Université PARIS DIDEROT (Paris 7)

SORBONNE PARIS CITE

Ecole Doctorale

Hématologie, Oncogenèse et Biothérapies ED nº 561

Doctorat de Recherche

Hématologie et Oncologie

p53-mediated control of mRNA translation during Endoplasmic Reticulum stress: mechanisms and physiological implications

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Soutenue le 5 septembre 2016

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Abstract

Physiological fluctuations of protein production/folding and pathological processes like viral infection, aging and cancers can lead to Endoplasmic Reticulum (ER) stress. It is a state characterized by the accumulation of unfolded or misfolded proteins in the ER lumen that triggers the Unfolded Protein Response. In restoring ER proteostasis, the UPR inhibits global cap-dependent protein synthesis and promotes proteases and ER chaperons, notably BiP, which also functions as the main UPR sensor. Our group has previously shown that during ER stress, a selective induction of the p53 tumour suppressor protein isoform p53ΔN40 (also known as p53/47, Δ40p53, ΔNp53, p47) by PERK leads to G2 arrest, and that this depends on a suppression of $p21^{CDKN1A}$ expression by p53 full-length (p53FL) and p53ΔN40 acting at transcription and translation.

The main topic of my work has been to understand how p53 promotes apoptosis during prolonged ER stress. I could show that this depends on the down regulation of BiP expression via a direct interaction between a restricted region of the *bip* mRNA's coding sequence and p53 protein. The trans-suppression is mediated by a 7-aa domain of the p53 protein present in both p53FL and p53ΔN40. The inhibition of BiP expression during ER stress leads to an increase in apoptosis via activation of the BH3-only BIK protein by liberating it from a repressive interaction with BiP. Moreover, BIK is further activated during ER stress by transcription induction mediated by p53FL and/or p53ΔN40. These results links for the first time the RNA-binding capacity of p53 and the control of mRNA translation with a particular cellular response.

The work also shows that p53 controls the translation of two additional mRNAs in what appears to be two different mechanisms. Both mechanisms rely on sequences present in the mRNAs but differ in the requirement of a direct interaction with the p53 protein. Like with BiP, the RNA-binding capacity of p53 shuts down the translation of *fgf-2* and *p53* mRNAs. To this category we can now also include the *mdmx* mRNA. On the other hand, suppression of $p21^{CDKN1A}$ translation was not shown to require an interaction with p53 and this is also the case for suppression of MDM2. The physiological implications of MDMX and MDM2 suppression are discussed.

These data illustrate that p53-mediated mRNA translation suppression plays a physiological role during the UPR and further supports the specific role of the p53ΔN40 during ER stress.

Résumé

Contrôle de la traduction des ARNm médiée par p53 au cours du stress du Réticulum Endoplasmique : Mécanismes et implications physiologiques

Des fluctuations physiologiques lors de la production et du repliement des protéines, ainsi que des processus pathologiques comme l'infection virale, le vieillissement et les cancers peuvent conduire à un stress du Réticulum Endoplasmique (RE). Il s'agit d'un état caractérisé par l'accumulation de protéines non/mal repliées dans la lumière du RE, qui déclenche la réponse aux protéines dépliées, dite UPR (Unfolded Protein Response). Pour rétablir l'équilibre protéique, la réponse UPR va inhiber la synthèse protéique globale cap-dépendante et favoriser la production de protéases et de chaperonnes associées au RE, notamment la protéine BiP qui joue également le rôle de senseur principal de l'UPR. Notre groupe a précédemment montré que lors d'un stress du RE, une isoforme particulière du suppresseur de tumeur p53, l'isoforme p53ΔN40 (également connue sous le nom de p53/47, Δ40p53, ΔNp53 ou p47) est induite sélectivement par PERK pour conduire à l'arrêt de la division cellulaire en G2 et que ceci dépend de la suppression de l'expression de $p21^{CDKN1A}$ par l'isoforme longue de p53 (p53FL) avec p53ΔN40 agissant notamment au niveau transcriptionnel et traductionnel.

Le sujet principal de mon travail a été de comprendre comment p53 induit l'apoptose en cas de stress prolongé du RE. J'ai pu montrer que ceci dépend de la diminution de l'expression de BiP, via une interaction directe de la protéine p53 avec une petite région de la séquence codante de l'ARNm de BIP. Cette trans-suppression de BiP est médiée par un domaine de 7 acides aminés présent dans p53FL et aussi dans p53ΔN40. Cette inhibition de l'expression de BiP pendant le stress du RE conduit à une augmentation de l'apoptose par l'activation de la protéine BIK ainsi libérée d'une interaction répressive avec BiP. De plus, BIK est également activée pendant le stress du RE par p53FL et/ou p53ΔN40 au niveau transcriptionnel. Mes résultats établissent pour la première fois un lien entre la capacité de liaison à l'ARNm de p53 et le contrôle de la traduction de cet ARNm avec une réponse cellulaire particulière.

Ce travail montre également que p53 contrôle la traduction de deux ARNm supplémentaires par ce qui semble être deux mécanismes d'action différents. Ces deux mécanismes reposent sur des séquences présentes dans l'ARNm, mais diffèrent sur la nécessité d'une interaction directe avec la protéine p53. Comme montré pour BiP, la capacité de liaison à l'ARNm de p53 bloque la traduction des ARNm de FGF-2 et de p53. Dans cette catégorie, nous pouvons maintenant également inclure l'ARNm de MDMX. D'un autre côté, la suppression de la traduction de $p21^{CDKN1A}$ n'a pas été associée à une interaction avec p53, ce qui est aussi le cas pour la suppression de l'expression de MDM2. Les implications physiologiques des suppressions d'expression de MDM2 et de MDMX sont discutées.

Ces résultats montrent que la suppression de la traduction de l'ARNm médiée par p53 joue un rôle physiologique majeur lors de l'UPR et soutient le rôle spécifique de la p53ΔN40 en réponse à du stress du RE.

Acknowledgments

The work described here has been accomplished with the support of many people from both the “academic world” as well as the “real one”.

First, I would like to thank the members of the committee, Professors Fabien Calvo and Marc Blondel and Doctors Eric Chevet, Morgane Le Bras and Martin Dutertre. Thank you very much for the time and interest in reading my thesis and the comments and suggestions that will undoubtedly improve the final result.

I am particularly grateful to Robin. I would like to thank him for having directed my thesis in the way he did, for having let me find my own way, for having helped me to be a step closer to the independence and for all the opportunities he has given me during this PhD and even before when I spent some months working in his lab. I would also thank Robin for been available at all times for scientific, personal, political, sport and even philosophical discussions, although I feel I did not take full advantage of that. I have learned a lot from him in all those areas.

I also thank all the members of the lab, those that are here now but also former members that have contributed a lot to this work and to the day-to-day life. I particularly thank Anne-Sophie and Laurence for letting me be part of a great team work and for all the technical help they have offered me with experiments but also with administration, writing and much more. Also, I would like to thank Anne for receiving me when I was almost lost in a new country and for showing me the first steps both in the lab and in France. I am also very thankful to Rodrigo, a particular Brazilian who has helped me both in scientific and personal life with great discussions and advices on both topics and that has changed the day-to-day life from his first day. Also, I specially thank Konstantinos for all the discussions and the (short) time spent outside the lab, Coraline for showing me the way the lab works as well as for initial discussions and Guy and Sarah for their assistance with imaging experiments. I also thank the rest of the members of the team as well the rest of the members of the unit. Last, I do not forget Marie-Christine who has helped me so many times I cannot even remember and made the french bureaucracy look a little bit “funny”. I kindly thank Dr. Borek Vojtesek for the constant supply of reagents.

A big part of the work presented here was done in collaboration with the IUH platform and therefore I am very grateful to its members, in particular I would like to thank Niclas, Antonio, María Marta and Sophie.

I would also thank members of my former lab in Uruguay, in particular to Mónica. She has taught me a lot about research and many other things and she gave me lot of opportunities and helped me enormously to be where I am today. I thank Marc Blondel and his team for receiving me in his lab for a few months during my master degree and for establishing the first bridge that allowed me to cross the Atlantic Ocean.

At this point I also remember my friends. Those that are here in France and that belong to “Argenguay”, a particular and equilibrated mixture of people from “Río de la Plata”. I specially thank Mariano and Martín for been there during a difficult period of my life and for having helped me to get back on my feet again. Also, those friends back in Uruguay. Those that I had grown up with and still are like brothers even though we see each other a few days per year; the “Luzbelito” and specially Coco. And of course, I particularly thank to the members of “Ídolos de la Gloria”, an exclusive group of scientists, philanthropists, business and religious men and many other things that have been there from the beginning of the scientific journey and that they still contribute every day to the solution of many and diverse problems related to the academia but most importantly, to life. I also think of them as brothers.

Infinite thanks to my family. To my parents that have not only let me but have motivated me to do what I wanted, that have believed in me and have supported my craziest ideas and wishes and than have taught me to live and most importantly, to be happy. To my sisters and brother; Chara, Ana and Agus. Those three that I often see as kids when I look at them and that I miss in a way I cannot put it in words. They have contributed incredibly during all my life and in particular during these last years and are a big support and an important part of who I am. A special thank for my grandparents for all the help they have given me from the moment I was born and for being always interested in “la p53”.

Finally, I would like to thank in the most special way to the person that has changed my life and that has helped the most during this thesis and during the last five years (and even before); María José Lista (Majo). Even though our paths are no longer together, I am sure I could not have achieved this without her. I thank her for the unlimited support, strength and love she has given me to pursue this work as well as for the scientific suggestions and ideas to improve it. I am very happy that I was able to share (almost) all this journey with her and I look forward to meeting her again, hoping that the future will find us close enough to be, in one way or another, part of each other’s life.

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State of the art

1. The tumour suppressor protein p53

1.1. p53 signalling pathway: The classical view

The tumour suppressor protein p53 (encoded in humans by *TP53* gene) is a central regulator of many and diverse cellular processes. p53 was discovered in the late 1970s and it was first described as an antigen associated with cellular transformation in SV40-transformed cell lines. p53 was first thought to be one of the SV40 antigens since it immunoprecipitated with an anti SV40 tumour serum along with SV40 large T and small t antigens (Lane & Crawford, 1979; Linzer & Levine, 1979). Later on, several studies relying on transfection of p53-encoding cDNAs isolated from transformed cells showed that p53 was able to cooperate with Ras in inducing cellular transformation. This observation changed the vision of p53 community towards p53's function and it was re-classified as an oncogene (Eliyahu et al, 1984; Jenkins et al, 1984; Parada et al, 1984). It was not until 1989 when the status of *TP53* was changed to that of a tumour suppressor gene. This was based on the observations that wild-type p53 cDNA transfection does not contain any oncogenic activity, but instead, it confers anti-proliferative capacity (Finlay et al, 1989) and to the demonstration of occurrence of inactivating p53 mutations in human cancers (Baker et al, 1989).

p53 is mainly known by its role as a transcription factor that both positively and negatively regulates the expression of a diverse multitude of genes. It becomes activated when different insults are infringed to cells, such as DNA damage, nutrient deprivation, viral infection or oncogene activation, among many others (Vousden & Lane, 2007). Due to its central role in cellular homeostasis and physiological processes, p53 is inactivated by mutations in over 50% of human cancers with most of the mutations located in its DNA-binding domain and resulting in a transactivation-deficient protein (Petitjean et al, 2007). Moreover, germline mutations in the human *TP53* gene are one cause of enhanced risk of developing cancer, as in a rare disorder called Li-Fraumeni syndrome. Patients harbouring this syndrome generally express both the mutant and the wild-type form of p53 in all tissues and the wild-type activity is often lost during cancer progression. This is due to the ability of mutant version to act as a dominant-negative inhibitor, to a gain of function (GOF) that favours cancer progression or to a direct loss of p53 wild-type allele, leading to loss of heterozygosity (LOH). In addition, and even though mutant version can co-exist with the normal protein without affecting its capacity, the exquisitely sensitivity of p53 pathway to small changes in levels or activity of p53 may impose an important handicap in Li-Fraumeni patients expressing half of the normal amount of fully active p53 (Vousden & Lane, 2007). The cancers most often associated with this syndrome include breast cancer, osteosarcoma and soft tissue sarcomas particularly in children and young adults. In addition, in tumours where *TP53* gene sequence is not changed, the protein activity might be inactivated trough different mechanisms. The most important are the binding with and inhibition by cellular regulators such as MDM2 (Momand et al, 1992) and MDMX (Shvarts et al, 1996) or the interaction with proteins from cancer-associated virus like T antigen from SV40 (Lane & Crawford, 1979; Linzer & Levine, 1979), adenovirus E1b (Sarnow et al, 1982) and the E6 protein from human papilloma virus (HPV) types 16 and 18 (Scheffner et al, 1990; Werness et al, 1990).

