates p53

Alterations in rRNA processing can lead to accumulation of unprocessed intermediate transcripts that can retard subunit assembly, therefore inducing nucleolar stress.

Bop1 is a nucleolar processing factor required for the cleavage of the ITS1 and ITS2 as well as the 3'-ETS of rRNA (Strezoska et al., 2002). The expression of a dominant negative form of Bop1 in mouse cells blocks 28S and 5.8S rRNA processing, leading to impaired ribosome large subunit assembly, and p53-dependent inhibition of cell cycle progression (Pestov et al., 2001). Bop1 together with Pes1 and WDR12 forms a nucleolar complex termed PeBoW complex. Similar to Bop1, depletion of WDR12 also induces rRNA processing defects and a p53 dependent cell cycle arrest (Holzel et al., 2005), thus suggesting that disruption of this functional complex involved in rRNA processing activates the RP-Mdm2-p53 checkpoint.

Ribosomal proteins imbalance activates p53

There are many clinical manifestations caused by imbalance in ribosomal proteins production, globally named ribosomopathies. These diseases are associated with mutations or haploinsufficiency in various RP genes, leading to impaired ribosome biosynthesis. For instance one of the most frequent ribosome biogenesis disease is Diamond Blackfan anemia (DBA), a genetic disease in which mutations in several different RP genes cause a congenital bone marrow failure syndrome with a striking erythroid defect (Narla and Ebert, 2010). In addition with chronic anemia and congenital abnormalities, DBA patients also harbor a predisposition to develop different forms of cancer (Freed et al., 2010).

It is commonly accepted that functional ribosome biogenesis requires a consistent supply of ribosomal proteins, so an imbalance in the stoichiometric ratio of RPs can alter the assembly of 40S or 60S subunits, inducing ribosomal stress (Deisenroth and Zhang, 2010) thereby activating p53.

It has been consistently reported that disruption of 60S ribosome biogenesis alters nucleolar structure, inducing the release in the nucleoplasm of RPs, Mdm2 inhibition, and p53 accumulation and activation (Sun et al., 2010).

Interestingly, a different mechanism for p53 accumulation upon inhibition of 40S ribosome biogenesis has been described. Indeed altered 40S ribosome biogenesis has no effect on nucleolar integrity, although p53 induction is still mediated by RPL11. Rather, upon impairment of 40S ribosome biogenesis, despite a decrease in global translation, cells upregulate the translation of mRNAs with a polypyrimidine tract at their 5' transcriptional start sites (5'-TOP mRNAs). Among those mRNAs is that encoding RPL11, thus providing a pool of L11 protein able to bind and inhibit Mdm2 functions (Fumagalli et al., 2009).

GTPBP4

GTPBP4, also known as Nog1, NGB or CRFG, is a nucleolar GTP-binding protein of the Obg family. Obg family proteins are characterized by a G-domain and have been implicated in diverse cellular processes, including stress response, chromosome replication, and differentiation (Lapik et al., 2007).

GTPBP4 was initially described as the product of the chronic renal failure gene CRFG, a gene expressed in the outer medulla of the kidney, which is lost with renal disease or injury (Laping et al., 2001). Subsequently, other studies demonstrated that GTPBP4 is involved in ribosome biogenesis, and in particular it is an essential factor required for biogenesis of the 60S ribosomal subunit (Jensen et al., 2003).

GTPBP4 is a 634 amino acid protein. At the N-terminus of the protein there is a nuclear localization signal and a GTP binding domain, while at the C-terminus it contains coiled-coil domains. Moreover, GTPBP4 has five sequence motifs, G1 to G5, which are conserved in the GTPase superfamily. Apart from these characteristic motifs, the amino acid sequence of the protein differs substantially from other well characterized G-proteins suggesting the existence of a new subfamily of GTP-binding proteins (Lee et al., 2007).

GTPBP4 has been demonstrated to be linked to ribosome biogenesis in the early 2000s. In *T. Brucei*, GTPBP4 ortholog has been identified as a nucleolar protein (Park et al., 2001), and further studies in yeast and trypanosomes demonstrated that in these organisms GTPBP4 is essential for the biogenesis of 60S ribosomal subunits. Indeed, yeast cells depleted of GTPBP4 or harboring temperature sensitive alleles of the gene

display defects in pre-rRNA processing and in the release of nascent pre-60S subunits from the nucleolus (Kallstrom et al., 2003; Saveanu et al., 2003).

Proteomic analysis in yeast showed the association of GTPBP4 ortholog with multiple components of pre-60S ribosomes (Fuentes et al., 2007; Lebreton et al., 2006) suggesting a role of GTPBP4 in promoting different steps in ribosome biogenesis. These studies demonstrated that GTPBP4 accompanies the pre-60S ribosomal precursor in the late stages of maturation, shuttling between nucleoplasm and cytoplasm. In the cytoplasm, GTPBP4 is released from nascent 60S subunit along with other shuttling proteins, and is rapidly re-imported in the nucleus (Pertschy et al., 2007).

GTPBP4 functions seem to be regulated by nutrient availability. Indeed mTOR, beside its known involvement in regulating early stages of ribosome maturation in the nucleolus, has been reported in yeast to regulate also late stages of ribosome biogenesis via GTPBP4. Upon nutrient starvation, or mTOR pharmacological inhibition (Rapamycin treatment), GTPBP4 is first tethered in the nucleolus and then is transcriptionally reduced leading to cessation of ribosome maturation (Honma et al., 2006).

Interestingly, a further link between nutrient availability and GTPBP4 functions has been recently provided. In *C. elegans* knockdown of the GTPBP4 ortholog resulted in smaller broodsize, slower growth, increased lifespan, and more fat storage. Since TOR is involved in regulating lifespan and fat storage in this animal model, the authors proposed GTPBP4 as an important player in the insulin/IGF pathway (Kim et al., 2014).

Targeting ribosome biogenesis in cancer therapy

Classical DNA-damage chemotherapy, being mutagenic, can cause secondary tumors, especially in survivors of childhood cancers; therefore, alternative drugs are of particular interest. Since ribosome biogenesis is a critical target of classical chemotherapy, drugs that specifically inhibit rRNA synthesis without genotoxic side effects would be desirable. Indeed, in the last years some steps in ribosome biogenesis have been reported to be potentially therapeutically targeted (Burger and Eick, 2013).

RNA polymerase I, for example, has been considered as a therapeutic target in cancer, and molecules have been designed to target specifically rDNA transcription. CX-3543, for example, is a molecule that disrupts the interaction of the nucleolar processing factor nucleolin with rDNA-quadruplex complexes in the nucleolus and induces apoptosis in cancer cells (Drygin et al., 2009). Another molecule, CX-5461 has been reported to selectively kill B-lymphoma cells *in vivo* while maintaining a viable wild type B cell population. The therapeutic effect of CX-5461 is achieved by blocking the recruitment of SL1 to Pol I promoter, leading to nucleolar disruption and activation of p53 dependent apoptotic signaling (Drygin et al., 2011). Thus, non-genotoxic inhibition of Pol I transcription could be an important therapeutic strategy for cancer specific activation of p53 in cancer cells.

The nucleolar protein Nucleophosmin (NPM1) promotes rRNA processing during ribosome biogenesis, and its expression is found frequently altered in tumors; notably, overexpression of NPM drives leukemia initiation (Grisendi et al., 2006; Vassiliou et al., 2011). Two small compound inhibitors have been found for NPM1, NSC348884 and avrainvillamide. The first molecule, NSC348884, inhibiting NPM1 oligomerization, leads to p53 activation. This compound has been reported to synergize with the cytotoxicity of doxorubicin in various cancer cells (Qi et al., 2008). The second compound, avrainvillamide, is an alkaloid that was identified in an affinity screen with NPM1. Avrainvillamide also stabilizes p53 and induces apoptosis in cancer cells (Wulff et al., 2007).

These encouraging results emphasize that inhibition of ribosome biogenesis can be a promising approach for cancer therapy; within this area, a better understanding of the molecular mechanisms involved and the identification of novel potential targets might help to broaden available therapeutic options.

PART 1: Exploring the mechanism of wild type p53 accumulation upon GTPBP4 depletion

The p53 tumor suppressor protein is a transcription factor capable of sensing a wide range of stress signals, and coordinating a complex cellular response that can lead to cell cycle arrest, DNA repair, apoptosis or senescence (Vousden and Lu, 2002).

p53 activation and function requires a complex repertoire of posttranslational modifications and protein-protein interactions that are essential to integrate stress signals to a proper cellular outcome (Kruse and Gu, 2009).

Many years of research have now clearly shown that the biology of p53 is very complex, and that p53 is a highly interconnected protein, interfacing with different pathways (Collavin et al., 2010). Indeed, more than 150 p53-binding proteins have been described, and the list is increasing.

To gain novel insights on p53 interaction profile, our laboratory took a phylogenetic perspective, reasoning that the identification of new p53 interacting proteins in a simpler organism, such as *D. melanogaster*, could help identify conserved interactions of p53 and p53-family proteins in man. Using a genome-scale *in vitro* expression cloning approach, 91 previously unreported interactors of *Drosophila* p53 were identified. Looking for evolutionary conservation of these interactions, 41 mammalian orthologues were tested for binding to human p53 and p53 related proteins p63 and p73. Novel identified p53 interacting proteins were then tested in a functional screen in cells treated with DNA damaging drugs or Nutlin, leading to the validation of five such interactors as potential modulators of p53-dependent growth arrest (Lunardi et al., 2010).

Among the five novel functionally validated p53 interactors, GTPBP4, a monomeric GTPase that plays a crucial role in 60S ribosome biogenesis, was of particular interest. The interest derived from the observation that GTPBP4 was the only identified p53 interactor that did not bind p63 or p73, and that its depletion by siRNA induced accumulation and activation of the p53 protein.

Given the role of GTPBP4 in ribosome biogenesis, and the well-established link between altered ribosome biogenesis and p53 functions, further experiments were

performed to explore whether GTPBP4 depletion could affect p53 stability via the nucleolar stress response pathway.

All currently known mechanisms linking altered ribosome biogenesis to p53 activation rely directly or indirectly on the binding of ribosomal proteins to Mdm2, thus leading to its inhibition and consequent p53 stabilization (Hein et al., 2013). Interestingly, GTPBP4 depletion did not alter nucleolar structure and p53 accumulation did not correlate with increased protein half-life in the cell model used (fig. 10), thus suggesting the possible involvement of an alternative mechanism (Lunardi et al., 2010).

Considering that Mdm2 is not conserved in *Drosophila*, GTPBP4 might therefore be an ancient point of intersection between ribosome biogenesis and the p53 pathway. To dissect this potentially novel regulatory axis, we therefore decided to analyze in more detail the mechanism of p53 accumulation upon GTPBP4 depletion.

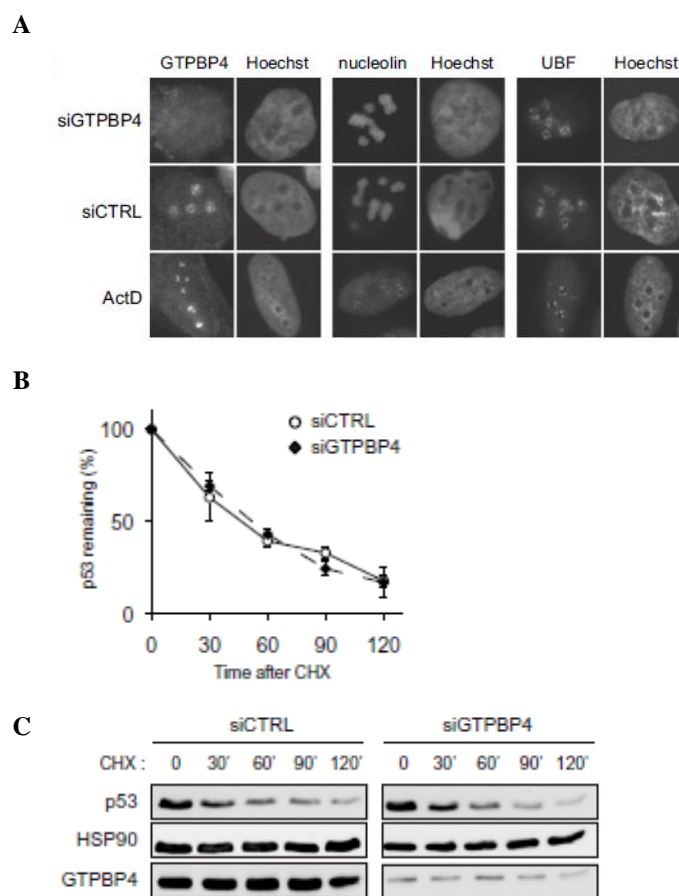


Figure 10. GTPBP4 knockdown does not alter nucleolar structure and does not promote p53 stabilization.

A) GTPBP4 knockdown does not affect localization of key nucleolar proteins. Immunofluorescence analysis is shown for GTPBP4, Nucleolin, and UBF in U2OS cells transfected for 48 hours with either control or GTPBP4 siRNA. The same proteins were analyzed in U2OS cells treated with 5 nM Actinomycin D for 12 hours. Nuclei were counterstained with Hoechst. **B)** GTPBP4 knockdown does not induce p53 stabilization. Quantification is shown of the ratio of p53 to Hsp90 (measured by densitometry on Western blots) in HCT116 WT cells transfected with GTPBP4 siRNA (solid diamonds) or control siRNA (open circles) for 48 hours and treated for the indicated times with 50 μ M Cycloheximide (CHX). Data are means \pm SD for three independent experiments. **C)** Representative Western blot used for the analysis of p53 half-life. To obtain a comparable p53 signal, the gel was loaded with 15 μ g lysate of control siRNA samples (Left), and 7.5 μ g lysate of GTPBP4 knockdown samples (Right). Immunoblotting for GTPBP4 confirmed efficient knockdown of the endogenous protein (Lunardi et al., 2010).

PART 1: RESULTS

Generation of HCT116 cell line inducible for GTPBP4 silencing

To analyze the mechanism of p53 accumulation upon GTPBP4 depletion, we decided to generate stable cell lines for inducible knock-down of GTPBP4 in the HCT116 colon cancer cell model (bearing wild-type p53). Such system allows GTPBP4 depletion without the side effects of transient siRNA transfection.

HCT116shC and HCT116shGTPBP4 cell lines were generated through infection of lentiviral vectors expressing the corresponding Tet-inducible shRNAs (purchased from Thermo Scientific), and upon treatment with doxycycline, effective GTPBP4 silencing and p53 accumulation were verified by western blot. As shown in fig. 11A, 48 hours of doxycycline treatment reduced GTPBP4 expression, and as expected, GTPBP4 depletion correlates with a marked p53 accumulation. Treatment with Doxy of the HCT116shC cells resulted in no alteration in GTPBP4 and p53 levels, thus confirming the specificity of GTPBP4 depletion on p53 accumulation, and also excluding non specific effects of doxycycline on p53 (or GTPBP4).

Induced cells were also stained with propidium iodide (PI) and were subjected to FACS analysis (fig. 11B). In these cells, GTPBP4 depletion promoted a significant reduction in the proliferation rate (measured as the percentage of cells in S- and G1-phase), thus suggesting that increased p53 is active.

To further characterize this cellular system, we analyzed p53 and GTPBP4 mRNA levels by RT-qPCR in the same conditions. As expected, doxycycline treatment resulted in a strong reduction in GTPBP4 expression while p53 mRNA levels remained unaffected (fig. 11C).

All these results are in line with our previous observations (Lunardi et al., 2010), so this inducible cellular system represents a reliable tool to study the mechanism of p53 accumulation upon GTPBP4 depletion.

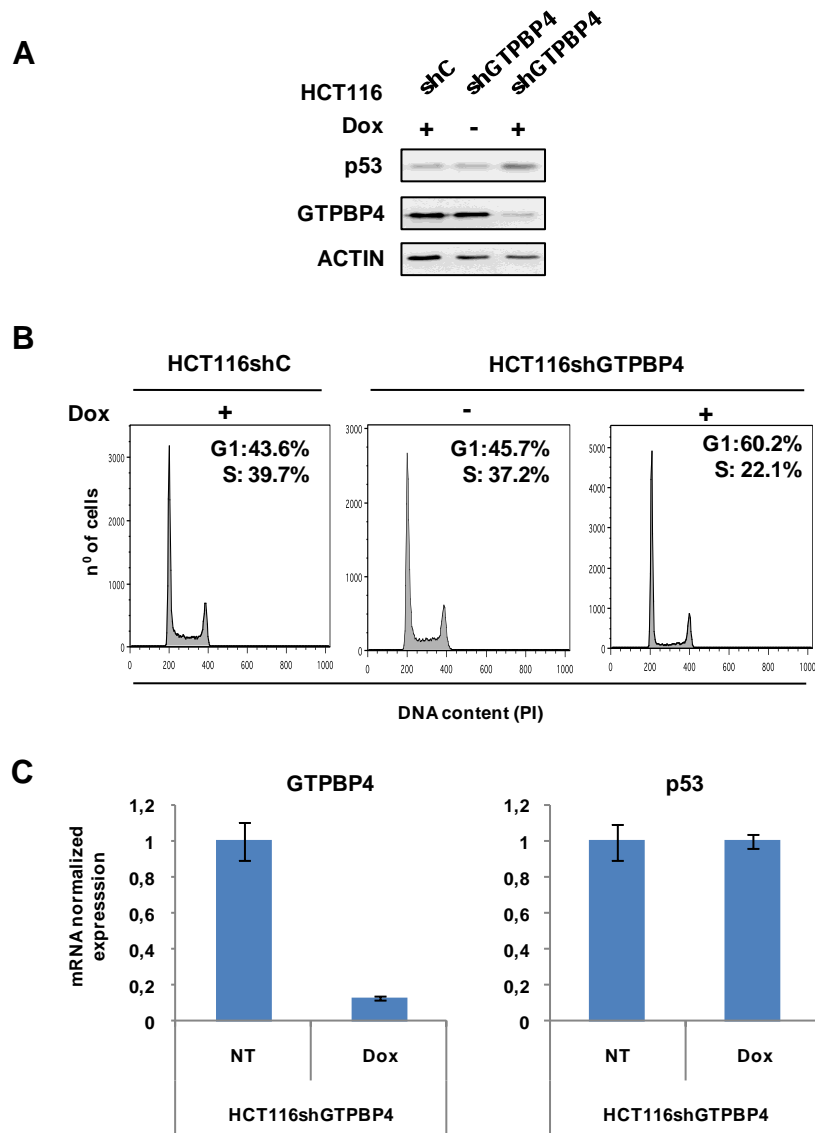


Figure 11. Generation of HCT116 cell line inducible for GTPBP4 silencing.

A) GTPBP4 depletion promotes p53 accumulation. HCT116shC and shGTPBP4 cells were treated for 48 hours with doxycycline (1 μ g/ml) or left untreated, and subjected to western blot analysis. GTPBP4 was blotted to confirm knockdown and Actin was detected as loading control. **B)** GTPBP4 depletion inhibits cell proliferation. HCT116shC and shGTPBP4 cells were treated as in A. Cells were analyzed by flow cytometry and DNA content was evaluated by Propidium Iodide (PI) staining. Cell cycle analysis was performed with FlowJo software. **C)** GTPBP4 depletion does not affect p53 mRNA levels. HCT116shGTPBP4 cells were treated as in A. GTPBP4 and p53 expression levels were measured by RT-qPCR. Data were normalized to GAPDH, and compared to expression levels in untreated cells.

Analysis of p53 translation in GTPBP4 depleted cells

Since previous experiments from our laboratory excluded the involvement of GTPBP4 in controlling p53 stability or p53 mRNA expression, we asked whether GTPBP4 depletion might promote p53 accumulation at the translational level.

There are in fact examples of ribosomal proteins controlling p53 translation: indeed RPL26 and nucleolin were found to bind the 5' untranslated region (UTR) of p53 mRNA and to control p53 with translation and induction after DNA damage (Takagi et al., 2005).

To determine whether GTPBP4 depletion could increase p53 levels by promoting its mRNA translation, we used two experimental approaches.

In one approach, we labeled newly synthesized proteins with [³⁵S]methionine. HCT116shGTPBP4 cells were treated for 48 hours with doxycycline or left untreated, and then incubated in medium with labeled methionine and cysteine in the presence of proteasome inhibitor. After 1 hour, p53 and HSP90 (used as a control), were immunoprecipitated and analyzed by SDS-PAGE and autoradiography. As shown in fig. 12A, no significant differences in the rate of p53 protein synthesis were detected with or without doxycycline treatment (i.e. GTPBP4 depletion).

In an alternative approach, in collaboration with Dr. Quattrone from the University of Trento, we analyzed the polysome distribution of p53 mRNA in cells depleted for GTPBP4. Polysomes profiles of untreated and doxycycline induced HCT116shGTPBP4 cells were generated by sucrose-gradient centrifugation and are shown in fig. 12B and C. The first observation is that doxycycline treatment strongly impacts on sub-polysomal and light polysomal fractions (see fractions 7 and 8); this might be due to the growth arrest of these cells (see Fig. 11B), or might indicate that GTPBP4 depletion is altering ribosome neo-synthesis.

We then analyzed by RT-qPCR the distribution of p53 and GAPDH mRNAs on polysomal fractions. As shown in fig. 12D, doxycycline treatment does not significantly affect p53 mRNA distribution on polysomal fractions; in particular it does not increase p53 mRNA loading on larger polysomes, thus suggesting that GTPBP4 depletion does not increase p53 translation.

These two different approaches lead to the conclusion that GTPBP4 depletion does not promote increased p53 translation, at least in our inducible shRNA cellular model.

Moreover, the polysomial analysis allowed us to speculate that at the timing of p53 accumulation ribosome biogenesis might be already altered by GTPBP4 depletion, even if no effects on global protein synthesis are observed (See Fig. 12A).

GTPBP4 depletion promotes p53 stability

The above results prompted us to reconsider our initial hypothesis that GTPBP4 might be a regulator of p53 translation.

Thus, since we found no differences in transcription and translation of p53 mRNA upon GTPBP4 depletion, we had to go back and re-examine the possible role of p53 stabilization.

We therefore analyzed p53 half-life with a series of meticulous cycloheximide experiments in the inducible HCT116shGTPBP4 cell line.

In contrast to previous results from transient siRNA transfection, in this cellular system GTPBP4 depletion promotes a reproducible increase in p53 stability (fig. 13A and fig. 13B). Specifically, p53 half-life increased from about 30 to 60 minutes upon GTPBP4 knockdown.

These results completely subverted our initial model, and led us to reconsider the possible pathways leading to p53 accumulation after GTPBP4 depletion.

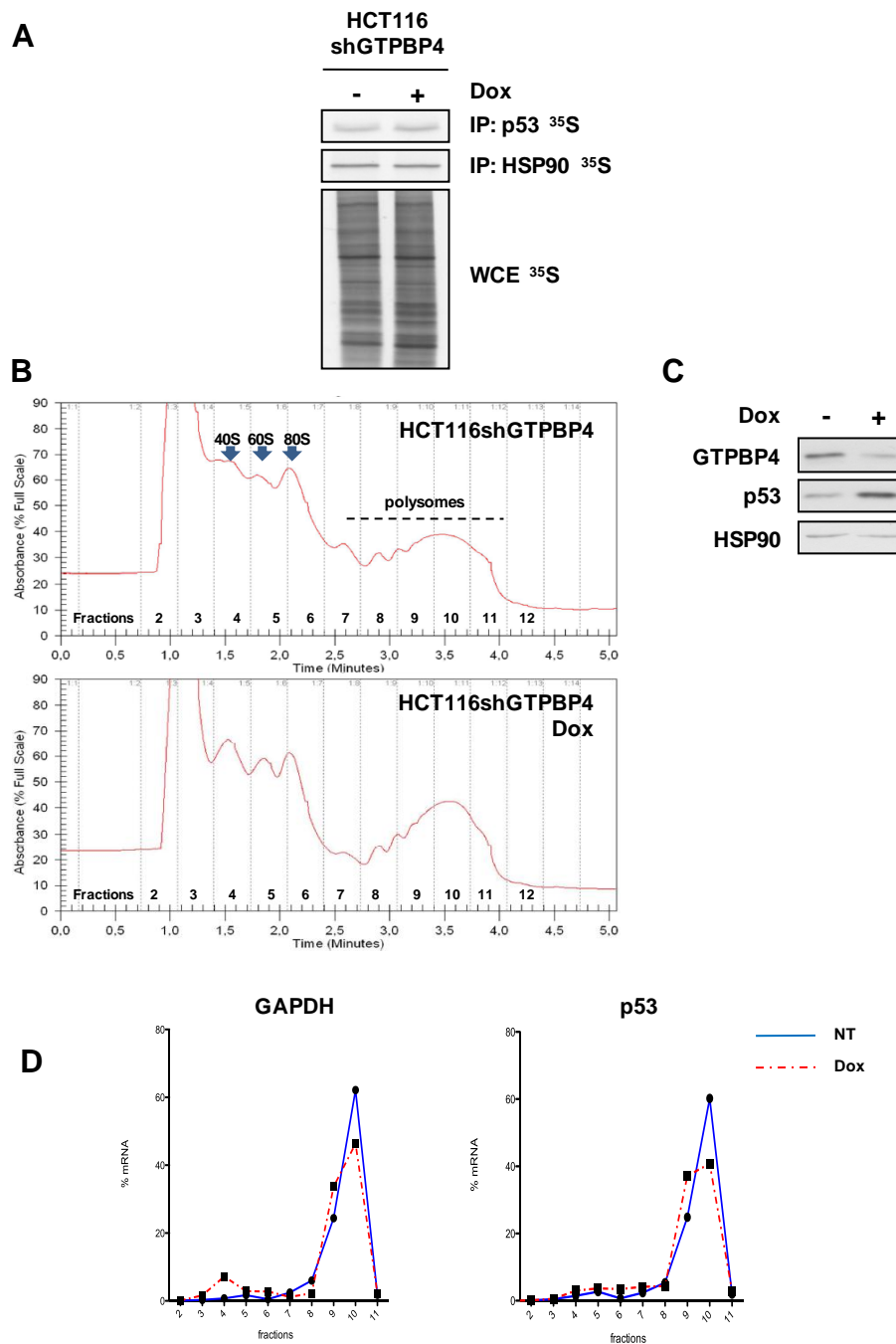


Figure 12. GTPBP4 depletion does not increase p53 translation.

A) GTPBP4 depletion does not increase p53 neosynthesis. HCT116shGTPBP4 cells were treated as indicated in fig. 11, pretreated with 50 μ M MG132, and incubated for 1 hour with [³⁵S] methionine and cysteine. p53 and HSP90 proteins were immunoprecipitated from labeled cells, and analyzed by autoradiography after SDS-PAGE. Analysis of whole-cell extracts (WCE) showed comparable incorporation of [³⁵S] methionine into dox-treated and untreated cells (bottom panel). B) Analysis of p53 mRNA distribution on polysomal fractions in HCT116shGTPBP4 cells treated as in fig. 11. Absorbance profile of RNA separated by velocity sedimentation through sucrose gradient. The position of the 40S, 60S, 80S, and polysomal fractions are indicated. C) Western blot analysis confirmed GTPBP4 depletion and p53 accumulation. Hsp90 protein has been analyzed as loading control. D) GAPDH and p53 mRNA distribution in sub- and polysomal fractions from untreated (blue line) or dox treated (red dashed) cells. In each fraction, mRNA expression levels measured by RT-qPCR were normalized on the total volume of the fraction.

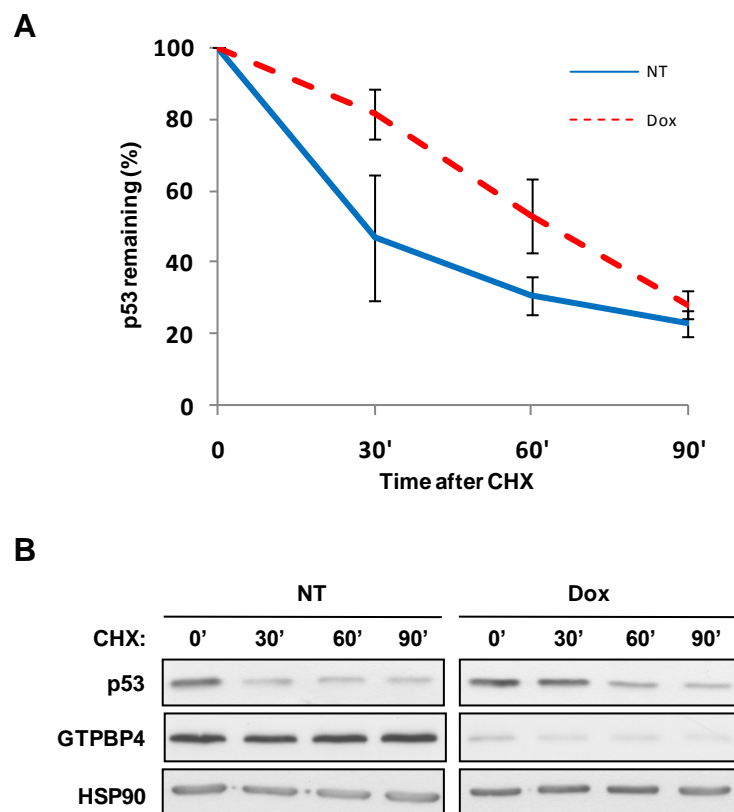


Figure 13. GTPBP4 depletion promotes p53 stabilization in HCT116 cells.

A) Quantification is shown of the ratio of p53 to HSP90 (measured by densitometry on Western blots) in HCT116shGTPBP4 cells treated for 48 hours with Doxycycline (red dashed) or left untreated (blue line). 50 μ M Cycloheximide (CHX) was added for the indicated times. Data are means \pm SD for three independent experiments. **B)** Representative Western blot used for the analysis of p53 half-life. The gel was loaded with a reduced volume of Dox-treated lysates to obtain a comparable p53 signal.

Impact of GTPBP4 depletion in ribosome biogenesis, and correlation with p53 accumulation

GTPBP4 is an essential factor in ribosome biogenesis (Jensen et al., 2003). The observation that GTPBP4 depletion can promote p53 stabilization and affects polysome profile, lead us to analyze the impact of GTPBP4 knockdown on ribosome biogenesis - and its correlation with p53 accumulation.

To measure the impact of GTPBP4 depletion on processing of ribosomal RNA (rRNA) we first determined the kinetic of ribosomal processing in uninduced HCT116shGTPBP4 cells.

Cells were metabolically labeled with [32 P] orthophosphate following the protocol illustrated in fig. 14B, and labeled total RNA was separated by gel electrophoresis and visualized by autoradiography. A schematic representation of rRNA processing is given in fig. 14A: primary 47S rRNA transcript is first processed into intermediate products and finally into the mature 18S, 5.8S, and 28S rRNAs.

The kinetics of rRNA processing in HCT116shGTPBP4 cells are shown in fig. 14C (right panel): after 3 hours of chase, the 47S rRNA precursor is processed into the 28S and 18S forms. Hence, since GTPBP4 is involved in the processing of 60S ribosomal subunits (Jensen et al., 2003), we performed the next experiments using this time-frame.

HCT116shC and HCT116shGTPBP4 cells were treated with or without doxycycline for 24 and 48 hours, and then metabolically labeled with [32 P] orthophosphate as summarized in fig. 15A. Total RNA was extracted, separated by gel electrophoresis and visualized by autoradiography. In parallel, cells were also collected for western blot analysis of GTPBP4 and p53 levels.

In line with data obtained by others with GTPBP4/Nog1 mutants (Lapik et al., 2007), we observed that GTPBP4 depletion has a strong impact on ribosome biogenesis. A reduction in newly synthesized 28S rRNA is an early event following GTPBP4 knockdown, since 28S rRNA production is almost abolished after 24 hours of doxycycline treatment. Altered 28S rRNA processing is specifically associated with reduced GTPBP4 levels, since no effects are observed in control cells (fig. 15B). Interestingly, western blot analysis indicates that p53 accumulation is a later event, subsequent to the defect in 28S rRNA synthesis triggered by GTPBP4 depletion (fig. 15C).

Collectively, these results demonstrate that alteration in ribosome biogenesis is an early event induced by GTPBP4 depletion, and suggest that the subsequent p53 stabilization might be functionally linked to such condition.

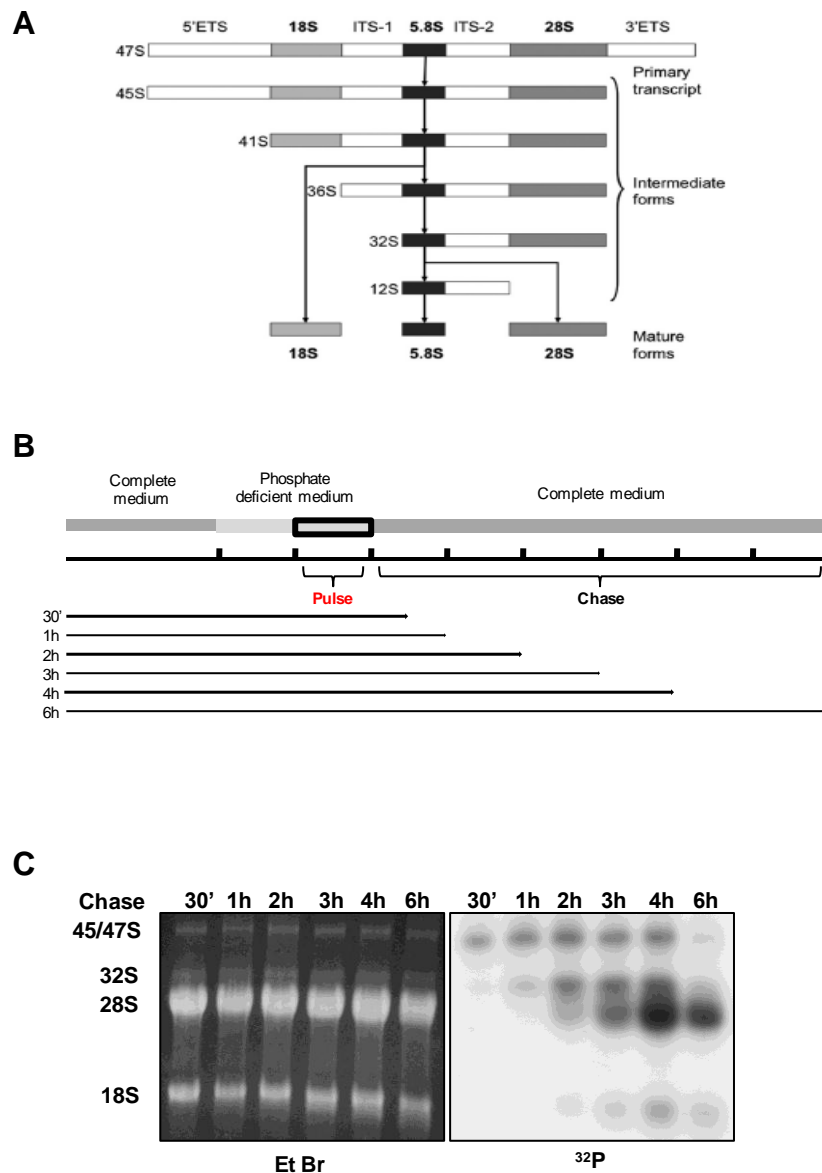


Figure 14. Analysis of rRNA processing in HCT116 cells.

A) Schematic representation of rRNA processing in mammalian cells. ETS, external transcribed spacer; ITS, internal transcribed spacer (adapted from Burger, 2010). **B)** Schematic representation of metabolic labeling experiment. Cells were cultured with complete medium, phosphate-depleted, and labeled with [^{32}P]orthophosphate for 1 hour (pulse) before addition of excess complete medium with unlabeled phosphate (chase). Total RNA was isolated at the indicated times. **C)** Kinetics of rRNA processing in HCT116shGTPBP4 cells. Cells were labeled with ^{32}P -orthophosphate for 1 h and chased with “cold” medium for the indicated time. RNA was extracted, and separated on denaturing agarose gel. Labeled RNA was visualized by autoradiography (right panel). Ethidium bromide staining of the gel confirmed equal loading of RNA (left panel).

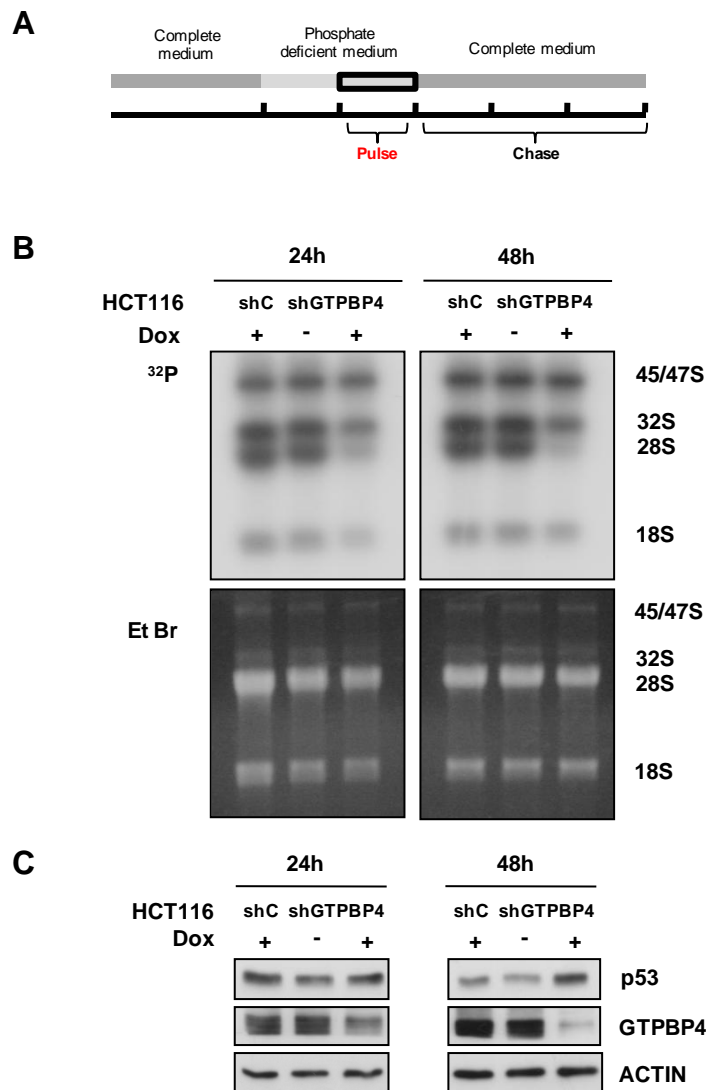


Figure 15. GTPBP4 depletion affects ribosome biogenesis, correlating with p53 accumulation.
A-B) GTPBP4 depletion affects rRNA processing. HCT116shC or HCT116shGTPBP4 cells were treated for 24 hours and 48 hours with Doxycycline or left untreated. Cells were labeled with ³²P-orthophosphate for 1 hour and chased with cold medium for 3 hours. Total RNA was extracted, separated on denaturing agarose gel and labeled RNA was visualized by autoradiography (top panels). Ethidium bromide staining of the gel confirmed equal loading of RNA (bottom panels). **C)** Expression levels of GTPBP4 and p53 were analyzed in the same cells by western blot. Actin was detected as loading control.