After p53 becomes activated, it induces different sets of gene products triggering particular biological effects that match the insults. These gene products are aimed at either preventing abnormal growth of compromised cells by reversible arrest of the cell cycle in G1 or G2 to facilitate repair processes, or at inducing irreversible outcomes including apoptosis or senescence (el-Deiry

et al, 1994; Kastan et al, 1992; Lanigan et al, 2011; Miyashita et al, 1994b; Miyashita & Reed, 1995) (Figure 1).

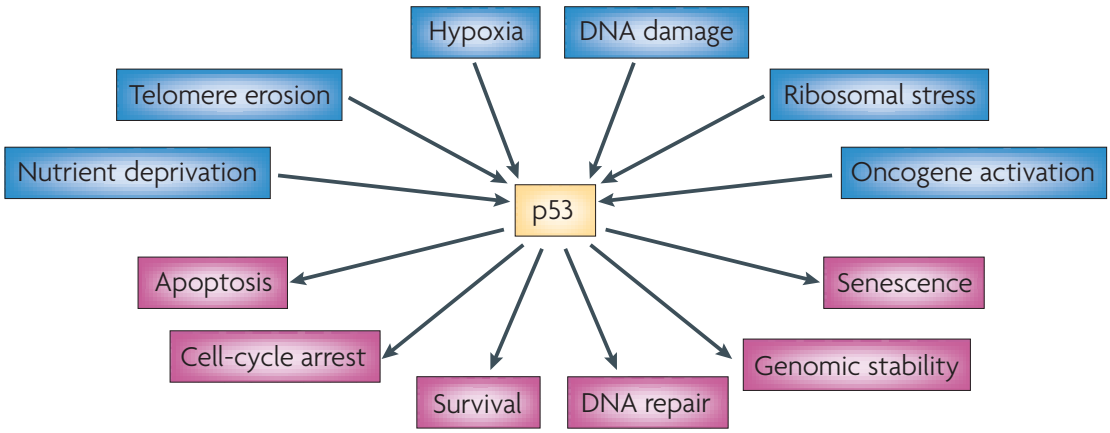

Figure 1. Activators of p53 and its responses.

Some of the vast insults that induce p53 activation (in blue) and the possible responses they may trigger (in pink). The final cellular output depends on the set of target genes induced/repressed by p53. Extracted from Vousden & Lane, 2007.

Two of the major and best-described p53 target genes following DNA-damage (the most well-studied inducer of p53) are *p21*^{CDKN1A} (*p21*$^{Cip1/Waf1}$) and *mdm2*, whose p53-dependent mRNA induction is mirrored by an increase in protein levels. Induction of p21^{CDKN1A} constitutes an important branch of the p53-dependent cancer protection. It is observed at early stages of DNA damage response due to its capacity to suppress both G1 and S phase cyclin and cyclin-dependent kinase (CDKs) activity and to prolong the G1 phase in order to prevent cells from entering replication carrying damaged DNA (Efeyan et al, 2007; el-Deiry et al, 1994). MDM2, however, plays a critical role both as negative and positive regulator of p53 in different scenarios and constitutes a fascinating loop of regulation (see further below).

In addition, p53 has been shown to induce many other genes in response to the insults named above. In order to simplify the reading (and mostly writing) processes, some of those targets will appear in different sections of this thesis and will not be listed at this point.

1.2. The intricate regulatory loops among p53, MDM2 and MDMX

The minute-deficient mouse (MDM) proteins MDM2 and MDMX (also called MDM4) are two non-redundant homologues and the main cellular regulators of p53. Altered expression of both proteins via post-transcriptional mechanism has been reported in melanoma, Ewing's sarcoma, colon carcinoma, retinoblastoma and breast cancer. Additionally, gene amplification of MDM genes was also detected in glioblastoma, liposarcoma, osteosarcomas and colorectal tumors. In many cases, the frequency of MDM protein deregulation is higher in tumours that retain wild-type p53 (Marine et al, 2007; Wade et al, 2013). Together, these observations point to a major oncogenic role of MDM proteins by suppressing p53 activity.

The similarity between these two proteins is remarkable; they share about 50% overall homology (identity), particularly in the N-terminal p53 binding and in the C-terminal domains, and both contain a RING domain (Figure 2) (Marine et al, 2007). It has been suggested that the oligomerisation state of MDM2 controls MDM2 and MDMX stability and therefore, the p53

ubiquitination state (Fahraeus & Olivares-Illana, 2014). Furthermore, both MDM2 and MDMX express isoforms that lack the N-terminal p53 binding domains but these retain the capacity to form hetero-oligomers via their respective RING domains and, thus, to regulate each other's activity (Perry et al, 2000; Tournillon et al, 2015).

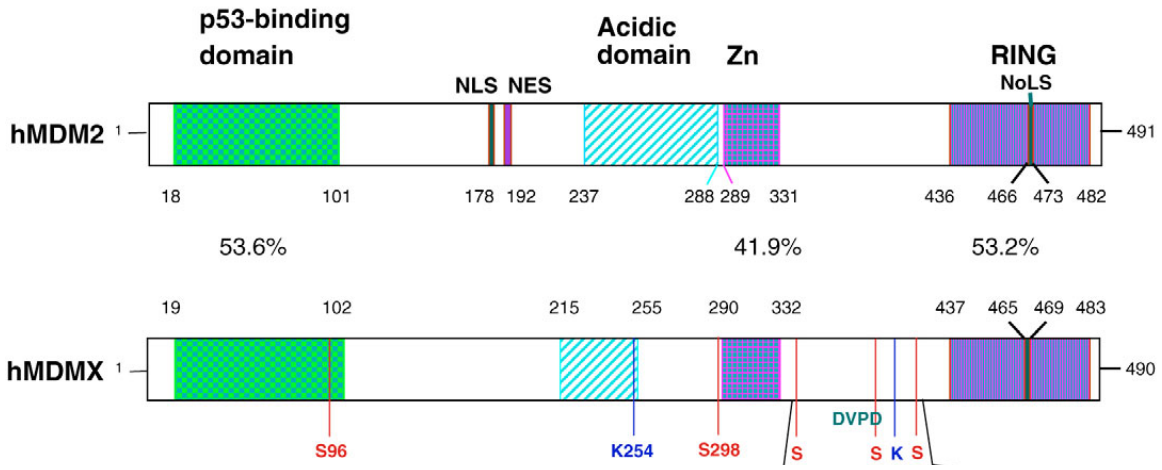

Figure 2. MDM2 and MDMX.
Schematic representation and comparison between the primary structure of MDM2 and MDMX. Most important domains are highlighted with their initiation and end positions shown, as well as the aa sequence identity between the most conserved ones: The p53-binding domain, zinc (Zn) finger and RING finger (that contains the nucleolar location signal (NoLS). Serines (S) and lysines (K) that are validated sites of phosphorylation and SUMO conjugation, respectively, are shown for MDMX. DVPD, caspase-3 cleavage site; NES, nuclear export signal; NLS, nuclear localization signal. The "h" before MDM2 and MDMX stands for human. However, this nomenclature was not used in the text. Extracted from Marine et al, 2007.

MDM2 binds the conserved BOX-I motif in the N-terminus of p53 and masks p53's transactivation domain (Kussie et al, 1996; Momand et al, 1992). Similarly, MDMX can also inhibit induction of transcription by p53 by binding its N-terminal transactivation domain via related but not identical N-terminal hydrophobic pockets (Kussie et al, 1996; Momand et al, 1992; Shvarts et al, 1996; Wade et al, 2013). Moreover, and despite of the high similarity of protein sequence between them, MDM2, but not MDMX, posses an E3-ubiquitin ligase activity that relies in its C-terminal RING domain (Fang et al, 2000). After binding to p53, MDM2 ubiquitinates p53 and targets it for 26S-dependent proteasomal degradation. This activity is more effective when MDMX forms heterodimers with MDM2 (Wade et al, 2013). In these ways, p53 abundance and activity are kept low in non-damaged cells.

Keeping low levels of p53 is an important feature during development and mice lacking either *Mdm2* or *Mdmx* die early during development in a non-redundant fashion that is rescued in either case by deletion of *TP53* (Jones et al, 1995; Montes de Oca Luna et al, 1995; Parant et al, 2001).

Interestingly, and although the P1 promoter of *Mdm2* is constitutive, the P2 promoter includes a p53-binding site that mediates induction of *mdm2* transcription, constituting a regulatory p53-MDM2 feed-back loop to keep p53 activity low during normal conditions (Barak et al, 1994; Barak et al, 1993). On the other hand, when cells face DNA damage, the negative effects of MDM2 and MDMX need to be suppressed in order to allow p53 activation. Firstly, the p53-MDM2 interaction is inhibited by phosphorylations on the p53 N-terminus by the ataxia-telangiectasia mutated (ATM) kinase. In addition, ATM-mediated phosphorylation of MDM2 and MDMX switches

these factors to become positive regulators of p53 by stimulating the rate of p53 protein synthesis and increasing its half-life (Candeias et al, 2008; Malbert-Colas et al, 2014). As the increase in MDM2 expression by p53 is one of the earliest events in the DNA damage response, this suggests that the p53-MDM2 negative feedback loop switches to become a positive loop during the DNA damage response. Although MDM2-dependent negative control of p53 activity is vital during mice development to subdue p53 activity, recent animal models indicate that the p53-MDM2 feed-back loop is important during the DNA damage response but is not required to suppress p53 activity during mice development (Pant et al, 2013).

MDM2 and MDMX's positive activity towards p53 was shown to depend on their capacity to bind p53's mRNA (Candeias et al, 2008; Naski et al, 2009). The interaction of MDM2 with the mRNA of p53 was proved to be co-transcriptional and dependent on a MDMX RNA chaperone capacity (Malbert-Colas et al, 2014). Indeed, our lab showed that MDMX binds and modifies p53's mRNA folding while been transcribed by the RNApolII, creating a platform to which MDM2 can bind in order to promote p53 protein synthesis, as shown in Figure 3 (Malbert-Colas et al, 2014). In both cases, an intact C-terminal RING domain was shown to be required for mRNA binding (Gajjar et al, 2012; Malbert-Colas et al, 2014; Naski et al, 2009).

Interestingly, the same mRNA region that serves as platform for MDM2 to bind the *p53* mRNA also codes for the peptide to which MDM2 binds in oder to suppress p53 activity. This opens for the possibility of a co-evolutionary scenario in which these two functions were shaped simultaneously towards time. However, it was recently shown that they evolved independently. Using the pre-vertebrate *Ciona intestinalis* as model, Karakostis et al showed that although the *p53* mRNA-MDM2 interaction is present in this organism, MDM2 is not able to interact with its p53 protein. Interestingly, the conserved BOX-I motif of p53 that binds a hydrophobic pocket in MDM2 is present in *C. intestinalis* but is prevented from interacting by a flanking motif that is not present in mammalian p53. Interestingly, deletion of this motif allows the interaction between *C. intestinalis*' MDM2 and p53 to occur (Karakostis et al, 2016).

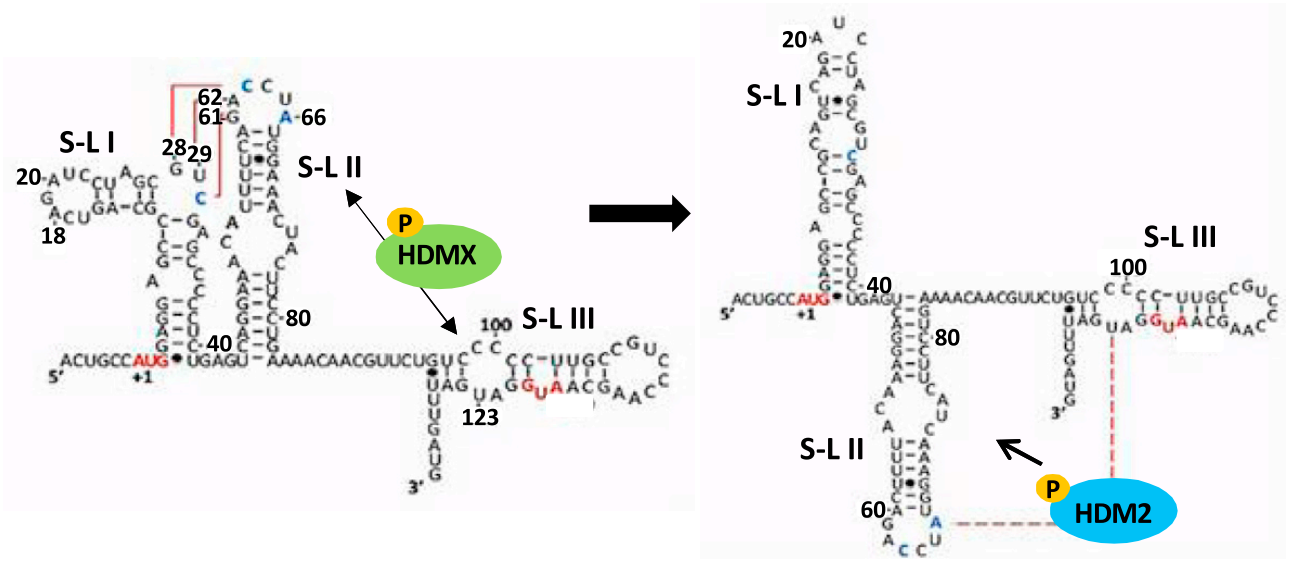

Figure 3. MDM2 and MDMX act as positive regulators of p53 under DNA damage by activating p53 mRNA translation.

Phosphorylation of MDMX at Ser403 by ATM following DMA damage provoques binding of MDMX to nascent *p53* mRNA and induces a conformational change on it. This new conformation constitutes the platform to which phosphorylated MDM2 (ATM-dependent phosphorylation on Ser 395) binds in order to stimulate p53 protein synthesis. This shows that both MDM2 and MDMX act as ITAFs for *p53* mRNA. S-L I, II and III are stem-loops I, II and III, respectively. p53FL and

p53ΔN40 initiation AUG codons are shown in red. Mutations reported in cancer at positions 30, 63 and 66 are shown in blue. mRNA structures were predicted based on RNA foot printing in cells pulsed with DMS. HDM2 and HDMX are MDM2 and MDMX, respectively. Extracted from Malbert-Colas et al, 2014.

When compared to the interplay between MDM2 and p53, little is still known on the regulatory feedback between p53 and MDMX even though it has been suggested that p53 can activate *Mdmx* gene transcription from a P2 promoter, similarly to that of *Mdm2*. Interestingly, the authors of this report claim that the P2-derived *mdmx* mRNA is more efficiently translated than that expressed from the constitutive P1 promoter, and thus, it can play a key role in the attenuation phase of the p53 response by effectively diminishing p53 abundance as cells recover from stress (Phillips et al, 2010). However, this finding remains controversial.

1.3. p53: Protein organization

The active form of p53 is constituted of 4 monomers that bind each other trough the tetramerization (TET) domain located in the C-terminus. In addition to the TET domain, p53 counts with 5 other domains. These are the N-terminal Trans-Activation domains (TAD I aa 1 to 40 and II aa 40-61), followed by the Proline Rich Region (PRR aa 62-92), the central DNA-binding domain (DBD aa 94 to 292), the nuclear localization and export signals (NLS and NES that localize to different regions of the protein) and the C-terminal (Ct aa 356 to 393) basic, regulatory and unstructured domain (Figure 4) (Joerger & Fersht, 2008).

Both TAD I and II interact with many factors. TAD I includes the BOX I motif that specifically binds MDM2 (Kussie et al, 1996) and it also interacts with transcription factors including the TATA-binding protein (TBP) (Chang et al, 1995) and other components of the initiation complex TFIID (Joerger & Fersht, 2008), as well as the pleiotropic cellular co-activator CREB-binding protein, CBP/p300 (Van Orden et al, 1999). On the other hand, the TAD II domain has been shown to interact with a single-stranded DNA-binding protein, the replication binding protein A (RPA) (Bochkareva et al, 2005) and with the transcription-associated factor TFIIH via binding to its p62 (or Tfb1 in yeast) subunit (Di Lello et al, 2006). Although the free TAD domains of p53 are flexible in solution, they may adopt transient secondary structures upon association with other factors, as it is exemplified by the p53-MDM2 interaction (Kussie et al, 1996). A stabilisation of the intrinsic disordered domain of TAD I is also likely to occur following phosphorylation events induced by ATM or kinases downstream of ATM such as Chk2. In the case of the secondary structure of the TAD II, it also becomes stabilised upon ligand binding and this might allow it to play different roles in terms of interaction with the partner proteins. In particular, Bochkareva et al, described two amphipathic helices; H1 (residues 41 to 44) and H2 (residues 47 to 55) with important differences in binding properties. Indeed, they claim that while helix H2 has the most extensive buried surface and therefore, appears to be the major determinant of the interaction, helix H1 has a smaller interaction surface and smaller changes in NMR resonance frequencies upon binding to RPA70N (the N-terminal domain of the RPA70 monomer of RPA used in the study), suggesting that H1 plays a secondary role in the interaction (Bochkareva et al, 2005). Moreover, Di Lello et al, also found that the helix H2 mediated the interaction with the p62 (Tfb1) subunit of TFIIH and although they did not attribute any function to the helix H1, they showed that p53 phosphorylated at Ser46 (just before the initial position of helix H2), enhanced binding of p53 to both p62 and Tfb1. The interaction was further enhanced by phosphorylation at Thr 55 (Di Lello et al, 2006). These results add evidence supporting a regulatory role for the region up-stream of helix H2.

Another putative protein-protein interaction platform is the PRR region that follows the TAD domains (Joerger & Fersht, 2008), where the PXXP sites might be involved in association with proteins via recognition of SH3 (Src Homology) domains. Although this domain is not necessary for transcriptional activation by p53, it impaired p53's ability to suppress tumor cell growth in culture, suggesting a critical role in the transmission of anti-proliferative signals downstream of the p53 protein and may link p53 to a signal transduction pathway that remains elusive (Walker & Levine, 1996). This domain is also the least conserved and it has been speculated that this region may represent a structural requirement. In this way, it would act as a spacer between two other functional domains (Joerger & Fersht, 2008).

The central DNA-binding domain (DBD) structure was first solved in complex with a consensus DNA sequence in 1994 (Cho et al, 1994). Later on, many others have been reported; both the crystal and in solution structure in its free form (Canadillas et al, 2006; Wang et al, 2007), in complex with different target DNAs and bound to domains of signaling proteins and to SV40 large T-antigen viral oncoprotein, as well as the structures of numerous mutants in their DNA-free form, as review elsewhere (Joerger & Fersht, 2008). These works showed that p53's DBD presents an immunoglobulin-like beta-sandwich structure from where the loop-sheet-helix and Zn-associated loops motifs emerge and mediate the interaction with the major and minor groove of the DNA, respectively (Joerger & Fersht, 2008).

It has been shown that the p53 protein is subjected to both nuclear import and export via a fast, energy-dependent pathway. In order to be imported, p53 relies on a bipartite nuclear localization signal (NLS) comprising the residues Lys305 and Arg306 along with the major and previously described NLS, the NLSI (PQPKKKP, from position 316 to 322) (Liang & Clarke, 1999). This sequence is accessible in both the DNA-free and DNA-bound form of p53. On the other hand, a C-terminal nuclear exporting signal (NES) (residues 340–351) regulates p53 nuclear export. This signal is concealed within the TET domain, thus requiring dissociation of the tetramer as part of the recognition process involved in nuclear export (Joerger & Fersht, 2008; Stommel et al, 1999).

Another important feature of p53 activity is that it binds DNA and control transcription as a tetramer. The formation of tetramers depends on the TET domain, expanding from residue 325 to 356, whose structure has been solved by X-ray crystallography and in solution by NMR. The structure points towards a dimer of dimers, where two monomers form a primary dimer stabilized via an anti-parallel intermolecular β-sheet and anti-parallel helix packing. Association of two of such dimers through their helices gives the formation of a four-helix bundle tetramer. Hydrophobic interactions between Leu 344 and 348 of each monomer stabilize the tetramer interface (Joerger & Fersht, 2008).

The C-terminal end is intrinsically unstructured, though it was shown that it can describe ordered-disordered transitions upon binding to proteins or to DNA on a non-specific way through electrostatic interactions. This part of the protein is the target of post-translational modifications such as acetylation, ubiquitination, phosphorylation, which regulate the activity, localization and level of the protein (Hupp et al, 1992; Joerger & Fersht, 2008).

1.4. p53 isoforms

p53 could be better described as a family of proteins. The existence of many isoforms offers one putative explanation for one of the main unanswered questions: How does p53 "decide" to trigger the pro-survival or cell death responses? Similarly to the p53-related proteins p63 and p73, that have main roles in epidermal morphogenesis and limb development in mice and humans, and neurogenesis and inflammation in mice, respectively, several p53 isoforms are derived from

alternative initiation of translation, usage of an internal promoter, and alternative splicing and they are shown in Figure 4 (Khoury & Bourdon, 2011).