The RPs-Mdm2-p53 stress response pathway promotes p53 stabilization in GTPBP4 depleted cells

Perturbations in any step of ribosome biogenesis have been demonstrated to induce a stress response leading to p53 activation and cell cycle arrest (Deisenroth and Zhang, 2010). The mechanism of p53 activation relies on the cooperative binding of ribosomal proteins (RPs) RPL5 and RPL11 to Mdm2, and this leads to Mdm2 inactivation and consequently p53 activation and cell cycle arrest (Zhou et al., 2012). This ribosome biogenesis surveillance mechanism has been defined the “RPs-Mdm2-p53 stress response pathway” (Deisenroth and Zhang, 2010).

We observed that GTPBP4 depletion affects ribosome biogenesis prior to p53 accumulation, thus we asked whether the “RPs-Mdm2-p53 stress response pathway” could be responsible for p53 stabilization.

To address this question we used RNAi to downregulate the ribosomal protein RPL11, a key mediator of the RPs-Mdm2-p53 axis. As shown in fig. 16A, RPL11 knockdown is sufficient to prevent p53 accumulation induced by GTPBP4 depletion, thus suggesting that p53 accumulation upon GTPBP4 silencing requires Ribosomal Protein L11.

Since the activation of p53 is mediated by the interaction of RPL11 with Mdm2, we asked whether GTPBP4 depletion would increase formation of such complex. Endogenous Mdm2 was immunoprecipitated and bound RPL11 was visualized by western blot upon doxycycline treatment of HCT116shGTPBP4 cells. We observed that GTPBP4 depletion promotes RPL11 binding to Mdm2 (fig. 16B), thus further suggesting that p53 stabilization upon GTPBP4 knockdown might derive from RP-mediated inhibition of Mdm2 functions.

To further clarify if GTPBP4 depletion promotes p53 accumulation through the RPs-Mdm2-p53 axis, we treated GTPBP4 depleted cells with two chemotherapeutic drugs, Actinomycin D (ActD) and 5-Fluorouracil (5-FU), at conditions known to specifically trigger ribosomal stress and activate the RPs-Mdm2-p53 axis (Bhat et al., 2004; Sun et al., 2007). As shown in fig. 16C, GTPBP4 depletion does not promote a further increase in p53 levels upon drug treatment, indicating that the same pathway might control p53 accumulation upon GTPBP4 knockdown and drug-induced ribosomal stress.

Collectively, these results strongly suggest that GTPBP4 depletion, by affecting ribosome biogenesis, activates the “RPs-Mdm2-p53 stress response pathway”, leading to p53 stabilization and cell cycle arrest.

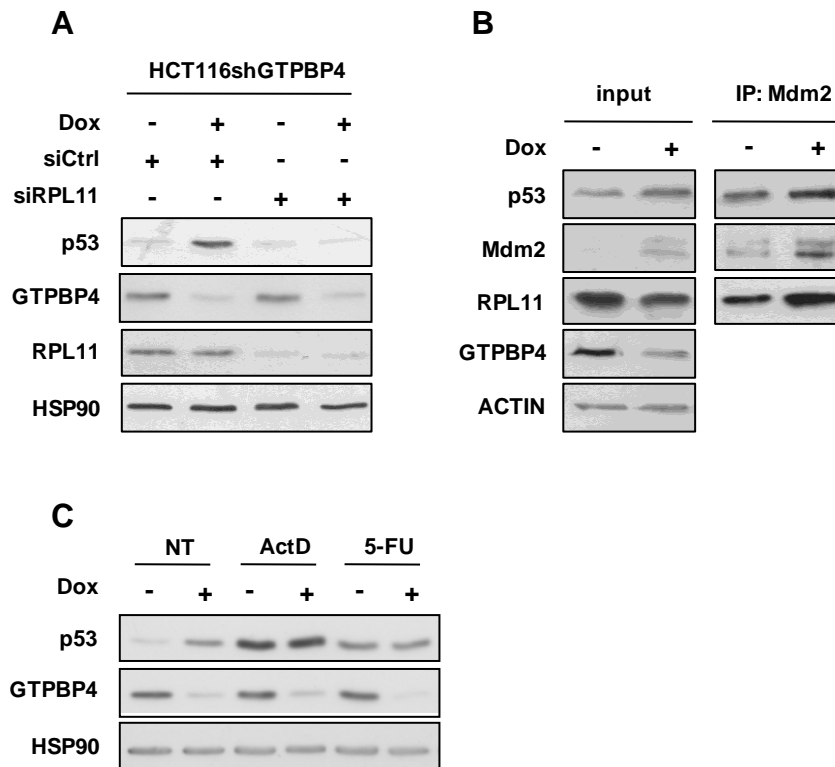


Figure 16. The RPs-Mdm2-p53 stress response pathway promotes p53 stabilization in GTPBP4 depleted cells.

A) RPL11 is required for p53 accumulation induced by GTPBP4 depletion. HCT116shGTPBP4 cells were silenced with control siRNA (siCtrl) or siRNA targeting RPL11 (siRPL11) for 16 hours. Cells were then collected and plated in presence or absence of Doxycycline for additional 48 hours. Western blot analysis shows p53 levels. Blot for GTPBP4 and RPL11 confirms depletion of the endogenous proteins. HSP90 was blotted as loading control. **B)** GTPBP4 depletion promotes the interaction of Mdm2 with RPL11. HCT116shGTPBP4 cells were treated with doxycycline for 48 hours or left untreated and subjected to immunoprecipitation with anti-MDM2 (SMP14) antibody. Immunoprecipitated proteins (IP, right panel) and 5% of total lysates (input, left panel) were subjected to western blot analysis with the indicated antibodies. **C)** GTPBP4 depletion does not cooperate with nucleolar stress to increase p53 levels. Doxycycline treated or untreated cells were exposed to 5nM Actinomycin D (ActD) and 50 μ M 5-Fluorouracil (5-FU) for 8 hours. Western blot for p53 and GTPBP4 is shown. HSP90 was blotted as loading control.

Impact of different ribosomal stresses on p53 stabilization

Currently, at least 3 types of nucleolar stress that can be experimentally induced in cells, have been reported to activate p53. 1) Inhibition of RNA Pol I, for example by Actinomycin D treatment; 2) depletion of selected ribosomal proteins, for example RPL30 and RPS6; 3) depletion or mutation of accessory factors necessary for ribosome biogenesis, such as Bop1 or Pes1. All these conditions have been reported to affect ribosome biogenesis and promote p53 accumulation. For the first two types of stress, it has been convincingly proven that p53 accumulation depends on the activation of the RPs-Mdm2-p53 axis (Deisenroth and Zhang, 2011; Zhou et al., 2012); for the third type of stress, several papers reported p53 accumulation after depletion of Bop1, Pes1, or other cofactors, but none of the studies formally demonstrated p53 stabilization, or proved the involvement of the aforementioned regulatory axis in governing p53 levels.

We demonstrated that the depletion of ribosomal processing factor GTPBP4 promotes a two-fold increase in p53 half-life and that such stabilization requires the activation of the RPs-Mdm2 axis.

Intriguingly, published data indicate that various ribosomal stressors promoting the activation of the RPs-Mdm2 axis have a much greater impact on p53 stability than what we observed with GTPBP4 depletion. Hence we asked whether the extent of p53 stabilization upon different types of ribosomal stress could be intrinsically dependent on the nature of the stress itself.

To answer this question, we analyzed the effects on ribosome biogenesis and p53 stability of the depletion of two proteins that induce different ribosomal stress features: RPL30, and Bop1. RPL30 depletion affects nucleolar structure and induces a consistent increase in p53 stability via the RPs-Mdm2-p53 axis (Sun et al., 2010). In contrast, Bop1 is a ribosome associated factor involved in the late phases of maturation of the 60S ribosome subunit, much like GTPBP4 (Strezoska et al., 2002). HCT116 cells were transfected with siRNAs targeting Bop1 or RPL30, and rRNA processing was analyzed by ³²P-orthophosphate labeling. As shown in fig. 17A, both Bop1 and RPL30 depletion strongly affects 28S rRNA biogenesis. Interestingly, the depletion of either protein seems to affect rRNA processing after the cleavage of pre-18S rRNA, since newly synthesized 18S rRNA is not affected. Western blot analyses confirmed that RPL30 or Bop1 depletion promotes p53 accumulation (fig. 17B).

After verifying the role of RPL30 and Bop1 in ribosome biogenesis, we analyzed the impact of RPL30 and Bop1 depletion on p53 stability through a cycloheximide experiment. Efficient knockdown of those proteins was controlled by RTqPCR as shown in the top panel of fig. 17C. Although the data are preliminary, depletion of either protein increases p53 half-life (fig. 17C, bottom panel), thus suggesting that in both cases altered ribosome biogenesis induces a checkpoint leading to p53 stabilization.

Performing a densitometric analysis of the bands, the extent of p53 stabilization upon GTPBP4, RPL30, and Bop1 depletion was quantified. As shown in fig. 17D, in RPL30 depleted cells p53 half-life is strongly increased. In GTPBP4 and Bop1 depleted cells, p53 half-life is also increased, but is sensibly shorter than in RPL30-depleted cells.

Notably, the increase in p53 half-life is remarkably similar in cells depleted of GTPBP4 or Bop1.

Although not definitive, these data suggest that the intensity of p53 stabilization might be different depending on the type of ribosomal stress. Defective ribosome processing in the absence of nucleolar disruption, induced in our case by GTPBP4 or Bop1 depletion, seems to intersect the p53 pathway with a lower impact than other ribosomal stressor, such as the depletion of ribosomal proteins, that induce nucleolar structure alterations. Nonetheless, at least for GTPBP4 depletion, I demonstrated that p53 accumulation requires the activation of the “RPs-Mdm2-p53 stress response pathway” which is also activated in response to nucleolar disruption.

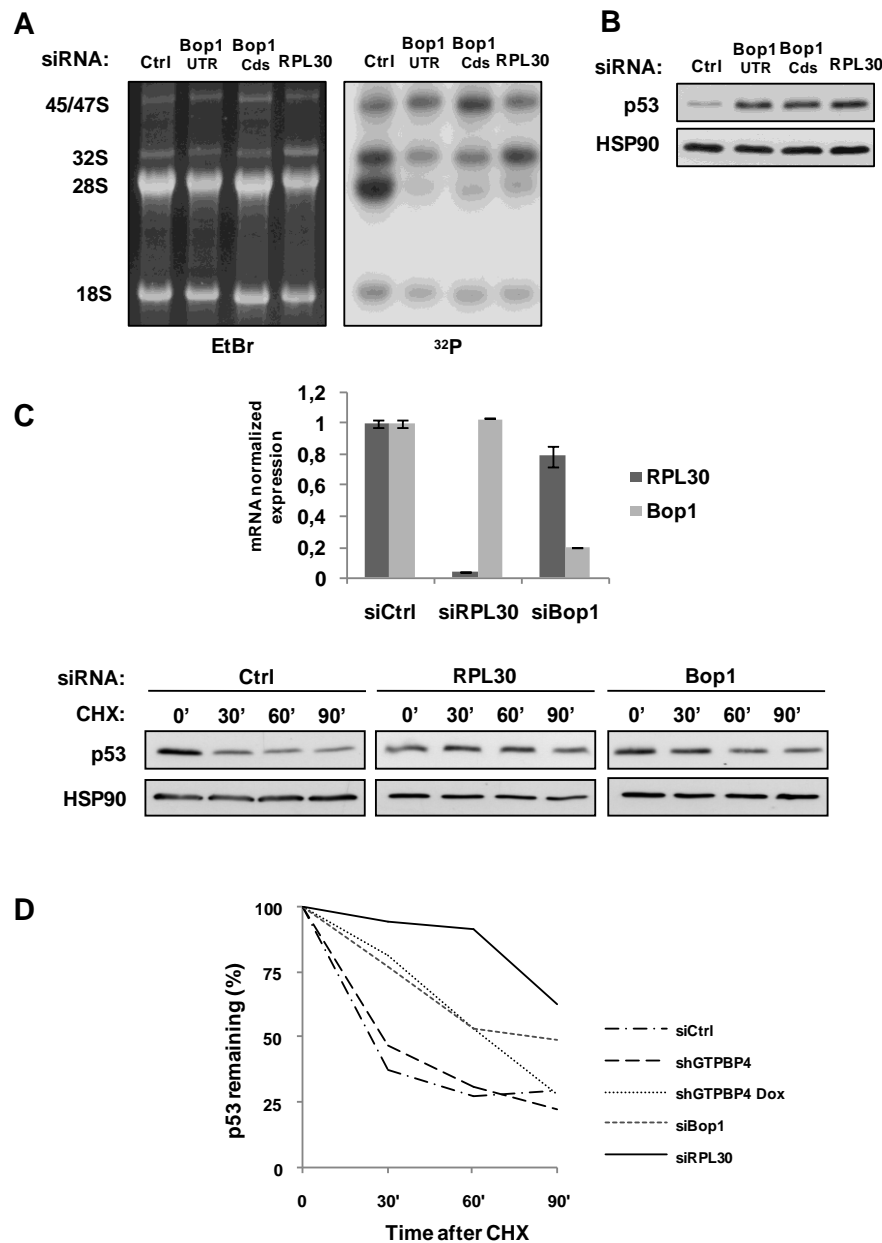


Figure 17. Impact of different ribosomal stress conditions on p53 stabilization.

A) Bop1 or RPL30 depletion affects 28S ribosome processing. HCT116 cells were treated for 48 hours with control siRNA (siCtrl), siRNA targeting RPL30, or two siRNAs targeting Bop1 (in the 3'-UTR and in the coding sequence, CDS). Cells were pulse-labeled (right panel) and rRNA processing was visualized as in fig. 15A. Ethidium bromide staining of the gel shows loading of RNA (left panel). **B)** Bop1 or RPL30 depletion triggers p53 accumulation. HCT116 cells were treated as in A, and analyzed by western blot with anti-p53 antibody. HSP90 was used as loading control. **C-D)** RPL30 or Bop1 depletion induces p53 stabilization. HCT116 cells were treated as in A (siRNA BOP1 3'-UTR was used). **C)** The efficiency of RPL30 and BOP1 depletion was measured by RT-qPCR (upper panel). Data were normalized to GAPDH, and compared to expression levels of untreated cells. Half-life of p53 was evaluated by immunoblotting after CHX treatment as described in fig. 13A. The gel was loaded with reduced amount of lysate from siRNA-treated cells to obtain a comparable p53 signal at the 0h point. **D)** Comparison of p53 half-life upon different ribosomal stresses. Quantification is shown of the ratio of p53 to HSP90 (measured by densitometry on Western blots). Data for RPL30 and Bop1 silencing are from the experiment in panel C; data for GTPBP4 silencing refer to the inducible HCT116shGTPBP4 cell line, and are extracted from fig. 13.

PART 1: DISCUSSION

Our group identified the protein GTPBP4 as a novel, evolutionary conserved interactor of wild-type p53 (Lunardi et al., 2010). The nucleolar protein GTPBP4 is an essential processing factor required for biogenesis of 60S ribosomal subunit (Jensen et al., 2003), indeed, yeast cells depleted of GTPBP4 or harboring temperature sensitive alleles of the gene display defects in pre-rRNA processing and in the release of nascent pre-60S subunits from the nucleolus (Kallstrom et al., 2003; Saveanu et al., 2003). Our primary interest in GTPBP4 derived from the observation that its depletion by siRNA induced accumulation and activation of p53, without apparently affecting nucleolar functions. Indeed we observed that GTPBP4 depletion did not alter nucleolar structure, and most importantly, p53 accumulation was apparently not due to increased protein half-life. Collectively these preliminary data, prompted us to initially exclude the ribosomal surveillance pathway as the mechanism promoting p53 accumulation upon GTPBP4 depletion (Lunardi et al., 2010) and incited us to explore a direct involvement of GTPBP4 in controlling p53 translation. To dissect this potentially novel regulatory axis, we generated a stable cell line engineered for inducible GTPBP4 knockdown. In this model system, ³⁵S methionine labeling experiments and analysis of RNA loading on polysomes, revealed no evidences of increased p53 translation in cells depleted of GTPBP4. Conversely, meticulous cycloheximide experiments, revealed that GTPBP4 depletion in fact increases p53 stability.

We then demonstrated that the mechanism responsible for such accumulation relies on the “RPs-Mdm2-p53 stress response pathway”, probably activated by a defect in rRNA processing triggered by GTPBP4 depletion (fig. 15).

In recent years, the depletion of many ribosomal proteins and accessory factors involved at different levels in ribosome biogenesis has been reported to activate the “RPs-Mdm2-p53 stress response pathway (Deisenroth and Zhang, 2011). Interestingly, depletion of accessory factors involved in late ribosomal processing, such as Bop1, Pes1, or others, has been reported to trigger p53 accumulation, but none of the studies rigorously demonstrated the mechanism involved. Our data, for the first time demonstrate that p53 accumulation upon impairment of late ribosomal

processing - triggered in our case by GTPBP4 depletion – also requires the activation of the RPs-Mdm2 axis.

Remarkably, the depletion of Bop1, a ribosomal associated factor, promotes p53 stability to a similar extent as GTPBP4 knockdown, while the depletion of RPL30 has a significantly greater impact on p53 half-life. Interestingly, when we analyzed how the depletion of these proteins affects rRNA processing, we found no relevant differences (fig. 17A). These preliminary observations raise the intriguing hypothesis that a distinct degree of p53 stabilization can be triggered by the RPs-Mdm2 axis, depending on the specific type of nucleolar stress. Indeed, in other conditions of nucleolar stress, all triggering the activation of the RPs-Mdm2-p53 axis, p53 half-life is increased to a greater extent than upon GTPBP4 or Bop1 depletion (Sun et al., 2007; Sun et al., 2010; Yu et al., 2011). One common instance is that a stronger p53 stabilization is probably observed upon treatments that provoke nucleolar disruption (i.e. ActD, Rpl30 knockdown), while in GTPBP4 depleted cells the nucleolar structure is preserved.