Alternative promoter usage can give a N-truncated p53 isoform called Δ133p53 that does not contain the TAD nor the PRR domains, and only part of the DBD, being the domains located further down-stream the same as the full-length p53 (p53FL) (Bourdon et al, 2005). Its corresponding mRNA was first detected in several (but not all) normal human tissues and the promoter controlling its expression was found to be located in the 3'-end of intron 4, a region that was proved to be able to drive the expression of a luciferase reporter gene in transfected cells. Expression at the protein level was also confirmed in cell culture (Bourdon et al, 2005). Later on, Δ133p53 was shown to reduce the level of p53-induced apoptosis in p53-null and p53-positive H1299 and U20S cell lines, respectively, as well as p53-dependent G1 arrest in U20S cells. Also, Δ133p53 was shown to inhibit p53-mediated replicative senescence and to promote cellular proliferation of normal human fibroblasts by inhibiting $p21^{CDKN1A}$ expression and repressing the expression of miR-34a. Interestingly, under genotoxic stress, p53 was shown to directly trans-activate its internal promoter driving the expression of Δ133p53, indicating that it may modulate the p53 response (Khoury & Bourdon, 2011).

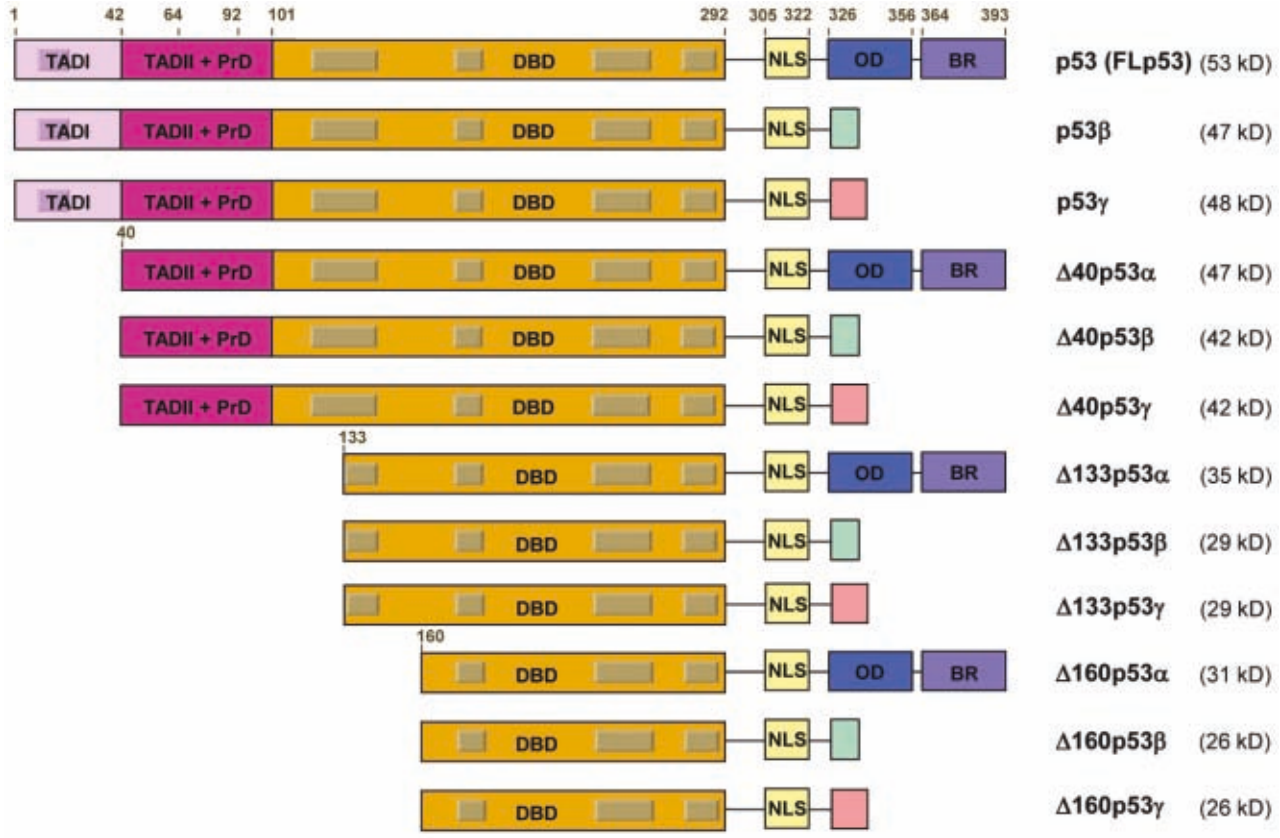

Figure 4. p53 isoforms.

The 12 putative isoforms of p53 described in the text with the domains they contain and the starting and ending positions for each of them. It shows they present differences both at the N- (Δ variants) and C-terminal (α, β and γ variants) regions. aa positions are signalled in protein domain boundaries and the molecular weight in kDa is shown on the right. TAD; trans-activation domain I and II, PrD; Proline rich region (PRR), DBD; DNA-binding domain, NLS; nuclear localization signal, OD; oligomerization domain (TET), BR; basic domain (C-terminal regulatory domain). The aa sequences of the C-terminal region of β and γ variants are presented in the text. Extracted from Khoury & Bourdon, 2011.

In addition to Δ133p53, the same mRNA produced from the P2 promoter codes for a translation variant named Δ160p53 whose translation is initiated at the AUG codon in the position 160. The surrounding nucleotide environment shows a quite conserved Kozak sequence among mammals that might be able to drive Δ160p53 protein expression. This isoform lacks the first 159 aa of p53, including the complete TAD and PRR, and contains a smaller DBD domain compared to Δ133p53. Although endogenous expression of Δ160p53 protein was detected in U2OS, T47D, and K562 cells, no physiological role was attributed to this short isoform (Marcel et al, 2010).

Another N-truncated isoform is produced by alternative splicing of intron 2 of p53 mRNA or by alternative initiation of translation. This isoform, called p53/47, Δ40p53, ΔNp53, p47 and from now on and for the convenience of this thesis, p53ΔN40, starts at the second in-frame AUG at position 40 (Courtois et al, 2002; Ghosh et al, 2004; Yin et al, 2002). Thus, p53ΔN40 lacks the first 39 aa of p53FL, which comprise the TAD I and the binding site for several proteins, most notably that of MDM2 (see above). Alternative splicing leading to retention of intron 2 of p53, originates an mRNA containing three stop codons that are in frame with the first and canonical translation initiation site of p53, offering a new initiation site at the Met40 that is surrounded at the nucleotide level by a consensus Kozak sequence (Matlashewski et al, 1987). The intron 2-containing *p53* mRNA was detected by reverse transcription and PCR amplification using both polyribosome-associated and total RNA and was shown to be less abundant than the prototype *p53* mRNA in different human cell lines and normal lymphocytes (Ghosh et al, 2004), though no difference in splicing was seen in HCT116 cell line by other group that was trying to address the origin of the same p53 isoform (Courtois et al, 2002). p53ΔN40 may also arise from a normal-spliced mRNA but from a second in-frame AUG codon, 40 positions down-stream of the first one. Expression of this protein was detected both from endogenous and exogenous *p53* mRNAs, and in the latter, its production was abolished when the Met40-coding codon was changed (Courtois et al, 2002; Yin et al, 2002). The nucleotide sequence up-stream of the 40th codon in the *p53* mRNA was shown to contain at least one internal ribosome entry site (IRES), which would mediate translation of p53 proteins when the cap-dependent general initiation of translation is inhibited, as it is under cytotoxic and Endoplasmic Reticulum (ER) stress (Candeias et al, 2006; Ray et al, 2006; Yang et al, 2006)(see future sections). Furthermore and in agreement with the IRES status, different IRES transacting factors (ITAFs) were shown to bind to this region and tune the expression of both p53 and p53ΔN40 isoforms (Khoury & Bourdon, 2011).

Although a functional property of p53ΔN40 was addressed by several groups, no consensus has been reached so far. On the hands of Courtois et al, p53ΔN40 was not able to activate the transcription of reporter constructs containing responsive elements (RE) from promoters of different p53 target genes (*Pig3*, *Gadd45*, *Cyclin-g*, *Mdm2* and *p21*^{CDKN1A}) to the same extent as p53FL. In line with this, they showed using clonogenic assays that p53ΔN40 did not suppress the growth of the p53-null cell line H358, but counteracted the suppressive effect of p53FL when present in 10-fold excess (Courtois et al, 2002). This observation was confirmed by Ghosh and collaborators. Using colony formation assays with p53-null tumor cells H1299 and Saos-2, they showed that cells could tolerate p53ΔN40 expression or p53 expression in the presence of p53ΔN40 but were unable to tolerate p53 expression in the absence of p53ΔN40, a condition where the cell viability was suppressed (Ghosh et al, 2004). Yin et al, however, reported that despite induction of MDM2 expression by p53ΔN40 was lower when compared to that induced by p53FL, induction of pro-apoptotic BAX protein was similar in p53-null H1299 cells, an observation that was supported by the fact that both isoforms were able to induce apoptosis with similar efficiency when the sub-G0 population was estimated by fluorescence-activated cell sorting (FACS) analysis (Yin et al, 2002). On the contrary, they showed that p53-dependent induction of

p21^{CDKN1A} expression was counteracted by increasing amounts of p53ΔN40, which contradicts the data from Courtois et al since *p21^{CDKN1A}* promoter (in the context of a reporter construct) was the one showing the most similar results between p53FL and p53ΔN40 (Courtois et al, 2002; Yin et al, 2002). Suppression of p53FL-dependent induction of p21^{CDKN1A} by p53ΔN40 was later on confirmed by Ghosh et al, and more recently by our group where it was correlated with a physiological role under ER stress conditions (see future sections) (Ghosh et al, 2004; Mlynarczyk & Fahraeus, 2014). Despite all the contradictory data, one important fact is common in all the works cited above: p53ΔN40 is able to form hetero-oligomers with p53FL on a TET domain-dependent manner, and this interaction prevents degradation of p53 by MDM2 (Courtois et al, 2002; Ghosh et al, 2004; Yin et al, 2002).

The four isoforms listed so far represent three different variants involving their C-terminal region that changes depending on the splicing of exon 9, giving the variants α, β and γ. The α forms include a complete and functional TET and C-terminal domains, which are replaced by new residues DQTSFQKENC and MLLDLRWCYFLINSS in the shorter β and γ forms, respectively. Although the β and γ forms were detected for all p53FL, p53ΔN40, Δ133p53 and Δ160p53 forms, their biological activities have not been yet fully investigated (Khoury & Bourdon, 2011).

1.5. p53 is a DNA- and RNA-binding protein or DRBP

The consideration of DNA- and RNA-binding functions within proteins as separate entities is becoming outdated. This is in part due to the discovery of long non-coding RNAs (lncRNAs) that target DNA-binding proteins and the growing data showing that many transcription factors are capable of binding diverse types of RNA and that this relates to modulation of gene expression, cell survival and homeostasis (Hudson & Ortlund, 2014). A recent work analysing the results of high throughput studies has found that among more than 4000 human proteins hat directly interact with double-stranded DNA (dsDNA) *in vitro*, a high number of them gave an “RNA binding” result when Gene Ontology analysis was applied, indicating that RNA-binding may be a common feature of DNA-binding proteins. Similarly, but in the other sense, the 860 RNA-binding proteins constituting the mRNA interactome of HeLa cells include a non negligible (43.7%) number of independently characterized dsDNA-binding proteins (Hudson & Ortlund, 2014). Taken together, these findings suggest that DRBPs are widespread, perhaps constituting 2% of the human proteome. Undoubtedly, this number would increase if other cell types, stress signals and other types of DNA or RNA are included (Hudson & Ortlund, 2014).

DRBPs could constitute a functional advantage to the cell in terms of energy consumption since using one DRBP rather than two independent DNA-binding and RNA-binding proteins is more efficient. In addition, by binding to both mRNAs and their encoding promoters, DRBPs can exert a powerful, amplified effect on gene expression and in orchestrating cellular responses, as they can produce rapid effects on protein synthesis and impart long-acting changes by acting on transcription.

1.5.1. General overview on DRBPs

Based on reported *in vitro* protein-DNA interactions that were afterwards confirmed *in vivo*, Hudson and Ortlund generated a detailed list of of 149 human DRBPs, with comments on their nucleic acid-binding properties, structures and functions. They carried out Gene Ontology analysis to shed some light on the biological processes of those proteins, revealing the expected transcriptional regulation, mRNA processing and DNA replication and also some intriguing results as exemplified by the DNA-damage response, apoptosis and responses to extreme temperatures (Hudson & Ortlund, 2014).

They have distinguished three classes of transcription factors that control different cellular functions by a constitutive DNA- and RNA-binding capacity. The first group is composed for those transcription factors whose DNA-binding activity is inhibited by a decoy RNA, as is the case of nuclear factor-Y (NF-Y), which binds the lncRNA P21-associated ncRNA DNA-damage activated (*PANDA*) and NF-κB, which binds the mouse pseudogene-derived RNA *Lethe*. This is also the case of an elaborate mechanism of an analogous bacterial system: the sequestration of ribosomal RNA small subunit methyl-transferase E (RsmE) by the non-coding RNA *RsmZ*. The competition between DNA and RNA for protein binding has also been shown for nucleic acid-modifying enzymes, such as DNA methyltransferases (DNMT3A and DNMT1) and metabolic enzymes (lactate dehydrogenase, glyeraldehyde-3-phosphate dehydrogenase GAPDH and α-enolase ENO1) (Hudson & Ortlund, 2014).

Another group of DRBP can be defined with others factors that bind both the DNA and the mRNA of their target genes, thus, showing a powerful, combinatorial control over protein expression that might generate both immediate effects (through regulating RNA turnover and translation) and long-lasting effects (through regulating transcription). One protein fulfilling this is the glucocorticoid receptor GCR that can promote the transcription of anti-inflammatory genes and repress the transcription of pro-inflammatory genes. In addition, GCR binds and destabilizes the mRNA of pro-inflammatory genes such as the chemokine (C-C motif) ligand 2 (*CCL2*). Another example is the NF of activated T cells 90 kDa, NF90, that up-regulates the mRNA levels of interleukin-2 (*IL-2*) by both binding its promoter to activate its transcription and by stabilizing the *IL-2* mRNA through direct binding to its 3′UTR (Hudson & Ortlund, 2014).

Finally, some DRBPs bind RNA and DNA simultaneously to perform a single function, as it is the case of the SOX2 that requires the lncRNA rhabdomyosarcoma 2-associated transcript (*RMST*) for binding to neurogenic gene promoters and to up-regulate them having an important role in development, pluripotency and cell fate. Dual nucleic acid recognition also facilitates targeted gene repression through RNA-guided DNA methylation as shown in mice by formation of a complex of DNMT3A with *Tsix* RNA to promote methylation of the X-inactive-specific transcript (*Xist*) promoter (Hudson & Ortlund, 2014).

Although grouped in terms by the way they function, the structures underlying DRBP activities are linked to their evolution and are difficult to put together, in particular because few structural data is available for the majority of DRBPs in complex with their DNA and/or RNA target. Some DRBPs contain ancient domains that have long bound DNA or RNA; others contain multiple domains that separately confer DNA- and RNA-binding abilities and mediate their functional roles (see Supplementary Data of Hudson & Ortlund, 2014)(Hudson & Ortlund, 2014).

1.5.2. The curious case of p53

p53 is better known as a DNA-binding protein. p53 controls transcription of promotores harbouring a specific DNA sequence containing palindromic decamers showing a general arrangement of 5’ RRRCWWGYYY 3’ (where R = A, G; W = A, T; Y = C, T) separated by 1 to 13 bp. Each of these pair of decamers is recognized by a fully p53 tetramer (Joerger & Fersht, 2008). The affinity of p53 to the response elements (REs) present in different target genes depends mostly on the sequence. Although there is not a clear correlation between promoter affinity and target function, people have described a tendency in which cell cycle-related promoters are bound by p53 with high affinity whereas some of those involved in apoptosis induction showed lower affinity values (Weinberg et al, 2005). Interestingly, p53 also has an RNA-binding property. Even though this capacity is known for a relatively long time, less effort was done to characterize it

properly and to associate it with particular physiological outcomes as compared to the DNA-binding capacity and its implications.

p53 was first described as an RNA-binding protein in 1993 by Oberosler et al. In this work they first confirmed that wild-type p53 efficiently inhibited the T antigen DNA helicase activity and showed that purified p53 also inhibited the activity of DNA helicase II of *Escherichia coli*, as well as the RNA helicase activity of T antigen and the human p68. In all cases, a heterologous protein-protein interaction was ruled out (Oberosler et al, 1993). Secondly, they presented data showing that wild-type p53 actually binds RNA *in vitro*, whereas the Val143Ala mutant does not. Interestingly, this interaction can be competed with total RNA from HeLa cells, ssDNA and dsDNA, being the former the most effective, suggesting that p53's affinity for RNA is higher than for DNA (Oberosler et al, 1993). Finally, they also showed that p53 catalyses the annealing of complementary single-stranded nucleic acids (Oberosler et al, 1993). However, the specificity of all these activities was not addressed and, therefore, it remains difficult to integrate these results into the full p53 function and its related responses.

The first association between binding of p53 to an RNA and control of its translation was presented in 1995 by Mosner et al, when they described an auto-regulatory control of p53 expression by a negative feed-back loop in gamma-irradiated mouse fibroblasts. In this work, they were able to detect *in vitro*-synthesized p53 protein bound to *in vitro*-synthesized mRNA containing the 5'UTR of *p53*, and no interaction was detected when the mRNA produced was restricted to the p53 coding sequence. Interestingly, both p53wt and p53MethA mutant bound similarly to the mRNA (although the MethA mutation used is not specified) (Mosner et al, 1995). The binding described above was correlated with a decrease of protein synthesis on *in vitro* translation experiments where the *p53* mRNA and recombinant p53wt protein were used. However, purified p53MethA did not give any change in p53 production, suggesting that binding is necessary but not enough to give the effect (Mosner et al, 1995). The authors described this observation as a control step whereby p53 might maintain its own abundance at low concentrations in normal conditions. On the other hand, after DNA damage, p53 translocates into the cell nucleus resulting in a decrease of cytosolic p53 and a release of *p53* mRNA from its auto-catalysed translational block and thereby, up-regulating the production of p53 protein within a very short timescale (Mosner et al, 1995).

Similarly, Miller et al reported that down-regulation of *cdk-4* translation after TGF-β treatment in mink lung epithelial cells was dependent on the presence of p53wt (and not mutant p53) and the 5'UTR of *cdk-4* mRNA. Moreover, they were also able to show that a N-terminal truncated form of p53 lacking the first 39 aa (later on shown to be p53ΔN40, see section p53 isoforms) was able to down-regulate *cdk-4* mRNA translation on a 5'UTR-dependent manner. In addition, by using different deletion mutants they also excluded the possibility of a passive effect on translation by the simply binding of p53 to the mRNA. Finally, it is noteworthy to point out that although the authors did not probe a direct interaction between p53 protein and *cdk-4* mRNA, the data presented suggest that as a plausible explanation (Miller et al, 2000).

In line with these studies, Galy et al first showed that translation of *fgf-2* mRNA was also repressed by p53 and via its 5'UTR in SK-Hep-1 cells. They also showed that cancer hotspots mutants of p53 (Arg 175, 248 243 and Ala 143) were unable to induce such decrease on their *fgf-2*'s 5'UTR-containing reporter construct. This work also showed that mutations on the TAD of p53 (Gln22 and Ser23) did not exhibit any inhibitory effect when co-expressed with the *fgf-2* reporter gene (Galy et al, 2001b). Later on, the same group showed that down-regulation of FGF-2 expression is due to the binding of p53 protein to several regions of the 5'UTR of *fgf-2* mRNA. In addition, they showed that binding itself was not enough to give the effect, and that a

conformational change of the mRNA mediated by the nucleic acid annealing activity of p53 was suggested to be required to generate the translation blockade (Galy et al, 2001a).

On the other hand, binding of p53 protein to mRNAs was also described to stabilize the involved mRNA, and therefore, to induce the expression of the encoded protein. This is the case of plasminogen activator inhibitor *pai-1* mRNA. By using transfection of p53 into H1299 cells or down-regulation of endogenous p53 by siRNA in non-malignant human bronchial epithelial Beas2B cells, Shetty et al showed that C-terminal-dependent (residues 296 to 393) binding of p53 to a 70-nucleotide region of the *pai-1* mRNA leads to the stabilization of the mRNA. This effect was functionally linked to the TSE (tobaco smoke extract)-induced apoptosis of lung epithelial cells. Importantly, induction of transcription or enhancement of protein synthesis were ruled out by the authors (Shetty et al, 2008).

In addition to the role of p53 as a post-transcriptional regulator of the expression of a particular set of proteins, p53-RNA interaction was found to have another function: that of controlling the oligomerization of p53, and therefore, its capacity to interact with DNA. Indeed, Yoshida et al showed *in vitro* and *in cellulo* that the C-terminal domain of p53 binds RNA and prevents p53 oligomerization, while on the other hand, treatment of samples with RNaseA resulted in formation of more p53 complexes (Yoshida et al, 2004). The sequence-independent p53-RNA interaction was shown to control the recognition of p53's REs by p53 *in vitro*, since a p53 consensus oligonucleotide was able to compete with RNA for interaction with p53 protein while the consensus oligonucleotides of CRE or NF-κB were not (Yoshida et al, 2004). Interestingly, p53-RNA interaction was also found to be controlled by phosphorylation at Ser392 (but not by phosphorylation in other C-terminal positions)(Yoshida et al, 2004), a modification that was previously showed to enhance wild-type p53 binding to a consensus DNA sequence in *in vitro* studies (Hupp et al, 1992).

Despite all the data pointing out that p53-RNA interaction is specific, an unbiased and, as the authors say, "serendipitous" study based on yeast three-hybrid assay, suggests that binding of p53 to RNA is mostly (but not all) non-specific (Riley et al, 2006). Indeed, they used a library of 60 nt random RNAs and selected by yeast three-hybrid the four presenting the strongest binding to p53 protein. Their predicted structure presented some similarities but also profound differences. They also mutated the most effective RNA binder to abrogate the interaction with p53, and although in the yeast three-hybrid assay the p53 protein was able to distinguish the two RNAs, p53 bound equally to those RNA when analyzed by EMSA. These data confirm that p53 binds RNA, but suggest that the RNA specificity of p53 recognition in the yeast nucleus is not retained under the tested *in vitro* conditions (Riley et al, 2006). These *in vitro* and *in vivo* data could be reconciled if p53 was actually a non-specific RNA-binding protein, and if RNAs selected for p53 interaction in the yeast three-hybrid system were simply better displayed on the surface of the MS2 coat protein, an hypothesis that was supported by the data obtained when they compared the pattern of p53-RNA affinity to that of an artificial tetrameric form of the sequence nonspecific RNA-binding HIV-1 nucleocapsid protein (HIV-1 NC) (Riley et al, 2006; Riley & Maher, 2007; Riley et al, 2007). When they followed up the previous study and looked for p53 RNA partners in p53-positive and p53-negative cell lines by CLIP, they astonishingly found no difference in the pattern of precipitated RNA. In addition, by using antibodies against unrelated p53 proteins, they found a non-specific ribonucleoprotein that immunoprecipitated in all the cases giving false p53-target RNAs. This observation was taken as support to their previous idea that p53 binding to RNA is non-specific and that post-translational modifications occurring in cells may impair binding of p53 towards RNA (Riley & James Maher, 2007; Riley & Maher, 2007). However, this result may also be pointing out a technical issue that could impinge on proper interpretation.