It should be noted that we had identified GTPBP4 as a novel p53 interacting protein, but the mechanism of p53 accumulation upon GTPBP4 depletion seems to be independent from their physical interaction. Thus, the potential functional significance of the interaction between p53 and GTPBP4 is still to be elucidated.

One intriguing hypothesis is that p53 itself, through GTPBP4 interaction, might regulate the late steps of rRNA processing and the maturation of 60S ribosomes, in turn influencing the translational apparatus of the cell. Interestingly, p53 has been already demonstrated to control other steps of ribosome biogenesis, for instance by regulating RNA pol I and pol III activity (Krastev et al., 2011; Zhai and Comai, 2000). An inhibitory action of p53 on 60S ribosome maturation could be part of a tumor-suppressive response to activation of proto-oncogenes that promote ribosomal synthesis such as Ras and Myc.

Being independent of its transcriptional functions, interaction with cofactors involved in ribosome maturation could be a preferential activity of N-terminal truncated p53 isoforms (i.e. DN p53).

The dependency of cancer cells on increased ribosome biogenesis has been recently exploited to identify new drugs that specifically target this biological process. In fact, a specific inhibitor of RNA pol I has been recently developed and is now in clinical trial (Drygin et al., 2011). Accessory proteins involved in ribosome biogenesis, specifically deregulated in tumors, could expand the list of potential targets for this type of therapy.

High expression levels of GTPBP4 correlate with reduced survival in breast cancer bearing wild-type p53 (Lunardi et al., 2010). Interestingly, we observed that the expression of various other proteins involved in late ribosomal processing inversely correlates with survival in breast cancer tumors (fig. 18). It may be interesting to assess the addiction of tumor cells on the elevated expression of these ribosomal processing factors, and eventually find drugs that - by specifically targeting these cofactors - would activate the RPs-Mdm2-p53 axis, increasing p53 stability preferentially in tumor cells. Elevated p53 levels could then be exploited to sensitize cancer cells to conventional chemotherapy.

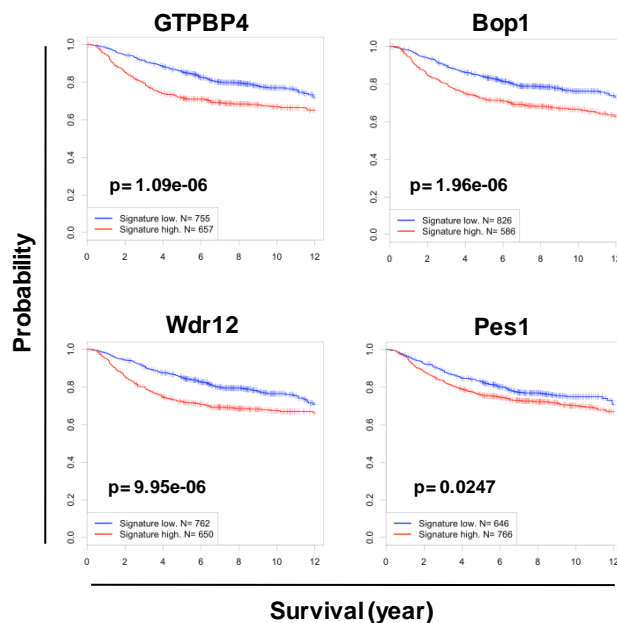


Figure 18. Cumulative survival (Kaplan–Meier) analysis showing that higher expression of ribosomal processing factors GTPBP4, Bop1, Wdr12, and Pes1, significantly correlates with reduced survival in a large dataset (circa 1400 samples) of breast cancer patients with 12 years clinical follow-up.

PART 2: Effect of altered ribosome biogenesis on mutant p53 stability

Deregulated ribosome biogenesis triggers a checkpoint leading to the activation of wild type p53 tumor suppressor activity (Macias et al., 2010). However, the p53 pathway is impaired in most human cancers, so the above checkpoint is frequently non-functional in tumor cells.

In fact a large proportion of human tumors carry p53 mutations, and most of these are missense mutations that result in production of a mutant protein that not only loses tumor-suppressing activities, but also acquires new pro-oncogenic activities, defined as gain of function. Specifically, mutant p53 enhances proliferation and survival of cancer cells, and increases tumorigenesis *in vivo* when compared with p53-null cells or mice (Brosh and Rotter, 2009).

In opposition to the wild type counterpart, which is highly unstable, mutant p53 is often found at high levels in tumors (Bartek et al., 1991), and high mutant p53 protein levels are thought to be responsible for most of its oncogenic functions (Muller and Vousden, 2013).

Mice models and studies with cultured cells highlighted the prominent role of Mdm2 in the regulation of mutant p53 stability (Li et al., 2011; Suh et al., 2011; Terzian et al., 2008), thus suggesting the hypothesis that cellular stimuli known to activate the tumor suppressive activity of wild type p53, could in turn promote the oncogenic functions of mutant p53.

Since ribosome biogenesis is almost invariably deregulated in tumors, we hypothesized that one of the regulatory mechanisms that contribute to mutant p53 stability might be represented by the inhibition of Mdm2 functions through the binding of unbalanced ribosomal proteins.

We therefore decided to analyze whether mutant p53 could be indeed stabilized by the “RPs-Mdm2-p53 stress response pathway”.

PART 2: RESULTS

Altered ribosome biogenesis promotes mutant p53 stability

Increased ribosome biogenesis is frequently observed in human cancers, mainly as a result of the amplified biosynthetic demand linked to uncontrolled cell proliferation. In cells with wtp53, a deregulated ribosome processing activates a cellular checkpoint, known as the “RPs-Mdm2-p53 stress response pathway” that promotes tumor suppression wild-type p53 (Deisenroth and Zhang, 2010).

Since mutant p53 retains some of the regulatory mechanisms of the wild type counterpart (Terzian et al., 2008), it is conceivable that signaling pathways promoting the activation of wt p53, could instead increase stability of mutant p53, thereby promoting its oncogenic potential. Among these double edged processes, we reasoned that altered ribosome biogenesis might contribute to increased mutant p53 expression levels.

More specifically, we asked whether the “RPs-Mdm2-p53 stress response pathway”, could promote mut-p53 protein stability. After a survey of available lines, we chose the ovarian carcinoma Ovar3 cell line, bearing the p53 R248Q mutation. The choice of this cell line is due to the relatively low levels of endogenous mutant p53, allowing studies aimed to detect a further increase in mutant p53 protein levels.

We treated cells with two chemotherapeutic drugs, Actinomycin D and 5-fluorouracil, at concentrations and timing known to specifically affect ribosome biogenesis (Perry and Kelley, 1968, 1970; Sobell et al., 1971; Sun et al., 2007). As shown in the left panel of fig.19A, drug treatments promote mutant p53 accumulation, indicating that mutp53 indeed responds to nucleolar stress.

To confirm this observation under conditions in which the DNA-damage response is certainly not involved, we induced nucleolar stress by siRNA mediated depletion of ribosomal protein RPL30, and ribosomal processing factors GTPBP4 and Bop1. As shown in the right panel of fig.19A, also in these conditions mutant p53 levels are increased, thus confirming that altered ribosome biogenesis can promote mut-p53 accumulation.

Since ribosomal stress promotes the inhibition of Mdm2 E3 ubiquitin ligase activity (Deisenroth and Zhang, 2010), we asked whether increased mut p53 levels are the

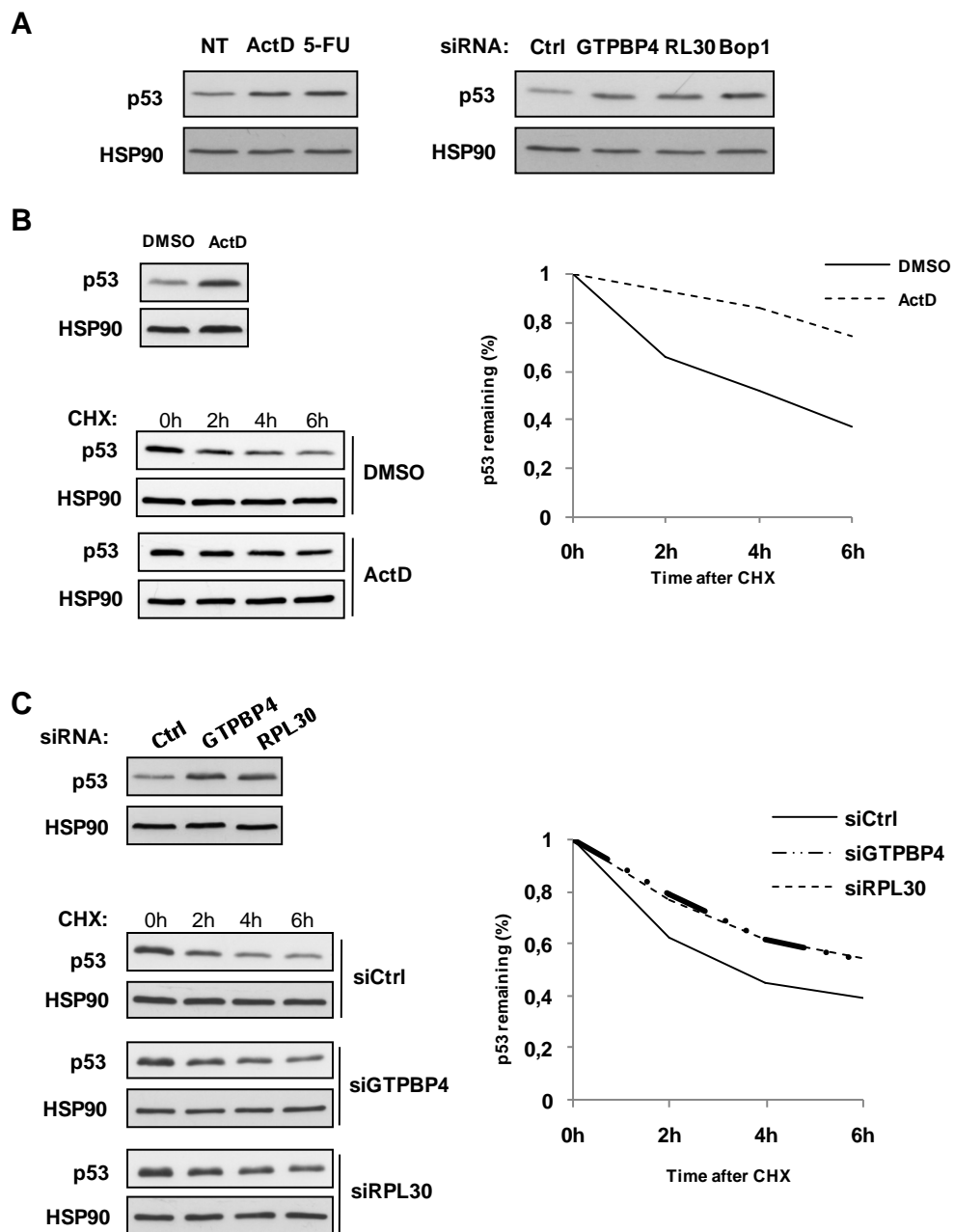


Figure 19. Altered ribosome biogenesis increases stability of mutant p53.

A) Various ribosomal stress conditions induce mutant p53 accumulation. Ovar-3 cells were exposed to 5nM ActD or 50 μ M 5-FU for 8 hours (left panel), or were treated for 48 hours with control siRNA or siRNA targeting GTPBP4, RPL30, or Bop1 (right panel). Western blots of p53 are shown. HSP90 was used as loading control. **B)** Nucleolar stress increases mutant p53 half-life. Ovar-3 cells were treated with Actinomycin D as in panel A, and 50 μ M Cycloheximide (CHX) was added for the indicated times. Analysis of p53 half-life is shown by western blot (bottom left panel) and quantification by densitometry (right graph). The gel in the figure was loaded to obtain a comparable p53 signal at 0h time point; accumulation of mutant p53 under these conditions was confirmed by an ad hoc western blot using identical quantities of lysate (top left panel). **C)** RPL30 or GTPBP4 knockdown increases mutant p53 stabilization. Ovar-3 cells were treated with siRNA as described in panel A. Analysis of mutant p53 half-life after CHX treatment was performed as described for panel B.

result of increased mutant p53 half-life. To this aim, we performed cycloheximide experiments upon Actinomycin D treatment, and RPL30 or GTPBP4 silencing.

As shown in fig. 19B, Actinomycin D treatment strongly promotes mutant p53 stabilization. Similarly, GTPBP4 and RPL30 knockdown also increase mutant p53 half-life, although with lower efficiency (fig. 19C).

These results indicate that ribosomal stress can stabilize and activate mutant p53, as well as wild type p53.

The RPs-Mdm2 axis promotes mutant p53 stabilization upon altered ribosome biogenesis

Since we observed that different ribosomal stressors promote mutant p53 stabilization, we asked whether the RPs-Mdm2 axis is responsible of this effect. Critical mediators of this axis are RPL5 and RPL11, that cooperatively bind Mdm2 and inhibit its functions.

We therefore treated Ovar3 cells with Actinomycin D (ActD) and 5-fluorouracil (5-FU), and analyzed Mdm2-bound RPL11 by co-immunoprecipitation. We observed that ribosomal stress induced by ActD and 5FU treatments promotes RPL11 binding to Mdm2 (fig. 20A), thus suggesting that mutant p53 stabilization is induced by RPs-mediated Mdm2 inhibition.

To further confirm the role of RPs in mutant p53 stabilization upon ribosomal stress, we silenced RPL11, and analyzed mutant p53 accumulation upon ActD and 5FU treatment. As shown in fig 20B, RPL11 silencing prevents p53 accumulation upon ribosomal stress, thus confirming the role of the RPs-Mdm2 axis in promoting mutant p53 stabilization.

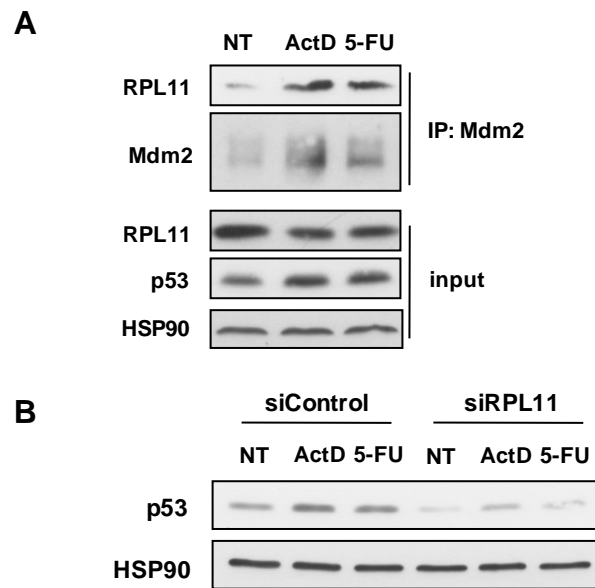


Figure 20. The RPs-Mdm2 axis promotes mutant p53 stabilization upon altered ribosome biogenesis.

A) ActD and 5-fluorouracil treatments promote RPL11 binding to Mdm2. Ovar-3 cells were treated with ActD and 5-FU as in fig. 19A, and subjected to immunoprecipitation with anti-MDM2 (SMP14) antibody. Immunoprecipitated proteins (IP, top panel) and 5% of each total cell extract (input, bottom panel) were subjected to western blot analysis with the indicated antibodies. **B)** RPL11 knockdown prevents p53 accumulation induced by nucleolar stress. Ovar-3 cells were silenced with control siRNA (siCtrl) or siRNA targeting RPL11 (siRPL11) for 48 hours, and then treated with ActD and 5-FU for 8 hours. Levels of mutant p53 were analyzed by western blot; HSP90 was detected as loading control.

Potential biological impact of mutp53 accumulation by ribosomal stress in cancer cells

An increase in stabilization and accumulation of mutp53 may amplify its gain-of-function properties (Muller et al., 2009), eventually augmenting its oncogenic potential. This might have a relevant impact in the use of ribosomal-damaging drugs for chemotherapy of cancers bearing p53 mutations.

To examine the potential significance of increasing mutant p53 levels by ribosomal stress, we analyzed the motility of cancer cells by performing an *in vitro* migration assay. Ovar3 cells were transfected with control siRNA or p53 siRNA for 48 hours and then treated for additional 8 hours with ActD or left untreated. Cells were then collected and used for transwell migration assays in the absence of drug. Treatment with ActD augmented mutant p53 levels (fig. 21, right panel) and promoted a significant increase in cell migration (fig. 21, left panel), confirming that

accumulation of mutant p53 may have biologically relevant consequences. Importantly, the observed increase in cell migration is dependent on mutant p53, since p53 knockdown abolishes this phenotype.

Collectively, these results indicate that altered ribosome biogenesis can increase stability of mutant p53 via the same pathway that stabilizes wild type p53. Moreover, such increased mutp53 levels can be biologically relevant, since they promote a classical mutant p53 phenotype such as cell migration.

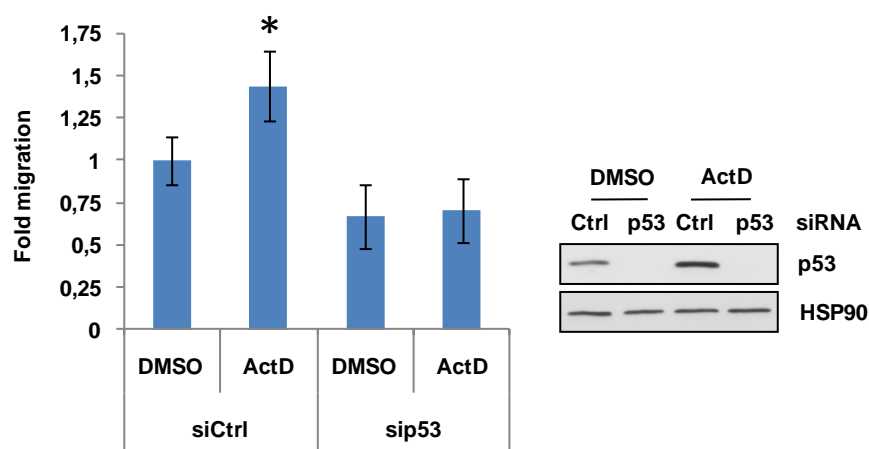


Figure 21. Mutant p53 stabilization by nucleolar stress can promote cell migration.

ActD treatment increases motility of cancer cells through a mechanism that requires mutp53. Ovar-3 cells were transfected with control siRNA or p53 siRNA for 48 hours, and treated for additional 8 hours with ActD or DMSO. Cells were then collected and used for transwell migration assays in the absence of drug. Error bars indicate standard deviation (n=3, *p<0.05 one-tailed t-test). p53 accumulation upon ActD treatment and efficiency of p53 silencing were controlled by western blot of the same cells (right panel).

Development of a tool for studying the RPs-Mdm2-mutp53 axis in tumorigenesis

Mutp53 can be stabilized in tumors by various mechanisms; here we have shown that widely used chemotherapeutic drugs such as 5-FU can induce mutp53 stabilization through ribosomal stress. To experimentally evaluate the specific impact of deregulated ribosome biogenesis in the oncogenic functions of mutant p53, however, we need to specifically inhibit the RPs-Mdm2 axis, thus uncoupling ribosomal stress from Mdm2 functions, without affecting other pathways.

Preventing mutant p53 accumulation by altered ribosome biogenesis can be achieved by depleting those ribosomal proteins responsible for Mdm2 inhibition. We biochemically demonstrated in fig. 20B that RPL11 is an essential mediator of the RPs-Mdm2 axis, since its knockdown completely abolishes mut-p53 accumulation upon ribosomal stress. However, RPL11 is a constitutive ribosomal protein, and its depletion could in the long time globally affect cell homeostasis, regardless of mutant p53.

Indeed, experiments with RPL11 knock-down should be done over short time frames, and in any case the potential impact on the ribosome pool must be considered when analyzing the results.

We took advantage of previous studies that determined the crucial importance of RPL5 and RPL11 for this checkpoint, and mapped the region of Mdm2 where these proteins bind (Zhang et al., 2003).

We then reasoned that to prevent mutant p53 accumulation by the RPs-Mdm2 axis, it might be sufficient to sequester the nuclear pool of free RPL11 that is presumably increased upon ribosomal stress. So, overexpression of a polypeptide corresponding to the specific region of Mdm2 that binds RPL11 would prevent its interaction with endogenous full-length Mdm2.

Thus we cloned in an HA-tag expression vector the Mdm2 region between aa 284 and 374, that has been reported to specifically interact with RPL11. Moreover, since RPL11 has been reported to bind and cooperate with RPL5 in promoting Mdm2 inhibition upon ribosomal stress (Bursac et al., 2012; Donati et al., 2013), we also cloned a larger Mdm2 region, from aa 216 to 374, that binds both RPL5 and RPL11 (Zhang et al., 2003) (fig. 22A).

We first compared the ability of these two peptides to bind RPL11. We cotransfected myc-tagged RPL11 alone or with HA-tagged peptides, and analyzed bound RPL11 co-immunoprecipitated with the peptide. As shown in fig. 22B, the 216-274 Mdm2 fragment binds more efficiently to myc-tagged RPL11, than the 278-374 peptide. Moreover, we could easily observe interaction between endogenous RPL11 and HA-tagged 216-374 peptide (fig. 22C).

It is widely assumed that ribosomal stress causes an increase in free RPL11. In order to verify the ability of the Hdm2(216-374) peptide to detect such increased free RPL11, we treated Hdm2(216-374)-transfected cells with Actinomycin D, and

analyzed RPL11 binding to the peptide by HA-tag immunoprecipitation. As shown in fig. 22D ribosomal stress enhances RPL11 binding to the peptide. Interestingly, it appears that the Hdm2-peptide may be used as an efficient probe to detect free RPL11 in cells.

In conclusion, the use of this peptide, if validated, could be an alternative way to study the role of RP-Mdm2 axis, in promoting the oncogenic functions of mutant p53 upon deregulated ribosome biogenesis.

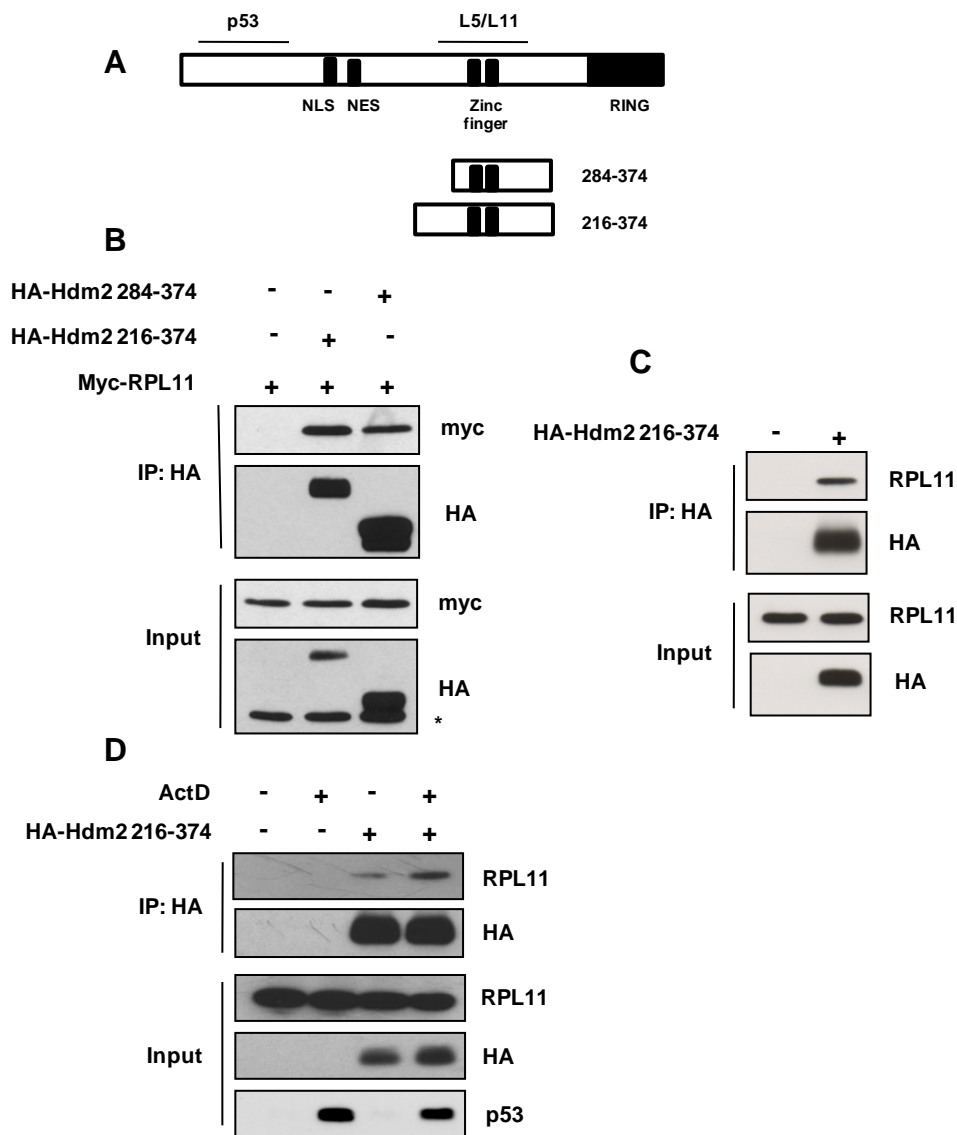


Figure 22. Development of a Mdm2-derived expression construct as a tool for studying the RPs-Mdm2-mutp53 axis in tumorigenesis.

A) Schematic representation of the Hdm2 protein and the two peptides used in this study (adapted from Zhang et al., 2003). **B)** The peptide Hdm2(216-374) binds more efficiently than Hdm2(284-374) to overexpressed RPL11. H1299 cells were transfected with the indicated plasmids for 48 hours and subjected to immunoprecipitation with anti-HA (12CA5) antibody. Immunoprecipitated proteins (IP) and 5% of each total cell extract (input) were subjected to western blot analysis with the indicated antibodies. **C)** The Hdm2(216-374) construct binds endogenous RPL11. H1299 cells were transfected with a plasmid encoding HA-Hdm2(216-374) or empty vector for 48 hours, and subjected to immunoprecipitation and immunoblot as in B. **D)** Actinomycin D treatment enhances RPL11 binding to the Hdm2(216-374) peptide. U2OS cells were transfected with plasmid encoding for HA-Hdm2(216-374) or an empty vector for 24 hours. Cells were then collected and splitted into two plates. 24 hours later cells were treated with 5nM Actinomycin D or DMSO for additional 8 hours, and subjected to immunoprecipitation with anti-HA (12CA5) antibody. Immunoprecipitated proteins (IP) and 5% of total cell extracts (input) were subjected to western blot analysis with the indicated antibodies.

PART 2: DISCUSSION

Increased mutant p53 levels are a common feature of high grade tumors (Bartek et al., 1991). Many evidences suggest that high levels of mutant p53 protein are responsible for most of its oncogenic functions (Muller and Vousden, 2013); indeed, its precocious stabilization in knock-in mice is associated with enhanced aggressiveness (Suh et al., 2011; Terzian et al., 2008). However, in contrast to the plethora of information available for wild type p53, the mechanisms dictating mutant p53 stability are much less characterized (Brosh and Rotter, 2009).

We found that the “RPs-Mdm2-p53 stress response pathway” can promote mutant p53 stabilization upon impaired ribosome biogenesis in cancer cells. Importantly, we observed that increased mutp53 levels may have a relevant functional impact, since they foster a mutant p53-dependent phenotype such as cell migration.

These results raise two interesting considerations.

The first concerns the mechanism of mutp53 stabilization in tumor cells. In fact, since altered ribosome biogenesis is a common feature to cancer cells, it is possible that a “constitutive ribosomal stress” generated by oncogenic transformation might be responsible for increased mutant p53 levels by activating the RPs-Mdm2 axis. To test this hypothesis, it would be necessary to interfere with the Rp-Mdm2-p53 axis without affecting the protein synthesis apparatus. This could be elegantly approached by generating double transgenic mice with the knock-in of mutant p53 in the context of the Mdm2^{C305F} point mutation (Macias et al., 2010); this mutation uncouples the interaction between free ribosomal proteins and Mdm2, and would allow the study of mutp53 stability (and tumorigenicity) in the absence of a functional Rp-Mdm2-p53 axis. Less elegant, but more accessible, is the generation of cell lines with stable expression of the Hdm2(216-374) peptide described in Fig. 22. High levels of the peptide might in fact reduce the interaction between free ribosomal proteins and Mdm2, thus interfering with the Rp-Mdm2-p53 axis. This is currently in progress.

The second consideration regards the possible implications in the choice of chemotherapy. In fact, many commonly used chemotherapeutic drugs have been reported to impinge on ribosome biogenesis (Burger et al., 2010), and it is possible that activation of the nucleolar stress response pathway could be detrimental in tumors

that bear TP53 missense mutation. In fact, we have shown that this pathway triggers mutant p53 stabilization, and increased mutp53 levels promote chemoresistance and metastasis (Muller et al., 2009) . Thus, the use of drugs such as 5-FU or ActD, that inhibit ribosome biogenesis at low concentrations, could be counterproductive in treating tumors that carry p53 mutations. Our data provide yet another evidence that the status of p53 should probably be taken into consideration when defining a chemotherapy regimen.

CONCLUSIONS

Ribosome biogenesis is a very complex process involving transcription as well as many post-transcriptional steps to produce functional ribosomes.

Cells actively monitor the process of ribosome biogenesis, consequently activating cellular checkpoints when this process is deregulated (Boulon et al., 2010). The best understood of such checkpoints is the nucleolar stress pathway (also called the ribosomal surveillance pathway). The key event of this pathway is the modulation of the E3 ubiquitin ligase Mdm2 by a subset of RPs that, upon altered ribosome biogenesis, are released in the nucleoplasm (Zhang and Lu, 2009) where they bind Mdm2, inhibiting its ubiquitin ligase activity (Lee and Gu, 2010).

Part of the work presented in this Thesis demonstrates that the RPs-Mdm2 axis is responsible for wild-type p53 stabilization upon depletion of the nucleolar protein GTPBP4. Specifically, we observed that GTPBP4 depletion affects 28S rRNA processing, and this correlates with p53 stabilization mediated by the inhibitory effect of RPL11 on Mdm2.

In this Thesis we also report that the RPs-Mdm2 axis—normally responsible for the tumor suppressive functions of wild-type p53 upon deregulated ribosome biogenesis—is also implicated in the regulation of mutant p53 stability, with potentially oncogenic consequences.

Indeed, we observed that various nucleolar stress conditions can promote stabilization of wild-type p53 as well as mutant p53, very likely through the same molecular mechanism based on the inhibitory binding of RPL11 to Mdm2.

Collectively, these results support the notion that the process of ribosome biogenesis has an active role in tumorigenesis. We propose that upon deregulated ribosome biogenesis, or nucleolar stress, the TP53 status may be critical to drive the nucleolar surveillance pathway towards a tumor suppressive or oncogenic outcome.

MATERIALS AND METHODS

Cell Culture, Transfections, Retroviral Transductions and treatments. HCT116, HCT116shC, HCT116shGTPBP4, 293T, U2OS cells were cultured in DMEM medium supplemented with 10% FCS (ECS0180L, Euroclone), and antibiotics (DE17-602E, Lonza). H1299 were cultured in RPMI medium supplemented with 10% FCS and antibiotics. OVCAR-3 were cultured in RPMI medium supplemented with 20% FCS, insulin (10 μ g/ml), and antibiotics.

For siRNA transfections, cells were transfected with 40 nM siRNA oligonucleotides using Lipofectamine RNAiMax (Invitrogen), following manufacturer's instructions.

siRNAs used in this work are listed in the following table:

siRNA	Sequence	Purchase from/ Reference
Control siRNA (SiQ)	Unknown	All star negative control (1027281, Qiagen)
si53 ORF	GACUCCAGUGGUAUUCUAC	Eurofins MWG
siRPL30	AACTGGTGTCCATCACTACAG	Eurofins MWG
siBOP1 UTR	UCGUGCUGAAGUCAACAGA	Eurofins MWG
siBOP1 CDS	AUGGCAUGGUGUACAAUGA	Eurofins MWG

For DNA transfection, cells were transfected with Lipofectamine 2000 (Invitrogen), following manufacturer's instructions.

For lentiviral production, manufactures procedures were followed (ViraPower™ Lentiviral Expression Systems, Invitrogen MAN0000273).

Plasmids. pLPC-HDM2 peptides were generated by clonig PCR products generated using specific primers with AccuPrime™ Taq DNA Polymerase High Fidelity (Invitrogen), following manufacturer's instructions. The pcDNA3-myc3-RPL11 plasmid was purchased from Addgene (#19972).

Western blot analysis and antibodies. Total cell extracts were prepared in RIPA buffer without SDS (150mM NaCl, 50mM Tris-HCl pH8, 1mM EDTA, 1% NP-40,

0,5% Na-deoxycholate) supplemented with 1 mM PMSF, 5 mM NaF, 1 mM Na₃VO₄, 10µg/ml CLAP, 1µM TSA and 5µM nicotinamide. Protein concentration was determined with Bio-Rad Protein Assay Reagent (#500-0006, Bio-Rad). Lysates were resolved by SDS/PAGE and transferred to nitrocellulose (Millipore). Western blot analysis was performed according to standard procedures using the following primary antibodies:

Target	Antibody
p53 (DO1)	sc-126 (Santa Cruz)
Actin	#A9718 (Sigma)
HSP90	sc-13119 (Santa Cruz)
GTPBP4	13897-1-AP (protein tech)
RPL11	ab79352 (Abcam)
Mdm2	2A10 home made preparation
Mdm2	SMP14 (Santa Cruz)
Myc-tag	Cell Signaling #2272
HA-tag	12CA5 home made preparation
HA-tag	Y-11 (sc-805 santa Cruz)

FACS analysis. For FACS analysis, adherent and floating cells were harvested, permeabilized with 0.1% NP-40 in PBS containing RNase A (200 µg/ml) and then stained with 50 µg/ml Propidium Iodide (#P4865, Sigma). At least 20.000 cells were counted in each experiment, using a FACSCalibur flow cytometer (Becton-Dickinson). Cell cycle analysis was performed with FlowJo software, (<http://www.flowjo.com/>).

RNA extraction and RT-qPCR. Total RNA was extracted with QIAzol lysis reagent (Qiagen) following manufacturer's instructions. 1µg of total RNA was reverse-transcribed with QuantiTect Reverse Transcription (Qiagen). The genes of interest were amplified with Fast SYBR® Green Master Mix (Applied Biosystems) following manufacturer's instructions. A CFX96 Touch™ Real-Time PCR System (Biorad) was used for qPCRs. List of primers used:

Target	Sequence
p53	Fw 5'-CTCCTCTCCCCAGCCAAAGA-3' Rev 5'-GGAACATCTCGAAGCGCTCA-3'
GAPDH	Fw 5'-CATGCCATCACTGCCACCC-3' Rev 5'-ACCTGGTCCTCAGTGTAGC-3'
GTPBP4	Fw 5'-CTGGAGGATAGGAACACC-3' Rev 5'-TGGCTACAACCTATGAGAG-3'
RPL30	Fw 5'-AGTGGGAAGTACGTCCTGGGGT-3' Rev 5'-TCCGCATGCTGTGCCAGTT-3'
BOP1	Fw 5'-AGCCCTTGATGACGAGGGCCA-3' Rev 5'-GCCACCGTGTTCGGATGT-3'
H3	Fw 5'-GAAGAAACCTCATCGTTACAGGCCTGGT- 3' Rev 5'-CTGCAAAGCACCAATAGCTGCACTCTGGAA-3'

³²P-orthophosphate in vivo labeling and RNA analysis. Cells were incubated with phosphate-free medium supplemented with 10% dialyzed FBS (labeling medium) for 1 h before labeling with ³²P-orthophosphate (15 mCi/ml) in labeling medium for 1 h (pulse). Medium was then replaced with complete medium (chase). Doxycycline was maintained where necessary, for the entire time of the experiment. Total RNA was extracted using TRIZOL reagent (Invitrogen) and 1mg of purified RNA was separated on 1% agarose–formaldehyde gel. After electrophoresis, 28S and 18S rRNA were controlled under UV light and gels were dried.

[³⁵S]protein labeling and immune precipitation. For metabolic labeling, HCT116 cells were preincubated in DMEM without methionine and cysteine with 5% dialyzed FCS for 30 minutes. Preincubated cells were treated with proteasome inhibitor, 50 μM MG132, and labeled with 100 μCi/ml of [³⁵S]methionine and cysteine (NEG772014MC Perkin Elmer) for 1 hour. Immune precipitation was performed using RIPA buffer, with protease inhibitors. Samples were cleared by centrifugation for 30 min at 13,000g at 4 °C and incubated for 1 h at 4 °C with anti-p53 antibody (sc-126, Santa Cruz) and anti-HSP90 antibody (sc-13119, Santa Cruz). After 1 h incubation with protein G-Sepharose (GE Healthcare), immunoprecipitates were washed three times in Co-IP buffer, resuspended in sample buffer, and analyzed by by autoradiography.

Co-immune precipitation. Co-IP experiments with Mdm2 or overexpressed proteins were performed using Co-IP buffer (NaCl 120mM, Tris-HCl pH8 20mM, EDTA

1mM, NP40 0,5%) with protease inhibitors. Samples were cleared by centrifugation for 30 min at 13,000g at 4 °C and incubated for 16 h at 4 °C with anti-Mdm2 antibody (SMP14; Santa Cruz), anti-HA (Y-11 sc-805, Sigma). After 1 h incubation with protein G-Sepharose (GE Healthcare), immunoprecipitates were washed three times in Co-IP buffer, resuspended in sample buffer, and analyzed by immunoblotting.