According to the information given above, p53 can be classified into a fourth category according to Hudson & Ortlund, 2014 ; a protein that binds both DNA and RNA and it shows a powerful, combinatorial control over protein expression that might generate both immediate and long-lasting effect. Even though binding of p53 to the DNA and mRNA of the same gene has not yet been described, its capacity to control transcription of some genes and translation of others might constitute a very powerful tool to coordinate a broader cellular response to a concise insult. However, and although some studies have analyzed the capacity of p53 to bind RNA and control some steps of their metabolism and were able to show that the interaction does exist, p53-RNA interaction has not been studied as well as the p53-DNA counterpart. Thus, despite all the work done, we still do not completely understand its functions and, most importantly, its physiological relevance in normal and pathological scenarios remains obscure.

2. mRNA translation

2.1. Mechanistic concepts

The mRNA translation in eukaryotic cells is a very intricate and fascinating process whose final end is the *de novo* synthesis of proteins. It is regulated at different steps and provides to the cell the plasticity that is needed to respond to rapid changes in the environment as in several cellular stresses such as heat shock, hypoxia, nutrient deprivation and ER stress and its deregulation is the basis of several diseases. The importance of translation regulation is highlighted by the lack of correlation between the mRNA and protein levels of numerous genes, according to several studies using comparative genomic and proteomic profiling (Holcik & Sonenberg, 2005). For practical reasons, the continuos translation process is divided in three different stages: initiation, elongation and termination.

2.1.1. Initiation

Translation initiation is the process of assembly of elongation-competent 80S ribosomes in which the initiation codon is base-paired with the anticodon loop of initiator tRNA (Met-tRNA^{Meti}) in the ribosomal P-site. It comprises two steps: the formation of 48S initiation complexes with established codon-anticodon base-pairing in the P-site of the 40S ribosomal subunit and the joining of 48S complexes with 60S subunits. It begins with the interaction of the cap-binding protein complex or eukaryotic initiation factor-4F (eIF4F) with the mRNA 5′-end cap structure (m^{7}GpppN; where N is any nucleotide). eIF4F comprises three subunits: eIF4E, which is the cap-binding protein; eIF4A, which is an RNA helicase; and the scaffolding protein eIF4G that bridges the mRNA and the ribosome through eIF3. eIF3 and eIF1A bind to the 40S ribosome subunit that is further bound by the ternary complex consisting of eIF2, initiator Met-tRNA^{Meti} and GTP and together form a 43S pre-initiation complex. Once the 40S ribosomal subunit is bound to the mRNA, it is thought to scan the mRNA in the 5′→3′ direction until it locates an initiation codon (most often AUG) in a favourable sequence context (GCC(A/G)CCAUGG, with a purine at the –3 and a G at the +4 positions, relative to the A of the AUG codon, which is designated +1) establishing the 48S complex. At this point, eIF5 and eIF5B promote the hydrolysis of eIF2-bound GTP, the displacement of eIFs and the joining of a 60S subunit to form an 80S initiation complex when the polypeptide-elongation step of translation commences (Holcik & Sonenberg, 2005; Jackson et al, 2010).

In addition to the scanning mechanism, initiation on a sizeable proportion of cellular mRNAs is mediated by internal ribosome entry sites or IRESs and constitutes a cap-independent translational mechanism. The IRES directly recruits ribosomes, thereby bypassing the requirement for the mRNA 5'-cap structure and eIF4E. These structures were first identified in picornaviruses and later on in cellular mRNAs, mostly in their 5' structured UTR but also in the coding sequence. However, there are no common discernible features that indicate the presence of an IRES and the detection of IRESs remains largely empirical. IRES-mediated translation is independent of several canonical initiation factors and instead, it relies on auxiliary cellular proteins that are known as IRES *trans*-acting factors, ITAFs. Therefore, it provides a means for escaping the global decline in protein synthesis and allows the selective translation of specific mRNAs in conditions of cellular stress (Holcik & Sonenberg, 2005). A summary depicting the main events during initiation of translation is presented in Figure 5.

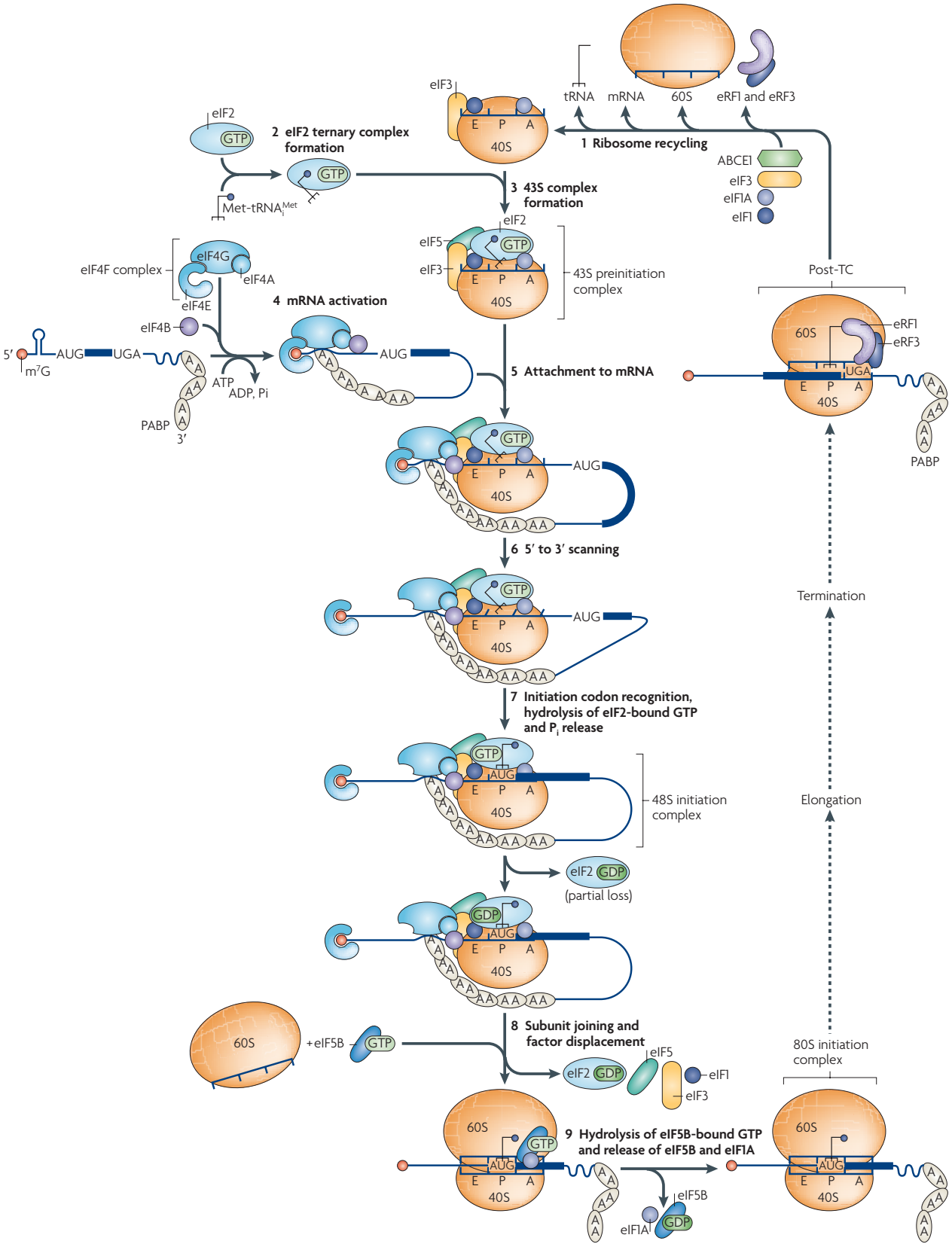

Figure 5. Canonical process of initiation of translation in eukaryotes.
The different stages constituting the initiation of translation are shown along with the main actors involved and principal accessory factors. Taken from Jackson et al, 2010.

2.1.2. Elongation

Once translation is initiated, the fully competent 80S ribosome is poised on the mRNA establishing base-paired interactions between the Met-tRNA^{Meti} anticodon and the start codon of the mRNA in the P-site. In eukaryotes, the elongation factor eEF1A binds amino-acyl-tRNA in a GTP-dependent fashion forming a ternary complex and guides it to the A-site on the ribosome, facilitating the codon recognition by the tRNA. This triggers GTP hydrolysis by eEF1A, release of eEF1A-GDP and accommodation of the aminoacyl-tRNA into the A-site. At this point, peptide bond formation occurs rapidly at the peptidyl transferase center (PTC) between the peptide hold by the peptidyl-tRNA located at the P-site and aminoacyl-tRNA at the A-site and this is accompanied by transition of the A- and P-site tRNAs into hybrid states with the acceptor ends of the tRNAs moving to the P- and E-sites, respectively. Binding of eEF2-GTP promotes translocation of the tRNAs into the canonical P- and E-sites and is followed by release of eEF2-GDP. In the post-translocation state of the ribosome, a deacylated tRNA occupies the E-site and is later on released, the peptidyl-tRNA is in the P-site and the A-site is vacant and available for binding of the next eEF1A-GTP-aminoacyl-tRNA ternary complex. These basic mechanisms of translation elongation and peptide bond formation are conserved between bacteria and eukaryotes, though additional factors and unique features were described in eukaryotes (Dever & Green, 2012).

2.1.3. Termination

The above-described cycle of protein elongation is repeated until a stop codon enters into the A-site. Eukaryotic termination relies on the high-fidelity stop codon recognition and peptidyl-tRNA hydrolysis by release factor eRF1 acting in collaboration with the translational GTPase eRF3. On recognition of a stop codon, the eRF1-eRF3-GTP ternary complex binds to the A-site of the ribosome in a pre-accommodated state, GTP hydrolysis occurs and eRF3 is released, leading to the deposition of the M domain of eRF1 in the PTC inducing peptide release, followed by dissociation of the translation machinery and engagement of their components into the downstream events of recycling (Dever & Green, 2012).

2.2. Translation regulation

Although all three phases are subjected to regulatory mechanisms, under most circumstances the rate-limiting step in translation is regulated: The initiation.

2.2.1. Initiation

Mechanisms that regulate initiation fall into two broad categories: those that impact on the eIFs (or ribosomes), and therefore affect virtually all scanning-dependent initiation events and those that impact on the mRNA itself, either through sequence-specific RNA-binding proteins or microRNAs (miRNAs) and are therefore, potentially selective for certain mRNAs.

Decline of global translation during stress relies mostly, but not only, on phosphorylation of Ser51 of eIF2α by four distinct protein kinases in response to different stresses: haem-regulated inhibitor kinase (HRI, low haem or treatment with arsenite, osmotic or heat shock), protein kinase RNA (PKR, double-stranded RNA, viral infection), PKR-like endoplasmic reticulum (ER) kinase (PERK, ER stress) and general control non-derepressible-2 (GCN2, amino-acid starvation and UV irradiation). Phosphorylation of eIF2α does not impinge on eIF2's capacity to form competent eIF2–Met-tRNA^{Meti}–GTP ternary complex, but instead, it blocks the rate-limiting step of GDP-GTP exchange of eIF2 catalyzed by eIF2B. Phosphorylated eIF2–GDP tightly binds to and sequesters the guanine nucleotide-exchange factor eIF2B, abrogating its activity. eIF2-ternary complexes

levels consequently fall and most mRNA translation is reduced (Holcik & Sonenberg, 2005; Jackson et al, 2010).