Polysomal fraction and RNA extraction. Cells were washed once with phosphate buffer saline (PBS + cycloheximide 10 µg/ml) and treated directly on the plate with 300 µl lysis buffer (10 mM NaCl, 10 mM MgCl₂, 10 mM Tris-HCl, pH 7.5, 1% Triton X-100, 1% sodium deoxycholate, 0.2 U µl⁻¹ RNase inhibitor, cycloheximide 10 µg/ml and 1 mM dithiothreitol) and transferred to an Eppendorf tube. After a few minute incubation on ice with occasional vortexing, the extracts were centrifuged for 5 min at 12,000 g at 4 °C. The supernatant was stored at -80 °C or loaded directly onto a 15–50% linear sucrose gradient containing 30 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, and centrifuged in an Sorvall rotor for 100 min at 180,000 g. Fractions (polysomal and subpolysomal) were collected monitoring the absorbance at 254 nm and treated directly with proteinase K. After phenol–chloroform extraction and isopropanol precipitation, polysomal RNA was resuspended in 30 µl of water. RNA quality was assessed by agarose gel electrophoresis and by the Agilent 2100 Bioanalyzer platform (Tebaldi et al., 2012).

Migration assay. For analysis of cell migration, transfected cells ($0,75-1 \times 10^5$) were plated on 24 well PET inserts (8.0 µm pore size, Falcon). After 16 h cells on the upper part of the membrane were removed with a cotton swab and cells that had passed through the filter were fixed in 4% PFA, stained with 0.05% crystal violet and counted.

BIBLIOGRAPHY

- Adorno, M., Cordenonsi, M., Montagner, M., Dupont, S., Wong, C., Hann, B., Solari, A., Bobisse, S., Rondina, M.B., Guzzardo, V., *et al.* (2009). A Mutant-p53/Smad complex opposes p63 to empower TGFbeta-induced metastasis. *Cell* 137, 87-98.
- Ahmad, Y., Boisvert, F.M., Gregor, P., Cobley, A., and Lamond, A.I. (2009). NOPdb: Nucleolar Proteome Database--2008 update. *Nucleic Acids Res* 37, D181-184.
- Ahn, J., Byeon, I.J., Byeon, C.H., and Gronenborn, A.M. (2009). Insight into the structural basis of pro- and antiapoptotic p53 modulation by ASPP proteins. *J Biol Chem* 284, 13812-13822.
- Albrechtsen, N., Dornreiter, I., Grosse, F., Kim, E., Wiesmuller, L., and Deppert, W. (1999). Maintenance of genomic integrity by p53: complementary roles for activated and non-activated p53. *Oncogene* 18, 7706-7717.
- Arabi, A., Wu, S., Ridderstrale, K., Bierhoff, H., Shiue, C., Fatyol, K., Fahlen, S., Hydbring, P., Soderberg, O., Grummt, I., *et al.* (2005). c-Myc associates with ribosomal DNA and activates RNA polymerase I transcription. *Nat Cell Biol* 7, 303-310.
- Balint, E.E., and Vousden, K.H. (2001). Activation and activities of the p53 tumour suppressor protein. *Br J Cancer* 85, 1813-1823.
- Bartek, J., Bartkova, J., Vojtesek, B., Staskova, Z., Lukas, J., Rejthar, A., Kovarik, J., Midgley, C.A., Gannon, J.V., and Lane, D.P. (1991). Aberrant expression of the p53 oncoprotein is a common feature of a wide spectrum of human malignancies. *Oncogene* 6, 1699-1703.
- Bartel, F., Taubert, H., and Harris, L.C. (2002). Alternative and aberrant splicing of MDM2 mRNA in human cancer. *Cancer Cell* 2, 9-15.
- Bartkova, J., Horejsi, Z., Koed, K., Kramer, A., Tort, F., Zieger, K., Guldborg, P., Sehested, M., Nesland, J.M., Lukas, C., *et al.* (2005). DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. *Nature* 434, 864-870.
- Bergamaschi, D., Samuels, Y., O'Neil, N.J., Trigiante, G., Crook, T., Hsieh, J.K., O'Connor, D.J., Zhong, S., Campargue, I., Tomlinson, M.L., *et al.* (2003). iASPP oncoprotein is a key inhibitor of p53 conserved from worm to human. *Nat Genet* 33, 162-167.
- Bertrand, P., Saintigny, Y., and Lopez, B.S. (2004). p53's double life: transactivation-independent repression of homologous recombination. *Trends Genet* 20, 235-243.
- Bhat, K.P., Itahana, K., Jin, A., and Zhang, Y. (2004). Essential role of ribosomal protein L11 in mediating growth inhibition-induced p53 activation. *EMBO J* 23, 2402-2412.
- Boisvert, F.M., van Koningsbruggen, S., Navascues, J., and Lamond, A.I. (2007). The multifunctional nucleolus. *Nat Rev Mol Cell Biol* 8, 574-585.
- Bond, G.L., Hu, W., Bond, E.E., Robins, H., Lutzker, S.G., Arva, N.C., Bargonetti, J., Bartel, F., Taubert, H., Wuerl, P., *et al.* (2004). A single nucleotide polymorphism in the MDM2 promoter attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans. *Cell* 119, 591-602.

- Bossi, G., Marampon, F., Maor-Aloni, R., Zani, B., Rotter, V., Oren, M., Strano, S., Blandino, G., and Sacchi, A. (2008). Conditional RNA interference in vivo to study mutant p53 oncogenic gain of function on tumor malignancy. *Cell Cycle* 7, 1870-1879.
- Boulon, S., Westman, B.J., Hutten, S., Boisvert, F.M., and Lamond, A.I. (2010). The nucleolus under stress. *Mol Cell* 40, 216-227.
- Branzei, D., and Foiani, M. (2005). The DNA damage response during DNA replication. *Curr Opin Cell Biol* 17, 568-575.
- Branzei, D., and Foiani, M. (2010). Maintaining genome stability at the replication fork. *Nat Rev Mol Cell Biol* 11, 208-219.
- Brooks, C.L., and Gu, W. (2004). Dynamics in the p53-Mdm2 ubiquitination pathway. *Cell Cycle* 3, 895-899.
- Brooks, C.L., Li, M., Hu, M., Shi, Y., and Gu, W. (2007). The p53--Mdm2--HAUSP complex is involved in p53 stabilization by HAUSP. *Oncogene* 26, 7262-7266.
- Brosh, R., and Rotter, V. (2009). When mutants gain new powers: news from the mutant p53 field. *Nat Rev Cancer* 9, 701-713.
- Bullock, A.N., and Fersht, A.R. (2001). Rescuing the function of mutant p53. *Nat Rev Cancer* 1, 68-76.
- Burger, K., and Eick, D. (2013). Functional ribosome biogenesis is a prerequisite for p53 destabilization: impact of chemotherapy on nucleolar functions and RNA metabolism. *Biol Chem* 394, 1133-1143.
- Burger, K., Muhl, B., Harasim, T., Rohrmoser, M., Malamoussi, A., Orban, M., Kellner, M., Gruber-Eber, A., Kremmer, E., Holzner, M., *et al.* (2010). Chemotherapeutic drugs inhibit ribosome biogenesis at various levels. *J Biol Chem* 285, 12416-12425.
- Bursac, S., Brdovcak, M.C., Pfannkuchen, M., Orsolich, I., Golomb, L., Zhu, Y., Katz, C., Daftuar, L., Grabusic, K., Vukelic, I., *et al.* (2012). Mutual protection of ribosomal proteins L5 and L11 from degradation is essential for p53 activation upon ribosomal biogenesis stress. *Proc Natl Acad Sci U S A* 109, 20467-20472.
- Campisi, J. (2005). Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. *Cell* 120, 513-522.
- Carter, S., and Vousden, K.H. (2009). Modifications of p53: competing for the lysines. *Curr Opin Genet Dev* 19, 18-24.
- Cavanaugh, A.H., Hempel, W.M., Taylor, L.J., Rogalsky, V., Todorov, G., and Rothblum, L.I. (1995). Activity of RNA polymerase I transcription factor UBF blocked by Rb gene product. *Nature* 374, 177-180.
- Chan, T.A., Hermeking, H., Lengauer, C., Kinzler, K.W., and Vogelstein, B. (1999). 14-3-3Sigma is required to prevent mitotic catastrophe after DNA damage. *Nature* 401, 616-620.
- Chau, B.N., and Wang, J.Y. (2003). Coordinated regulation of life and death by RB. *Nat Rev Cancer* 3, 130-138.

- Chicas, A., Molina, P., and Bargonetti, J. (2000). Mutant p53 forms a complex with Sp1 on HIV-LTR DNA. *Biochem Biophys Res Commun* 279, 383-390.
- Cicalese, A., Bonizzi, G., Pasi, C.E., Faretta, M., Ronzoni, S., Giulini, B., Brisken, C., Minucci, S., Di Fiore, P.P., and Pelicci, P.G. (2009). The tumor suppressor p53 regulates polarity of self-renewing divisions in mammary stem cells. *Cell* 138, 1083-1095.
- Clarke, A.R., Purdie, C.A., Harrison, D.J., Morris, R.G., Bird, C.C., Hooper, M.L., and Wyllie, A.H. (1993). Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature* 362, 849-852.
- Clegg, H.V., Itahana, K., and Zhang, Y. (2008). Unlocking the Mdm2-p53 loop: ubiquitin is the key. *Cell Cycle* 7, 287-292.
- Collavin, L., Lunardi, A., and Del Sal, G. (2010). p53-family proteins and their regulators: hubs and spokes in tumor suppression. *Cell Death Differ* 17, 901-911.
- d'Adda di Fagagna, F., Reaper, P.M., Clay-Farrace, L., Fiegler, H., Carr, P., Von Zglinicki, T., Saretzki, G., Carter, N.P., and Jackson, S.P. (2003). A DNA damage checkpoint response in telomere-initiated senescence. *Nature* 426, 194-198.
- Dai, C., and Gu, W. (2010). p53 post-translational modification: deregulated in tumorigenesis. *Trends Mol Med* 16, 528-536.
- Dai, M.S., and Lu, H. (2004). Inhibition of MDM2-mediated p53 ubiquitination and degradation by ribosomal protein L5. *J Biol Chem* 279, 44475-44482.
- Danovi, D., Meulmeester, E., Pasini, D., Migliorini, D., Capra, M., Frenk, R., de Graaf, P., Francoz, S., Gasparini, P., Gobbi, A., *et al.* (2004). Amplification of Mdmx (or Mdm4) directly contributes to tumor formation by inhibiting p53 tumor suppressor activity. *Mol Cell Biol* 24, 5835-5843.
- Das, S., Raj, L., Zhao, B., Kimura, Y., Bernstein, A., Aaronson, S.A., and Lee, S.W. (2007). Hzf Determines cell survival upon genotoxic stress by modulating p53 transactivation. *Cell* 130, 624-637.
- Dawson, R., Muller, L., Dehner, A., Klein, C., Kessler, H., and Buchner, J. (2003). The N-terminal domain of p53 is natively unfolded. *J Mol Biol* 332, 1131-1141.
- de Las Heras-Rubio, A., Perucho, L., Paciucci, R., Vilardell, J., and Lleonart, M.E. (2013). Ribosomal proteins as novel players in tumorigenesis. *Cancer Metastasis Rev.*
- de Stanchina, E., McCurrach, M.E., Zindy, F., Shieh, S.Y., Ferbeyre, G., Samuelson, A.V., Prives, C., Roussel, M.F., Sherr, C.J., and Lowe, S.W. (1998). E1A signaling to p53 involves the p19(ARF) tumor suppressor. *Genes Dev* 12, 2434-2442.
- Debbas, M., and White, E. (1993). Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. *Genes Dev* 7, 546-554.
- Deisenroth, C., and Zhang, Y. (2010). Ribosome biogenesis surveillance: probing the ribosomal protein-Mdm2-p53 pathway. *Oncogene* 29, 4253-4260.
- Deisenroth, C., and Zhang, Y. (2011). The Ribosomal Protein-Mdm2-p53 Pathway and Energy Metabolism: Bridging the Gap between Feast and Famine. *Genes Cancer* 2, 392-403.

- Derenzini, M., Montanaro, L., and Trere, D. (2009). What the nucleolus says to a tumour pathologist. *Histopathology* 54, 753-762.
- Derenzini, M., and Ploton, D. (1991). Interphase nucleolar organizer regions in cancer cells. *Int Rev Exp Pathol* 32, 149-192.
- Dhar, G., Banerjee, S., Dhar, K., Tawfik, O., Mayo, M.S., Vanveldhuizen, P.J., and Banerjee, S.K. (2008). Gain of oncogenic function of p53 mutants induces invasive phenotypes in human breast cancer cells by silencing CCN5/WISP-2. *Cancer Res* 68, 4580-4587.
- Di Como, C.J., Gaiddon, C., and Prives, C. (1999). p73 function is inhibited by tumor-derived p53 mutants in mammalian cells. *Mol Cell Biol* 19, 1438-1449.
- Dittmer, D., Pati, S., Zambetti, G., Chu, S., Teresky, A.K., Moore, M., Finlay, C., and Levine, A.J. (1993). Gain of function mutations in p53. *Nat Genet* 4, 42-46.
- Donati, G., Peddigari, S., Mercer, C.A., and Thomas, G. (2013). 5S ribosomal RNA is an essential component of a nascent ribosomal precursor complex that regulates the Hdm2-p53 checkpoint. *Cell Rep* 4, 87-98.
- Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A., Jr., Butel, J.S., and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* 356, 215-221.
- Dornan, D., Shimizu, H., Burch, L., Smith, A.J., and Hupp, T.R. (2003). The proline repeat domain of p53 binds directly to the transcriptional coactivator p300 and allosterically controls DNA-dependent acetylation of p53. *Mol Cell Biol* 23, 8846-8861.
- Dornan, D., Wertz, I., Shimizu, H., Arnott, D., Frantz, G.D., Dowd, P., O'Rourke, K., Koeppen, H., and Dixit, V.M. (2004). The ubiquitin ligase COP1 is a critical negative regulator of p53. *Nature* 429, 86-92.
- Drygin, D., Lin, A., Bliesath, J., Ho, C.B., O'Brien, S.E., Proffitt, C., Omori, M., Haddach, M., Schwaebe, M.K., Siddiqui-Jain, A., *et al.* (2011). Targeting RNA polymerase I with an oral small molecule CX-5461 inhibits ribosomal RNA synthesis and solid tumor growth. *Cancer Res* 71, 1418-1430.
- Drygin, D., Siddiqui-Jain, A., O'Brien, S., Schwaebe, M., Lin, A., Bliesath, J., Ho, C.B., Proffitt, C., Trent, K., Whitten, J.P., *et al.* (2009). Anticancer activity of CX-3543: a direct inhibitor of rRNA biogenesis. *Cancer Res* 69, 7653-7661.
- Efeyan, A., and Serrano, M. (2007). p53: guardian of the genome and policeman of the oncogenes. *Cell Cycle* 6, 1006-1010.
- el-Deiry, W.S., Kern, S.E., Pietenpol, J.A., Kinzler, K.W., and Vogelstein, B. (1992). Definition of a consensus binding site for p53. *Nat Genet* 1, 45-49.
- Esser, C., Scheffner, M., and Hohfeld, J. (2005). The chaperone-associated ubiquitin ligase CHIP is able to target p53 for proteasomal degradation. *J Biol Chem* 280, 27443-27448.
- Fatica, A., and Tollervey, D. (2002). Making ribosomes. *Curr Opin Cell Biol* 14, 313-318.

- Felton-Edkins, Z.A., Fairley, J.A., Graham, E.L., Johnston, I.M., White, R.J., and Scott, P.H. (2003). The mitogen-activated protein (MAP) kinase ERK induces tRNA synthesis by phosphorylating TFIIB. *EMBO J* 22, 2422-2432.
- Fernandez-Medarde, A., and Santos, E. (2011). Ras in cancer and developmental diseases. *Genes Cancer* 2, 344-358.
- Freed-Pastor, W.A., Mizuno, H., Zhao, X., Langerod, A., Moon, S.H., Rodriguez-Barrueco, R., Barsotti, A., Chicas, A., Li, W., Polotskaia, A., *et al.* (2012). Mutant p53 disrupts mammary tissue architecture via the mevalonate pathway. *Cell* 148, 244-258.
- Freed, E.F., Bleichert, F., Dutca, L.M., and Baserga, S.J. (2010). When ribosomes go bad: diseases of ribosome biogenesis. *Mol Biosyst* 6, 481-493.
- Fridman, J.S., Hernando, E., Hemann, M.T., de Stanchina, E., Cordon-Cardo, C., and Lowe, S.W. (2003). Tumor promotion by Mdm2 splice variants unable to bind p53. *Cancer Res* 63, 5703-5706.
- Fuentes, J.L., Datta, K., Sullivan, S.M., Walker, A., and Maddock, J.R. (2007). In vivo functional characterization of the *Saccharomyces cerevisiae* 60S biogenesis GTPase Nog1. *Mol Genet Genomics* 278, 105-123.
- Fumagalli, S., Di Cara, A., Neb-Gulati, A., Natt, F., Schwemberger, S., Hall, J., Babcock, G.F., Bernardi, R., Pandolfi, P.P., and Thomas, G. (2009). Absence of nucleolar disruption after impairment of 40S ribosome biogenesis reveals an rPL11-translation-dependent mechanism of p53 induction. *Nat Cell Biol* 11, 501-508.
- Gaidon, C., Lokshin, M., Ahn, J., Zhang, T., and Prives, C. (2001). A subset of tumor-derived mutant forms of p53 down-regulate p63 and p73 through a direct interaction with the p53 core domain. *Mol Cell Biol* 21, 1874-1887.
- Girardini, J.E., Napoli, M., Piazza, S., Rustighi, A., Marotta, C., Radaelli, E., Capaci, V., Jordan, L., Quinlan, P., Thompson, A., *et al.* (2011). A Pin1/mutant p53 axis promotes aggressiveness in breast cancer. *Cancer Cell* 20, 79-91.
- Gohler, T., Jager, S., Warnecke, G., Yasuda, H., Kim, E., and Deppert, W. (2005). Mutant p53 proteins bind DNA in a DNA structure-selective mode. *Nucleic Acids Res* 33, 1087-1100.
- Golomb, L., Bublik, D.R., Wilder, S., Nevo, R., Kiss, V., Grabusic, K., Volarevic, S., and Oren, M. (2012). Importin 7 and exportin 1 link c-Myc and p53 to regulation of ribosomal biogenesis. *Mol Cell* 45, 222-232.
- Gomez-Roman, N., Grandori, C., Eisenman, R.N., and White, R.J. (2003). Direct activation of RNA polymerase III transcription by c-Myc. *Nature* 421, 290-294.
- Grandori, C., Gomez-Roman, N., Felton-Edkins, Z.A., Ngouenet, C., Galloway, D.A., Eisenman, R.N., and White, R.J. (2005). c-Myc binds to human ribosomal DNA and stimulates transcription of rRNA genes by RNA polymerase I. *Nat Cell Biol* 7, 311-318.
- Grisendi, S., Mecucci, C., Falini, B., and Pandolfi, P.P. (2006). Nucleophosmin and cancer. *Nat Rev Cancer* 6, 493-505.
- Hammond, E.M., Denko, N.C., Dorie, M.J., Abraham, R.T., and Giaccia, A.J. (2002). Hypoxia links ATR and p53 through replication arrest. *Mol Cell Biol* 22, 1834-1843.

- Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. *Cell* 100, 57-70.
- Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. *Cell* 144, 646-674.
- Hannan, K.M., Brandenburger, Y., Jenkins, A., Sharkey, K., Cavanaugh, A., Rothblum, L., Moss, T., Poortinga, G., McArthur, G.A., Pearson, R.B., *et al.* (2003). mTOR-dependent regulation of ribosomal gene transcription requires S6K1 and is mediated by phosphorylation of the carboxy-terminal activation domain of the nucleolar transcription factor UBF. *Mol Cell Biol* 23, 8862-8877.
- Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K., and Elledge, S.J. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* 75, 805-816.
- Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997). Mdm2 promotes the rapid degradation of p53. *Nature* 387, 296-299.
- Hein, N., Hannan, K.M., George, A.J., Sanij, E., and Hannan, R.D. (2013). The nucleolus: an emerging target for cancer therapy. *Trends Mol Med* 19, 643-654.
- Hermeking, H., Lengauer, C., Polyak, K., He, T.C., Zhang, L., Thiagalingam, S., Kinzler, K.W., and Vogelstein, B. (1997). 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. *Mol Cell* 1, 3-11.
- Hirao, A., Kong, Y.Y., Matsuoka, S., Wakeham, A., Ruland, J., Yoshida, H., Liu, D., Elledge, S.J., and Mak, T.W. (2000). DNA damage-induced activation of p53 by the checkpoint kinase Chk2. *Science* 287, 1824-1827.
- Hofseth, L.J., Saito, S., Hussain, S.P., Espey, M.G., Miranda, K.M., Araki, Y., Jhappan, C., Higashimoto, Y., He, P., Linke, S.P., *et al.* (2003). Nitric oxide-induced cellular stress and p53 activation in chronic inflammation. *Proc Natl Acad Sci U S A* 100, 143-148.
- Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C.C. (1991). p53 mutations in human cancers. *Science* 253, 49-53.
- Holzel, M., Rohrmoser, M., Schlee, M., Grimm, T., Harasim, T., Malamoussi, A., Gruber-Eber, A., Kremmer, E., Hiddemann, W., Bornkamm, G.W., *et al.* (2005). Mammalian WDR12 is a novel member of the Pes1-Bop1 complex and is required for ribosome biogenesis and cell proliferation. *J Cell Biol* 170, 367-378.
- Honma, Y., Kitamura, A., Shioda, R., Maruyama, H., Ozaki, K., Oda, Y., Mini, T., Jen, P., Maki, Y., Yonezawa, K., *et al.* (2006). TOR regulates late steps of ribosome maturation in the nucleoplasm via Nog1 in response to nutrients. *EMBO J* 25, 3832-3842.
- Itahana, K., Campisi, J., and Dimri, G.P. (2004). Mechanisms of cellular senescence in human and mouse cells. *Biogerontology* 5, 1-10.
- James, M.J., and Zomerdijk, J.C. (2004). Phosphatidylinositol 3-kinase and mTOR signaling pathways regulate RNA polymerase I transcription in response to IGF-1 and nutrients. *J Biol Chem* 279, 8911-8918.
- Janz, C., and Wiesmuller, L. (2002). Wild-type p53 inhibits replication-associated homologous recombination. *Oncogene* 21, 5929-5933.

- Jensen, B.C., Wang, Q., Kifer, C.T., and Parsons, M. (2003). The NOG1 GTP-binding protein is required for biogenesis of the 60 S ribosomal subunit. *J Biol Chem* 278, 32204-32211.
- Jin, A., Itahana, K., O'Keefe, K., and Zhang, Y. (2004). Inhibition of HDM2 and activation of p53 by ribosomal protein L23. *Mol Cell Biol* 24, 7669-7680.
- Jin, S., Tong, T., Fan, W., Fan, F., Antinore, M.J., Zhu, X., Mazzacurati, L., Li, X., Petrik, K.L., Rajasekaran, B., *et al.* (2002). GADD45-induced cell cycle G2-M arrest associates with altered subcellular distribution of cyclin B1 and is independent of p38 kinase activity. *Oncogene* 21, 8696-8704.
- Junttila, M.R., and Evan, G.I. (2009). p53--a Jack of all trades but master of none. *Nat Rev Cancer* 9, 821-829.
- Kaesler, M.D., and Iggo, R.D. (2002). Chromatin immunoprecipitation analysis fails to support the latency model for regulation of p53 DNA binding activity in vivo. *Proc Natl Acad Sci U S A* 99, 95-100.
- Kallstrom, G., Hedges, J., and Johnson, A. (2003). The putative GTPases Nog1p and Lsg1p are required for 60S ribosomal subunit biogenesis and are localized to the nucleus and cytoplasm, respectively. *Mol Cell Biol* 23, 4344-4355.
- Kamal, A., Thao, L., Sensintaffar, J., Zhang, L., Boehm, M.F., Fritz, L.C., and Burrows, F.J. (2003). A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. *Nature* 425, 407-410.
- Kastan, M.B., Zhan, Q., el-Deiry, W.S., Carrier, F., Jacks, T., Walsh, W.V., Plunkett, B.S., Vogelstein, B., and Fornace, A.J., Jr. (1992). A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell* 71, 587-597.
- Kato, S., Han, S.Y., Liu, W., Otsuka, K., Shibata, H., Kanamaru, R., and Ishioka, C. (2003). Understanding the function-structure and function-mutation relationships of p53 tumor suppressor protein by high-resolution missense mutation analysis. *Proc Natl Acad Sci U S A* 100, 8424-8429.
- Khan, S.H., Moritsugu, J., and Wahl, G.M. (2000). Differential requirement for p19ARF in the p53-dependent arrest induced by DNA damage, microtubule disruption, and ribonucleotide depletion. *Proc Natl Acad Sci U S A* 97, 3266-3271.
- Kim, S., Li, Q., Dang, C.V., and Lee, L.A. (2000). Induction of ribosomal genes and hepatocyte hypertrophy by adenovirus-mediated expression of c-Myc in vivo. *Proc Natl Acad Sci U S A* 97, 11198-11202.
- Kim, Y.I., Bandyopadhyay, J., Cho, I., Lee, J., Park, D.H., and Cho, J.H. (2014). Nucleolar GTPase NOG-1 Regulates Development, Fat Storage, and Longevity through Insulin/IGF Signaling in *C. elegans*. *Mol Cells* 37, 51-58.
- Klinge, S., Voigts-Hoffmann, F., Leibundgut, M., Arpagaus, S., and Ban, N. (2011). Crystal structure of the eukaryotic 60S ribosomal subunit in complex with initiation factor 6. *Science* 334, 941-948.
- Kogan-Sakin, I., Tabach, Y., Buganim, Y., Molchadsky, A., Solomon, H., Madar, S., Kamer, I., Stambolsky, P., Shelly, A., Goldfinger, N., *et al.* (2011). Mutant p53(R175H) upregulates Twist1 expression and promotes epithelial-mesenchymal transition in immortalized prostate cells. *Cell Death Differ* 18, 271-281.

- Krastev, D.B., Slabicki, M., Paszkowski-Rogacz, M., Hubner, N.C., Junqueira, M., Shevchenko, A., Mann, M., Neugebauer, K.M., and Buchholz, F. (2011). A systematic RNAi synthetic interaction screen reveals a link between p53 and snoRNP assembly. *Nat Cell Biol* 13, 809-818.
- Kress, M., May, E., Cassingena, R., and May, P. (1979). Simian virus 40-transformed cells express new species of proteins precipitable by anti-simian virus 40 tumor serum. *J Virol* 31, 472-483.
- Kruse, J.P., and Gu, W. (2009). Modes of p53 regulation. *Cell* 137, 609-622.
- Kubbutat, M.H., Jones, S.N., and Vousden, K.H. (1997). Regulation of p53 stability by Mdm2. *Nature* 387, 299-303.
- Kumari, A., Schultz, N., and Helleday, T. (2004). p53 protects from replication-associated DNA double-strand breaks in mammalian cells. *Oncogene* 23, 2324-2329.
- Lafontaine, D.L., and Tollervey, D. (1998). Birth of the snoRNPs: the evolution of the modification-guide snoRNAs. *Trends Biochem Sci* 23, 383-388.
- Lane, D.P. (1992). Cancer. p53, guardian of the genome. *Nature* 358, 15-16.
- Lane, D.P., and Crawford, L.V. (1979). T antigen is bound to a host protein in SV40-transformed cells. *Nature* 278, 261-263.
- Lang, G.A., Iwakuma, T., Suh, Y.A., Liu, G., Rao, V.A., Parant, J.M., Valentin-Vega, Y.A., Terzian, T., Caldwell, L.C., Strong, L.C., *et al.* (2004). Gain of function of a p53 hot spot mutation in a mouse model of Li-Fraumeni syndrome. *Cell* 119, 861-872.
- Lanni, J.S., and Jacks, T. (1998). Characterization of the p53-dependent postmitotic checkpoint following spindle disruption. *Mol Cell Biol* 18, 1055-1064.
- Lapik, Y.R., Misra, J.M., Lau, L.F., and Pestov, D.G. (2007). Restricting conformational flexibility of the switch II region creates a dominant-inhibitory phenotype in Obg GTPase Nog1. *Mol Cell Biol* 27, 7735-7744.
- Laping, N.J., Olson, B.A., and Zhu, Y. (2001). Identification of a novel nuclear guanosine triphosphate-binding protein differentially expressed in renal disease. *J Am Soc Nephrol* 12, 883-890.
- Laplante, M., and Sabatini, D.M. (2013). Regulation of mTORC1 and its impact on gene expression at a glance. *J Cell Sci* 126, 1713-1719.
- Lebreton, A., Saveanu, C., Decourty, L., Jacquier, A., and Fromont-Racine, M. (2006). Nsa2 is an unstable, conserved factor required for the maturation of 27 SB pre-rRNAs. *J Biol Chem* 281, 27099-27108.
- Lee, H., Kim, D., Dan, H.C., Wu, E.L., Gritsko, T.M., Cao, C., Nicosia, S.V., Golemis, E.A., Liu, W., Coppola, D., *et al.* (2007). Identification and characterization of putative tumor suppressor NGB, a GTP-binding protein that interacts with the neurofibromatosis 2 protein. *Mol Cell Biol* 27, 2103-2119.
- Lee, J.T., and Gu, W. (2010). The multiple levels of regulation by p53 ubiquitination. *Cell Death Differ* 17, 86-92.
- Lee, T., Yao, G., Nevins, J., and You, L. (2008). Sensing and integration of Erk and PI3K signals by Myc. *PLoS Comput Biol* 4, e1000013.

- Lee, Y., and Nazar, R.N. (2003). Terminal structure mediates 5 S rRNA stability and integration during ribosome biogenesis. *J Biol Chem* 278, 6635-6641.
- Leger-Silvestre, I., Milkereit, P., Ferreira-Cerca, S., Saveanu, C., Rousselle, J.C., Choismel, V., Guinefoleau, C., Gas, N., and Gleizes, P.E. (2004). The ribosomal protein Rps15p is required for nuclear exit of the 40S subunit precursors in yeast. *EMBO J* 23, 2336-2347.
- Leng, R.P., Lin, Y., Ma, W., Wu, H., Lemmers, B., Chung, S., Parant, J.M., Lozano, G., Hakem, R., and Benchimol, S. (2003). Pirh2, a p53-induced ubiquitin-protein ligase, promotes p53 degradation. *Cell* 112, 779-791.
- Lessard, F., Morin, F., Ivanchuk, S., Langlois, F., Stefanovsky, V., Rutka, J., and Moss, T. (2010). The ARF tumor suppressor controls ribosome biogenesis by regulating the RNA polymerase I transcription factor TTF-I. *Mol Cell* 38, 539-550.
- Leu, J.I., Dumont, P., Hafey, M., Murphy, M.E., and George, D.L. (2004). Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex. *Nat Cell Biol* 6, 443-450.
- Li, D., Marchenko, N.D., Schulz, R., Fischer, V., Velasco-Hernandez, T., Talos, F., and Moll, U.M. (2011). Functional inactivation of endogenous MDM2 and CHIP by HSP90 causes aberrant stabilization of mutant p53 in human cancer cells. *Mol Cancer Res* 9, 577-588.
- Li, H.H., Cai, X., Shouse, G.P., Piluso, L.G., and Liu, X. (2007). A specific PP2A regulatory subunit, B56gamma, mediates DNA damage-induced dephosphorylation of p53 at Thr55. *EMBO J* 26, 402-411.
- Lin, P., and Medeiros, L.J. (2013). The impact of MYC rearrangements and "double hit" abnormalities in diffuse large B-cell lymphoma. *Curr Hematol Malig Rep* 8, 243-252.
- Linares, L.K., Hengstermann, A., Ciechanover, A., Muller, S., and Scheffner, M. (2003). HdmX stimulates Hdm2-mediated ubiquitination and degradation of p53. *Proc Natl Acad Sci U S A* 100, 12009-12014.
- Lindstrom, M.S., Jin, A., Deisenroth, C., White Wolf, G., and Zhang, Y. (2007). Cancer-associated mutations in the MDM2 zinc finger domain disrupt ribosomal protein interaction and attenuate MDM2-induced p53 degradation. *Mol Cell Biol* 27, 1056-1068.
- Linzer, D.I., and Levine, A.J. (1979). Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell* 17, 43-52.
- Liu, T., Laurell, C., Selivanova, G., Lundeberg, J., Nilsson, P., and Wiman, K.G. (2007). Hypoxia induces p53-dependent transactivation and Fas/CD95-dependent apoptosis. *Cell Death Differ* 14, 411-421.
- Longley, D.B., Harkin, D.P., and Johnston, P.G. (2003). 5-fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer* 3, 330-338.
- Lukashchuk, N., and Vousden, K.H. (2007). Ubiquitination and degradation of mutant p53. *Mol Cell Biol* 27, 8284-8295.
- Lunardi, A., Di Minin, G., Provero, P., Dal Ferro, M., Carotti, M., Del Sal, G., and Collavin, L. (2010). A genome-scale protein interaction profile of *Drosophila* p53 uncovers additional nodes of the human p53 network. *Proc Natl Acad Sci U S A* 107, 6322-6327.

- Luo, J., Nikolaev, A.Y., Imai, S., Chen, D., Su, F., Shiloh, A., Guarente, L., and Gu, W. (2001). Negative control of p53 by Sir2alpha promotes cell survival under stress. *Cell* 107, 137-148.
- Luo, J., Su, F., Chen, D., Shiloh, A., and Gu, W. (2000). Deacetylation of p53 modulates its effect on cell growth and apoptosis. *Nature* 408, 377-381.
- Machado-Silva, A., Perrier, S., and Bourdon, J.C. (2010). p53 family members in cancer diagnosis and treatment. *Semin Cancer Biol* 20, 57-62.
- Macias, E., Jin, A., Deisenroth, C., Bhat, K., Mao, H., Lindstrom, M.S., and Zhang, Y. (2010). An ARF-independent c-MYC-activated tumor suppression pathway mediated by ribosomal protein-Mdm2 Interaction. *Cancer Cell* 18, 231-243.
- Maden, B.E. (1990). The numerous modified nucleotides in eukaryotic ribosomal RNA. *Prog Nucleic Acid Res Mol Biol* 39, 241-303.
- Malkin, D., Li, F.P., Strong, L.C., Fraumeni, J.F., Jr., Nelson, C.E., Kim, D.H., Kassel, J., Gryka, M.A., Bischoff, F.Z., Tainsky, M.A., *et al.* (1990). Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. *Science* 250, 1233-1238.
- Mantovani, A. (2009). Cancer: Inflaming metastasis. *Nature* 457, 36-37.
- Mantovani, F., Piazza, S., Gostissa, M., Strano, S., Zacchi, P., Mantovani, R., Blandino, G., and Del Sal, G. (2004). Pin1 links the activities of c-Abl and p300 in regulating p73 function. *Mol Cell* 14, 625-636.
- Mantovani, F., Tocco, F., Girardini, J., Smith, P., Gasco, M., Lu, X., Crook, T., and Del Sal, G. (2007). The prolyl isomerase Pin1 orchestrates p53 acetylation and dissociation from the apoptosis inhibitor iASPP. *Nat Struct Mol Biol* 14, 912-920.
- Marchenko, N.D., Wolff, S., Erster, S., Becker, K., and Moll, U.M. (2007). Monoubiquitylation promotes mitochondrial p53 translocation. *EMBO J* 26, 923-934.
- Marchenko, N.D., Zaika, A., and Moll, U.M. (2000). Death signal-induced localization of p53 protein to mitochondria. A potential role in apoptotic signaling. *J Biol Chem* 275, 16202-16212.
- Marin, M.C., Jost, C.A., Brooks, L.A., Irwin, M.S., O'Nions, J., Tidy, J.A., James, N., McGregor, J.M., Harwood, C.A., Yulug, I.G., *et al.* (2000). A common polymorphism acts as an intragenic modifier of mutant p53 behaviour. *Nat Genet* 25, 47-54.
- Marine, J.C., and Lozano, G. (2010). Mdm2-mediated ubiquitylation: p53 and beyond. *Cell Death Differ* 17, 93-102.
- Mayer, C., and Grummt, I. (2006). Ribosome biogenesis and cell growth: mTOR coordinates transcription by all three classes of nuclear RNA polymerases. *Oncogene* 25, 6384-6391.
- Mayer, C., Zhao, J., Yuan, X., and Grummt, I. (2004). mTOR-dependent activation of the transcription factor TIF-IA links rRNA synthesis to nutrient availability. *Genes Dev* 18, 423-434.
- Mayo, L.D., and Donner, D.B. (2002). The PTEN, Mdm2, p53 tumor suppressor-oncoprotein network. *Trends Biochem Sci* 27, 462-467.

- Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P., and Moll, U.M. (2003). p53 has a direct apoptogenic role at the mitochondria. *Mol Cell* *11*, 577-590.
- Milner, J., and Medcalf, E.A. (1991). Cotranslation of activated mutant p53 with wild type drives the wild-type p53 protein into the mutant conformation. *Cell* *65*, 765-774.
- Miyashita, T., and Reed, J.C. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* *80*, 293-299.
- Muller, P.A., Caswell, P.T., Doyle, B., Iwanicki, M.P., Tan, E.H., Karim, S., Lukashchuk, N., Gillespie, D.A., Ludwig, R.L., Gosselin, P., *et al.* (2009). Mutant p53 drives invasion by promoting integrin recycling. *Cell* *139*, 1327-1341.
- Muller, P.A., and Vousden, K.H. (2013). p53 mutations in cancer. *Nat Cell Biol* *15*, 2-8.
- Mullineux, S.T., and Lafontaine, D.L. (2012). Mapping the cleavage sites on mammalian pre-rRNAs: where do we stand? *Biochimie* *94*, 1521-1532.
- Nakano, K., and Vousden, K.H. (2001). PUMA, a novel proapoptotic gene, is induced by p53. *Mol Cell* *7*, 683-694.
- Narla, A., and Ebert, B.L. (2010). Ribosomopathies: human disorders of ribosome dysfunction. *Blood* *115*, 3196-3205.
- Nissan, T.A., Bassler, J., Petfalski, E., Tollervey, D., and Hurt, E. (2002). 60S pre-ribosome formation viewed from assembly in the nucleolus until export to the cytoplasm. *EMBO J* *21*, 5539-5547.
- Ohnishi, K., Wang, X., Takahashi, A., and Ohnishi, T. (1998a). Contribution of protein kinase C to p53-dependent WAF1 induction pathway after heat treatment in human glioblastoma cell lines. *Exp Cell Res* *238*, 399-406.
- Ohnishi, T., Wang, X., Ohnishi, K., and Takahashi, A. (1998b). p53-dependent induction of WAF1 by cold shock in human glioblastoma cells. *Oncogene* *16*, 1507-1511.
- Oliner, J.D., Pietsenpol, J.A., Thiagalingam, S., Gyuris, J., Kinzler, K.W., and Vogelstein, B. (1993). Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. *Nature* *362*, 857-860.
- Olive, K.P., Tuveson, D.A., Ruhe, Z.C., Yin, B., Willis, N.A., Bronson, R.T., Crowley, D., and Jacks, T. (2004). Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. *Cell* *119*, 847-860.
- Oren, M., and Rotter, V. (2010). Mutant p53 gain-of-function in cancer. *Cold Spring Harb Perspect Biol* *2*, a001107.
- Ozenne, P., Eymin, B., Brambilla, E., and Gazzeri, S. (2010). The ARF tumor suppressor: structure, functions and status in cancer. *Int J Cancer* *127*, 2239-2247.
- Pabla, N., Huang, S., Mi, Q.S., Daniel, R., and Dong, Z. (2008). ATR-Chk2 signaling in p53 activation and DNA damage response during cisplatin-induced apoptosis. *J Biol Chem* *283*, 6572-6583.
- Palmero, I., Pantoja, C., and Serrano, M. (1998). p19ARF links the tumour suppressor p53 to Ras. *Nature* *395*, 125-126.

- Paquet, C., Schmitt, E., Beauchemin, M., and Bertrand, R. (2004). Activation of multidomain and BH3-only pro-apoptotic Bcl-2 family members in p53-defective cells. *Apoptosis* 9, 815-831.
- Park, J.H., Jensen, B.C., Kifer, C.T., and Parsons, M. (2001). A novel nucleolar G-protein conserved in eukaryotes. *J Cell Sci* 114, 173-185.
- Pederson, T. (1998). The plurifunctional nucleolus. *Nucleic Acids Res* 26, 3871-3876.
- Perona, R., Moncho-Amor, V., Machado-Pinilla, R., Belda-Iniesta, C., and Sanchez Perez, I. (2008). Role of CHK2 in cancer development. *Clin Transl Oncol* 10, 538-542.
- Perry, R.P., and Kelley, D.E. (1968). Persistent synthesis of 5S RNA when production of 28S and 18S ribosomal RNA is inhibited by low doses of actinomycin D. *J Cell Physiol* 72, 235-246.
- Perry, R.P., and Kelley, D.E. (1970). Inhibition of RNA synthesis by actinomycin D: characteristic dose-response of different RNA species. *J Cell Physiol* 76, 127-139.
- Pertschy, B., Saveanu, C., Zisser, G., Lebreton, A., Tengg, M., Jacquier, A., Liebminger, E., Nobis, B., Kappel, L., van der Klei, I., *et al.* (2007). Cytoplasmic recycling of 60S preribosomal factors depends on the AAA protein Drg1. *Mol Cell Biol* 27, 6581-6592.
- Pestov, D.G., Strezoska, Z., and Lau, L.F. (2001). Evidence of p53-dependent cross-talk between ribosome biogenesis and the cell cycle: effects of nucleolar protein Bop1 on G(1)/S transition. *Mol Cell Biol* 21, 4246-4255.
- Petitjean, A., Mathe, E., Kato, S., Ishioka, C., Tavtigian, S.V., Hainaut, P., and Olivier, M. (2007). Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database. *Hum Mutat* 28, 622-629.
- Piva, R. (2014). RB orchestrates fat cell and cell fate. *Cell Cycle* 13, 508.
- Qi, W., Shakalya, K., Stejskal, A., Goldman, A., Beeck, S., Cooke, L., and Mahadevan, D. (2008). NSC348884, a nucleophosmin inhibitor disrupts oligomer formation and induces apoptosis in human cancer cells. *Oncogene* 27, 4210-4220.
- Rabl, J., Leibundgut, M., Ataide, S.F., Haag, A., and Ban, N. (2011). Crystal structure of the eukaryotic 40S ribosomal subunit in complex with initiation factor 1. *Science* 331, 730-736.
- Restle, A., Farber, M., Baumann, C., Bohringer, M., Scheidtmann, K.H., Muller-Tidow, C., and Wiesmuller, L. (2008). Dissecting the role of p53 phosphorylation in homologous recombination provides new clues for gain-of-function mutants. *Nucleic Acids Res* 36, 5362-5375.
- Robertson, K.D., and Jones, P.A. (1998). The human ARF cell cycle regulatory gene promoter is a CpG island which can be silenced by DNA methylation and down-regulated by wild-type p53. *Mol Cell Biol* 18, 6457-6473.
- Roeder, R.G., and Rutter, W.J. (1970). Specific nucleolar and nucleoplasmic RNA polymerases. *Proc Natl Acad Sci U S A* 65, 675-682.
- Rouquette, J., Choismel, V., and Gleizes, P.E. (2005). Nuclear export and cytoplasmic processing of precursors to the 40S ribosomal subunits in mammalian cells. *EMBO J* 24, 2862-2872.

- Rubbi, C.P., and Milner, J. (2003). Disruption of the nucleolus mediates stabilization of p53 in response to DNA damage and other stresses. *EMBO J* 22, 6068-6077.
- Ruggero, D., and Pandolfi, P.P. (2003). Does the ribosome translate cancer? *Nat Rev Cancer* 3, 179-192.
- Sampath, J., Sun, D., Kidd, V.J., Grenet, J., Gandhi, A., Shapiro, L.H., Wang, Q., Zambetti, G.P., and Schuetz, J.D. (2001). Mutant p53 cooperates with ETS and selectively up-regulates human MDR1 not MRP1. *J Biol Chem* 276, 39359-39367.
- Samuels-Lev, Y., O'Connor, D.J., Bergamaschi, D., Trigiante, G., Hsieh, J.K., Zhong, S., Campargue, I., Naumovski, L., Crook, T., and Lu, X. (2001). ASPP proteins specifically stimulate the apoptotic function of p53. *Mol Cell* 8, 781-794.
- Saretzki, G. (2010). Cellular senescence in the development and treatment of cancer. *Curr Pharm Des* 16, 79-100.
- Saveanu, C., Namane, A., Gleizes, P.E., Lebreton, A., Rousselle, J.C., Noaillac-Depeyre, J., Gas, N., Jacquier, A., and Fromont-Racine, M. (2003). Sequential protein association with nascent 60S ribosomal particles. *Mol Cell Biol* 23, 4449-4460.
- Schlosser, I., Holzel, M., Murnseer, M., Burtscher, H., Weidle, U.H., and Eick, D. (2003). A role for c-Myc in the regulation of ribosomal RNA processing. *Nucleic Acids Res* 31, 6148-6156.
- Schmidt, E.V. (2004). The role of c-myc in regulation of translation initiation. *Oncogene* 23, 3217-3221.
- Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D., and Lowe, S.W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* 88, 593-602.
- Shan, B., Xu, J., Zhuo, Y., Morris, C.A., and Morris, G.F. (2003). Induction of p53-dependent activation of the human proliferating cell nuclear antigen gene in chromatin by ionizing radiation. *J Biol Chem* 278, 44009-44017.
- Shcherbik, N., and Pestov, D.G. (2010). Ubiquitin and ubiquitin-like proteins in the nucleolus: multitasking tools for a ribosome factory. *Genes Cancer* 1, 681-689.
- Shieh, S.Y., Taya, Y., and Prives, C. (1999). DNA damage-inducible phosphorylation of p53 at N-terminal sites including a novel site, Ser20, requires tetramerization. *EMBO J* 18, 1815-1823.
- Shimizu, H., Burch, L.R., Smith, A.J., Dornan, D., Wallace, M., Ball, K.L., and Hupp, T.R. (2002). The conformationally flexible S9-S10 linker region in the core domain of p53 contains a novel MDM2 binding site whose mutation increases ubiquitination of p53 in vivo. *J Biol Chem* 277, 28446-28458.
- Shiue, C.N., Berkson, R.G., and Wright, A.P. (2009). c-Myc induces changes in higher order rDNA structure on stimulation of quiescent cells. *Oncogene* 28, 1833-1842.
- Shvarts, A., Steegenga, W.T., Riteco, N., van Laar, T., Dekker, P., Bazuine, M., van Ham, R.C., van der Houven van Oordt, W., Hateboer, G., van der Eb, A.J., *et al.* (1996). MDMX: a novel p53-binding protein with some functional properties of MDM2. *EMBO J* 15, 5349-5357.
- Sobell, H.M., Jain, S.C., Sakore, T.D., and Nordman, C.E. (1971). Stereochemistry of actinomycin--DNA binding. *Nat New Biol* 231, 200-205.

- Song, H., Hollstein, M., and Xu, Y. (2007). p53 gain-of-function cancer mutants induce genetic instability by inactivating ATM. *Nat Cell Biol* 9, 573-580.
- Song, H., and Xu, Y. (2007). Gain of function of p53 cancer mutants in disrupting critical DNA damage response pathways. *Cell Cycle* 6, 1570-1573.
- Soussi, T., and Beroud, C. (2001). Assessing TP53 status in human tumours to evaluate clinical outcome. *Nat Rev Cancer* 1, 233-240.
- Stambolic, V., MacPherson, D., Sas, D., Lin, Y., Snow, B., Jang, Y., Benchimol, S., and Mak, T.W. (2001). Regulation of PTEN transcription by p53. *Mol Cell* 8, 317-325.
- Stommel, J.M., and Wahl, G.M. (2005). A new twist in the feedback loop: stress-activated MDM2 destabilization is required for p53 activation. *Cell Cycle* 4, 411-417.
- Strano, S., Fontemaggi, G., Costanzo, A., Rizzo, M.G., Monti, O., Baccharini, A., Del Sal, G., Levrero, M., Sacchi, A., Oren, M., *et al.* (2002). Physical interaction with human tumor-derived p53 mutants inhibits p63 activities. *J Biol Chem* 277, 18817-18826.
- Strezoska, Z., Pestov, D.G., and Lau, L.F. (2002). Functional inactivation of the mouse nucleolar protein Bop1 inhibits multiple steps in pre-rRNA processing and blocks cell cycle progression. *J Biol Chem* 277, 29617-29625.
- Suh, Y.A., Post, S.M., Elizondo-Fraire, A.C., Maccio, D.R., Jackson, J.G., El-Naggar, A.K., Van Pelt, C., Terzian, T., and Lozano, G. (2011). Multiple stress signals activate mutant p53 in vivo. *Cancer Res* 71, 7168-7175.
- Sun, X.X., Dai, M.S., and Lu, H. (2007). 5-fluorouracil activation of p53 involves an MDM2-ribosomal protein interaction. *J Biol Chem* 282, 8052-8059.
- Sun, X.X., Wang, Y.G., Xirodimas, D.P., and Dai, M.S. (2010). Perturbation of 60 S ribosomal biogenesis results in ribosomal protein L5- and L11-dependent p53 activation. *J Biol Chem* 285, 25812-25821.
- Tafforeau, L., Zorbas, C., Langhendries, J.L., Mullineux, S.T., Stamatopoulou, V., Mullier, R., Wacheul, L., and Lafontaine, D.L. (2013). The complexity of human ribosome biogenesis revealed by systematic nucleolar screening of Pre-rRNA processing factors. *Mol Cell* 51, 539-551.
- Takagi, M., Absalon, M.J., McLure, K.G., and Kastan, M.B. (2005). Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin. *Cell* 123, 49-63.
- Tebaldi, T., Re, A., Viero, G., Pegoretti, I., Passerini, A., Blanzieri, E., and Quattrone, A. (2012). Widespread uncoupling between transcriptome and translome variations after a stimulus in mammalian cells. *BMC Genomics* 13, 220.
- Terzian, T., Suh, Y.A., Iwakuma, T., Post, S.M., Neumann, M., Lang, G.A., Van Pelt, C.S., and Lozano, G. (2008). The inherent instability of mutant p53 is alleviated by Mdm2 or p16INK4a loss. *Genes Dev* 22, 1337-1344.
- Thomas, F., and Kutay, U. (2003). Biogenesis and nuclear export of ribosomal subunits in higher eukaryotes depend on the CRM1 export pathway. *J Cell Sci* 116, 2409-2419.

- Vassiliou, G.S., Cooper, J.L., Rad, R., Li, J., Rice, S., Uren, A., Rad, L., Ellis, P., Andrews, R., Banerjee, R., *et al.* (2011). Mutant nucleophosmin and cooperating pathways drive leukemia initiation and progression in mice. *Nat Genet* 43, 470-475.
- Vivanco, I., and Sawyers, C.L. (2002). The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer* 2, 489-501.
- Vogelstein, B., Lane, D., and Levine, A.J. (2000). Surfing the p53 network. *Nature* 408, 307-310.
- Vousden, K.H., and Lane, D.P. (2007). p53 in health and disease. *Nat Rev Mol Cell Biol* 8, 275-283.
- Vousden, K.H., and Lu, X. (2002). Live or let die: the cell's response to p53. *Nat Rev Cancer* 2, 594-604.
- Vousden, K.H., and Prives, C. (2009). Blinded by the Light: The Growing Complexity of p53. *Cell* 137, 413-431.
- Vousden, K.H., and Ryan, K.M. (2009). p53 and metabolism. *Nat Rev Cancer* 9, 691-700.
- Wang, J.C., and Chen, C. (1998). N-RAS oncogene mutations in patients with agnogenic myeloid metaplasia in leukemic transformation. *Leuk Res* 22, 639-643.
- Wang, S.P., Wang, W.L., Chang, Y.L., Wu, C.T., Chao, Y.C., Kao, S.H., Yuan, A., Lin, C.W., Yang, S.C., Chan, W.K., *et al.* (2009). p53 controls cancer cell invasion by inducing the MDM2-mediated degradation of Slug. *Nat Cell Biol* 11, 694-704.
- Warner, J.R. (1999). The economics of ribosome biosynthesis in yeast. *Trends Biochem Sci* 24, 437-440.
- White, R.J., Trouche, D., Martin, K., Jackson, S.P., and Kouzarides, T. (1996). Repression of RNA polymerase III transcription by the retinoblastoma protein. *Nature* 382, 88-90.
- Wulff, J.E., Siegrist, R., and Myers, A.G. (2007). The natural product avrainvillamide binds to the oncoprotein nucleophosmin. *J Am Chem Soc* 129, 14444-14451.
- Xue, W., Zender, L., Miething, C., Dickins, R.A., Hernando, E., Krizhanovsky, V., Cordon-Cardo, C., and Lowe, S.W. (2007). Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature* 445, 656-660.
- Yu, J., Zhang, L., Hwang, P.M., Kinzler, K.W., and Vogelstein, B. (2001). PUMA induces the rapid apoptosis of colorectal cancer cells. *Mol Cell* 7, 673-682.
- Yu, W., Qiu, Z., Gao, N., Wang, L., Cui, H., Qian, Y., Jiang, L., Luo, J., Yi, Z., Lu, H., *et al.* (2011). PAK1IP1, a ribosomal stress-induced nucleolar protein, regulates cell proliferation via the p53-MDM2 loop. *Nucleic Acids Res* 39, 2234-2248.
- Yuan, J., Luo, K., Zhang, L., Cheville, J.C., and Lou, Z. (2010). USP10 regulates p53 localization and stability by deubiquitinating p53. *Cell* 140, 384-396.
- Yuan, X., Zhou, Y., Casanova, E., Chai, M., Kiss, E., Grone, H.J., Schutz, G., and Grummt, I. (2005). Genetic inactivation of the transcription factor TIF-IA leads to nucleolar disruption, cell cycle arrest, and p53-mediated apoptosis. *Mol Cell* 19, 77-87.

- Zacchi, P., Gostissa, M., Uchida, T., Salvagno, C., Avolio, F., Volinia, S., Ronai, Z., Blandino, G., Schneider, C., and Del Sal, G. (2002). The prolyl isomerase Pin1 reveals a mechanism to control p53 functions after genotoxic insults. *Nature* *419*, 853-857.
- Zhai, W., and Comai, L. (2000). Repression of RNA polymerase I transcription by the tumor suppressor p53. *Mol Cell Biol* *20*, 5930-5938.
- Zhang, Y., and Lu, H. (2009). Signaling to p53: ribosomal proteins find their way. *Cancer Cell* *16*, 369-377.
- Zhang, Y., Wolf, G.W., Bhat, K., Jin, A., Allio, T., Burkhardt, W.A., and Xiong, Y. (2003). Ribosomal protein L11 negatively regulates oncoprotein MDM2 and mediates a p53-dependent ribosomal-stress checkpoint pathway. *Mol Cell Biol* *23*, 8902-8912.
- Zhao, J., Yuan, X., Frodin, M., and Grummt, I. (2003). ERK-dependent phosphorylation of the transcription initiation factor TIF-IA is required for RNA polymerase I transcription and cell growth. *Mol Cell* *11*, 405-413.
- Zheng, T., Wang, J., Zhao, Y., Zhang, C., Lin, M., Wang, X., Yu, H., Liu, L., Feng, Z., and Hu, W. (2013). Spliced MDM2 isoforms promote mutant p53 accumulation and gain-of-function in tumorigenesis. *Nat Commun* *4*, 2996.
- Zhou, X., Liao, J.M., Liao, W.J., and Lu, H. (2012). Scission of the p53-MDM2 Loop by Ribosomal Proteins. *Genes Cancer* *3*, 298-310.
- Zhu, J., Blenis, J., and Yuan, J. (2008). Activation of PI3K/Akt and MAPK pathways regulates Myc-mediated transcription by phosphorylating and promoting the degradation of Mad1. *Proc Natl Acad Sci U S A* *105*, 6584-6589.
- Zuckerman, V., Wolynec, K., Sionov, R.V., Haupt, S., and Haupt, Y. (2009). Tumour suppression by p53: the importance of apoptosis and cellular senescence. *J Pathol* *219*, 3-15.

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