Another eIF than can be controlled by phosphorylation, but this time indirectly, is the CAP-binding protein eIF4E by the activity of 4E-binding protein, 4E-BP. When hypophosphorylated, 4E-BP binds eIF4E and prevents the eIF4E from associating with eIF4G, but phosphorylation of the 4E-BP on multiple sites, mainly by mTOR, releases eIF4E for assimilation into eIF4F and for initiating translation (Holcik & Sonenberg, 2005; Jackson et al, 2010). Although eIF4E itself is also subject to phosphorylation (on Ser209) by MAP kinase interacting Ser/Thr kinase 1 (MNK1) and MNK2 on a eIF4G-dependent manner and it appears to fluctuate in parallel with changes in translation efficiency, it has been suggested that its phosphorylation–dephosphorylation cycles are not essential for translation. However, it appears that excessive eIF4E phosphorylation can promote malignancy, as shown by injection of haematopoietic stem cells stably expressing Myc into irradiated mice (Jackson et al, 2010).

Several other eIFs have been reported to be phosphorylated, such as eIF1, eIF2β, eIF2Bε, several eIF3 subunits, eIF4G, eIF4B, eIF4H, eIF5 and eIF5B. Despite the phosphorylation rate increases in many cases under conditions of activated translation (for example following serum addition to quiescent cells), no solid evidence that any of these phosphorylation events are the cause of such activation was reported so far, rendering these observations “merely” correlations (Jackson et al, 2010).

In addition to post-translational modification of translation factors to control the general translation process, initiation can be modified by binding of proteins to a particular mRNA, and this, in general, results in protein synthesis inhibition. Activation of translation of such mRNAs, therefore, requires removal of the inhibitory protein effect through different mechanisms. The most clear example is that of ferritin mRNA, whose translation is strongly inhibited by iron regulatory proteins (IRPs)–RNA interaction occurring at a cap-proximal location, which prevents loading of the 43S complex onto the mRNA but not eIF4F binding to the capped 5′ end (Muckenthaler et al, 1998). Interestingly, the mRNA translation control exerted by direct binding of p53 to *fgf-2*, *cdk-4* and its own mRNA reported before might be included in this group. Unfortunately, the molecular mechanisms involved in such repression remain largely unknown (Galy et al, 2001a; Galy et al, 2001b; Miller et al, 2000; Mosner et al, 1995).

Control of translation initiation by 3′ UTR–protein interactions is also known. Many examples fit into a generic model in which a sequence-specific binding protein interacts with the 3’UTR of the mRNA that result in an inhibitory closed loop involving two other proteins. The first of them is in some cases the canonical eIF4E1a, while the bridging protein is the eIF4E transporter 4E-T or EIF4ENIF1, an eIF4E-interacting protein that is charged of transporting eIF4E from the nucleus to the cytoplasm, where it also has other roles. These proteins have functional homologues in vertebrates and in *Drosophila* and this type of regulation is particularly important during development of both group of organisms (Jackson et al, 2010). Opposite, binding of poly(A)-binding protein, PABP, to the A-rich tail of of polyadenylated mRNAs might be seen as an activator of mRNA translation and constitutes a translational advantage over non-polyadenylated ones, particularly under conditions of strong competition for limiting eIFs and/or ribosomes. This suggests that the PABP–poly(A) effect is stimulatory rather than essential, as first thought (Jackson et al, 2010).

Finally, the 3’ UTR may be the target of miRNA repressors that can be acting in conjunction with sequence-specific RNA-binding proteins. The interaction typically involves perfect contiguous base pairing of miRNA seed residues followed by mismatch bulges in either the miRNA or mRNA (or both), and then irregular base pairing of the miRNA 3′ end to the mRNA. miRNAs, therefore,

act as adaptors that confer sequence-specific mRNA binding to Argonaute, AGO (a family of proteins that are characterized by the presence of two homology domains: PAZ and PIWI and that are essential for diverse RNA silencing pathways) that further recruit several effectors. The actual mechanism of translation inhibition remains controversial, though it seems to have two components: a true repression of mRNA translation and an accelerated rate of mRNA degradation through the normal deadenylation-dependent pathway (Jackson et al, 2010).

2.2.2. Elongation

It has been shown that a conserved His residue of eEF2 (His 715 and 699 in mammalian and yeast eEF2, respectively) is post-translationally modified to diphthamide. Diphthamide modification is conserved in eukaryotes and archaea and although it seems it is not essential for cell viability in yeast and CHO cells, knockout mice lacking the enzymes involved in its generation (DPH1(Ovca1), DPH3, or DPH4) were either embryonic lethal or showed severe developmental defects, perhaps suggesting a critical role of it at a specific time during development. Interestingly, the only known function of diphthamide is to serve as a site of ADP-ribosylation by diphtheria toxin and related toxins leading to eEF2 inactivation and translation blockade. Thus, it is presumed that the diphthamide modification somehow enhances eEF2 function (Dever & Green, 2012). Indeed, yeast mutants of eEF2 showed that substitutions at His699 block or impair yeast cell growth that was related to reduce translation, an observation further supported by an increased sensitivity to translational inhibitors and resistance to diphtheria toxin (Dever & Green, 2012).

In addition, eEF2 was also shown to be phosphorylated by a Ca^{2+}-activated protein kinase eEF2K that leads to block of total protein synthesis by impairing eEF2 binding to the ribosome. Interestingly, hyper-activation of eEF2K was seen in tumor cells that are faced to nutrient-depleted environments and confers cell survival by blocking translation elongation. This depends on AMP-kinase (AMPK)-dependent activation of eEF2K when cellular AMP:ATP or ADP:ATP ratios increase. eEF2K is known to be inhibited by mTORC1 and Ras-Erk-p90RSK pathways and was suggested as a valid target for anti-cancer treatment (Leprivier et al, 2013).

2.2.3. Termination

To end, the termination of translation is also the target of regulation and is mostly mediated by stop codon read-through, or nonsense suppression. This occurs when a ribosome positioned with a nonsense codon (stop codon) in its A-site incorporates an amino acid into the nascent polypeptide chain instead of terminating translation. This depends on the competition of eRF1 and near-cognate aminoacyl-tRNAs (either natural or mutated cellular tRNAs) to recognize that codon. Normal cellular tRNAs that allow to read a stop codon as a sense one are called natural suppressors. In addition, shifting on the reading frame might lead to misreading of a stop codon as a sense codon (Beier & Grimm, 2001). The action of natural suppressor tRNAs was shown to depend on a suitable nucleotide context particularly down-stream of the suppressed codon. This context may consist of a diverse of sequence and structure features. This is the case of selenocysteine (Sec) insertion into proteins, where recoding of an UGA stop codon is associated with a selenocysteine insertion sequence (SECIS): a stem-loop structure located immediately downstream of the in-frame UGA codon at which Sec is incorporated. In addition, particular associated factors are involved in Sec incorporation, named in eukaryotes SECIS binding protein 2 (SBP2) and the Sec-$tRNA^{[Ser]Sec}$-specific elongation factor, EFsec (Zavacki et al, 2003).

Translational read-through also provides a regulatory mechanism of gene expression by permitting the differential production of more than one polypeptide from a single gene, a

mechanism exploded by some RNA virus to expand the genetic information of their relatively small genome (Beier & Grimm, 2001).

3. Endoplasmic Reticulum stress and the Unfolded Protein Response (UPR)

The Endoplasmic Reticulum (ER) is the main sub-cellular compartment involved in protein folding and maturation, where around one-third of the total proteome is synthesized. The folding capacity of the ER highly differs depending on the tissue; while high protein-producing cells count with a very developed ER, as it is the case of secretory specialized plasmocytes, salivary glands and pancreatic β cells, other tissues non-specialized in protein production present less activity at the ER. The folding of proteins in the ER and their assembly into larger heteromeric complexes is guided by the same principles and processes used throughout the cell, but it is further complicated by the addition of large branched oligosaccharide moieties to nascent chains entering the ER, high ER concentrations of calcium, and an oxidizing environment combined with systems that catalyze the formation of intra- and inter-chain disulfide bonds (Behnke et al, 2015). When the folding capacity of the ER is reached, an imbalance between cellular demand for ER function and its capacity is stablished and the cells experience the so-called ER stress. There are several physiological and pathological conditions affecting protein folding and/or calcium homeostasis that can trigger ER stress, namely: glucose starvation, underglycosylation of glycoproteins, calcium flux across the ER membrane, elevated protein synthesis and secretion, and failure of protein folding, transport or degradation (Hetz et al, 2013; Zhao & Ackerman, 2006). ER stress might also be induced chemically and it serves as a tool in research laboratories. Some of those compounds are DTT, tunicamycin, thapsigargin, among others. Of particular interest in the context of this thesis is thapsigargin, which inhibits the sarcoplasmic/endoplasmic reticulum calcium ATPase pump, thereby preventing normal Ca^{2+} uptake into the ER from the cytosol and causing depletion of releasable ER Ca^{2+} by passive leak. Over time, this leads to induction of the UPR. A particularly interesting ER stress inducer is the aggregation or accumulation of misfolded proteins inside the ER and is usually reached when the cellular protein quality control mechanisms are overwhelmed.

3.1. Protein quality control mechanisms

Synthesis of proteins, particularly those secreted or with membrane destination, is tightly associated to quality control mechanisms. These proteins are first synthesized in ER-associated ribosomes and later on transported into the ER via the Sec61 translocon and then along the secretory pathway. Nascent and unfolded proteins are unstable because they tend to aggregate one to each other through inespecific and hydrophobic interaction involving their hydrophobic regions. And even once they achieve the mature status, they are exposed to different cellular stresses resulting in denaturation. Thus, the cell has evolved a comprehensive maintenance system for the crowded ambiance found inside the ER called ER protein quality control or ERQC, which is conserved in most eukaryotic organisms (Behnke et al, 2015; Morito & Nagata, 2015).

The ERQC posses three axes. The first is constituted by the protein folding machinery itself formed by molecular chaperons and folding enzymes. While being synthesized and folded, proteins are covalently modified with disulfide bonds and oligosaccharide chains to reinforce their structure. This group of enzymes includes for example, the classic heat shock protein 70 family, including BiP, that mediates folding, the oxidoreductases that form and isomerize disulfide bonds such as PDI and ERO1 and the lectin-like chaperones such as calnexin and calreticulin that recognize oligosaccharide chains attached to glycoproteins and promote their folding. The second axis is the Unfolded Protein Response or UPR, a three-branched pathway that aims at restoring the balance between newly-synthesized and misfolded proteins or at inducing apoptosis if the stress is too severe. The UPR initiates downstream events including translation attenuation and

up-regulation of folding enzymes and ER-associated degradation of proteins (ERAD) components, the third axis of the ERQC (Morito & Nagata, 2015). The UPR will be discussed in detail below.

The ERAD is a clearance system for misfolded, misassembled or metabolically regulated proteins in the ER that are selectively translocated to the cytosol via specific transporters and degraded by the ubiquitin-proteasome mechanism (UPS). The ERAD can be divided in three steps: recognition, dislocation and degradation. One way ERAD recognises its clients is via de N-glycosylation, a feature of most new proteins at the ER. Once glycosylated and first trimmed off resulting in mono-glucose forms, the N-glycans are recognized by lectin-like chaperons for proper folding. When the N-glycan is further processed and it arrives to the final form, the protein might continue through secretion if it is properly folded, but if the expected folding was not achieved, glucoses might be added again to try a new round of folding mediated by calnexin and calreticulin. However, if on the other hand, the oligosaccharide chain is further modified by mannosidases, the protein becomes terminally misfolded and is recruited by specific lectins for the ERAD, being osteosarcoma 9 OS9 and BIP itself some of them, which will recruit the substrate to the membrane penetration machinery. Although the identity of specific actors is matter of debate and the exact mechanism that links protein misfolding and mannose trimming remains unclear, the hypothetical “timer model” that was briefly described above may explain it (Morito & Nagata, 2015).

The second step or dislocation is mediated by a putative penetration channel protein conventionally called the ERAD complex, with several potential candidates that establish direct contact with the substrate. Some of those core components of the machinery are Derlin-1, -2 and -3, HRD1 ubiquitin ligase and the translocation channel for newly synthesized proteins Sec61. During this phase, the client protein is, very likely, ubiquitylated on the cytosolic face of the ER and finally translocated and degraded by the proteasome, constituting the third and final step (Morito & Nagata, 2015). Some of of the above-mentioned factors are direct targets of the UPR mostly acting at the transcription and translation steps of gene expression.

3.2. The UPR

When cells face stress on the ER they respond through the Unfolding Protein Response, or UPR, an adaptive three-branched pathway that aims at restoring the balance between newly synthesized and mature proteins. However, if the stress is too severe to handle, the UPR engages the cell into a pro-death cell signalling pathway that ultimately leads to mitochondria-dependent apoptosis (Hetz et al, 2013; Urra et al, 2013; Zhao & Ackerman, 2006). Recently, the UPR has been shown to play important roles besides protein quality control and folding, as exemplified by many physiological processes including energetic regulations, lipid and cholesterol metabolism, or inflammation and/or cell differentiation (Manie et al, 2014).

In mammals, the canonical UPR pathway counts on three branches represented by three transmembrane proximal sensors: inositol-requiring enzyme 1α (IRE1α) and IRE1β; protein kinase RNA-like ER kinase (PERK); and activating transcription factor 6 (ATF6; both α and β isoforms). When activated, the three sensors will transduce signals to the cytosol and nucleus to, at first, restore protein folding capacity by clearing the ER trough down-regulation of general protein synthesis and induction of protein quality control mechanisms such as chaperones and components of the ERAD and to restore the cellular homeostasis via antioxidant responses, amino acid metabolism, autophagy, and organelle biogenesis, as summarised in Figure 6. However, as mentioned before, prolonged or irreversibly stress in-defectively leads to apoptosis (Hetz et al, 2013; Urra et al, 2013; Zhao & Ackerman, 2006).

Activation of PERK involves dimerization and trans-autophosphorylation followed by formation of large clusters (Bertolotti et al, 2000). Activated PERK phosphorylates the translation

initiation factor eIF2α on Ser51 resulting in general inhibition of cap-dependent translation as discussed in previous sections (Holcik & Sonenberg, 2005; Jackson et al, 2010). This has a very important pro-survival and rapid impact on the loading of the ER by lowering the synthesis of proteins that are not needed for ER recovery. On the other hand, this opens for the translation of mRNAs containing short open reading frames in their 5′UTRs called upstream open reading frames or uORFs, such as activating transcription factor 4 (ATF4) (Harding et al, 2000). The human ATF4 contains three uORFs (1, 2 and 3) that are located 5' to the ATF4 start codon. These uORFs are translated in non-stressed conditions because the cell counts with sufficient eIF2-GTP to reinitiate translation from the uORF3 that overlaps ATF4 ORF in an out-frame manner. However, during ER stress, decreased levels of functional eIF2 complex prolongs the duration of the ribosome scanning, therefore bypassing the uORF3, and permitting translation to re-initiate at the ATF4 ORF, finally inducing ATF4 levels (Vattem & Wek, 2004), a model quite difficult to integrate. Induction of ATF4 leads to up-regulation of genes related to REDOX proceses, amino acid metabolism, ER chaperones and foldases. In addition, ATF4 also induces the expression of genes related to apoptosis, for example the transcription factor C/EBP-homologous protein CHOP and growth arrest and DNA damage-inducible 34 GADD34 (Hetz et al, 2013; Urra et al, 2013). Another protein induced upon phosphorylation of eIF2α by PERK is the p53 isoform p53ΔN40 (Bourougaa et al, 2010) that was presented before and whose induction at the translation initiation is discussed in future sections.

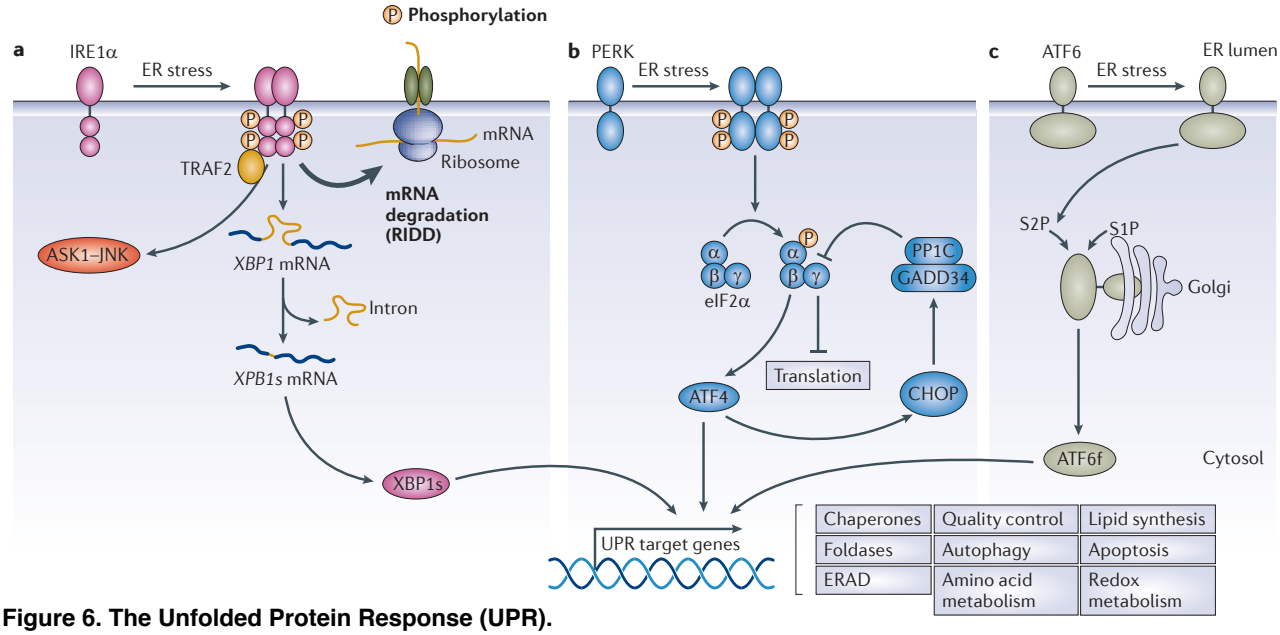

Figure 6. The Unfolded Protein Response (UPR).
When cells experiment ER stress they respond via the UPR, a cell signalling pathway that relies on the three proximal sensors IRE1, PERK and ATF6 to transduce signals from the ER lumen to the cellular cytoplasm and nucleus. All the actors shown in the figure are presented in the text along with their principal roles. Extracted from Hetz et al, 2013.

As a result of ER stress-induced oligomerization, trans-autophosphorylation and formation of high-molecular-mass complexes (Bertolotti et al, 2000), IRE1α describes a conformational change that activates its RNase domain which will splice out a 26-nucleotide intron from XBP1's mRNA via an unconventional mRNA splicing mechanism that shifts the ORF. Interestingly, the protein encoded by the unprocessed mRNA does not accumulate during the UPR whereas the one encoded by the processed *xbp1* mRNA, XBP1s, does (Calfon et al, 2002; Lee et al, 2002; Yoshida et al, 2001). XBP1s is an active transcription factor towards several genes involved in the UPR

response. In addition, IRE1α's RNase activity also degrades a subset of mRNAs through a process known as regulated IRE1-dependent decay (RIDD) of mRNA. The targets of such activity vary in different cell types and are mostly coding for proteins with a tendency to misfold and that are either secreted or located at the plasma membrane, showing they traffic through the ER (as shown by ER-targeting signal sequence dependency) but are not directly involved in ER function (Hetz et al, 2013; Hollien & Weissman, 2006). Accordingly, their coding mRNAs localize at the ER membrane (Hollien & Weissman, 2006). It was shown that RIDD targets harbour a consensus sequence (CUGCAG) accompanied by a stem-loop structure, according to an *in vitro* cleavage assay coupled with an exon microarray analysis (Oikawa et al, 2010).

The third actor, ATF6, translocates to the Golgi apparatus upon ER stress where it is processed by Site 1 and Site 2 proteases (S1P and S2P) to release the cytosolic N-terminal fragment ATF6f (Zhao & Ackerman, 2006). ATF6f is a transcription factor that contains a basic leucine zipper motif (bZIP) that migrates to the nucleus (Haze et al, 1999) and regulates the expression of genes with an ER stress response element (ERSE) in their promoters, such as those of the ERAD pathway, chaperones as BiP and PDI, and notably, XBP1 (Haze et al, 1999; Lee et al, 2002; Yoshida et al, 1998).

3.2.1. The protective arm of the UPR

The inhibition of protein translation by PERK represents a rapid protective action of the UPR because it decreases the load of nascent proteins arriving at the ER. In line with this, *Perk*$^{-/-}$ mouse embryonic fibroblasts, when challenged with ER stress-inducing agents, failed to block protein translation and exhibited increased cell death (Szegezdi et al, 2006). In addition, over-expression of non-phosphorylable form of eIF2α also generates hypersensitivity to ER stress highlighting the physiological role of translational attenuation in supporting cell survival (Urra et al, 2013). Also, the PERK-mediated inhibition of general cap-dependent translation also favours cell protection through ATF4 expression. ATF4 promotes cell survival by inducing genes involved in amino-acid metabolism, REDOX reactions, stress response and protein folding and secretion (Szegezdi et al, 2006).

The protective effect of IRE1 is mediated by the XBP-1-dependent expression of genes involved in protein folding, ERAD, protein translocation to the ER, and protein secretion. Also, the RIDD capacity of IRE1 to degrade a particular set of mRNAs coding for proteins with a tendency to misfold and that traffic through the ER but are not directly involved in ER function aids to alleviate the damage (Hetz et al, 2013).

ATF6 also contributes to cellular protection by regulating the transcription of genes involved in ER homeostasis, such as ER chaperones and ERAD components and biogenesis of ER and Golgi apparatus (Szegezdi et al, 2006; Urra et al, 2013).

In addition, autophagy has been described as a response to ER stress and has been described as a pro-survival mechanism by eliminating and degrading unfolded proteins and damaged organelles (Urra et al, 2013).

3.2.2. The killing face of the UPR

The molecular switches mediating the transition from adaptive to pro-apoptotic responses are not fully understood. However, some important actors have been identified and their roles have been suggested with certain confidentiality, as briefly depicted below. So far, the most accepted components mediating cell death under ER stress are down-stream of PERK and IRE1. The PERK arm of the UPR is involved in the up-regulation of CHOP (C/EBP homologous protein, also named growth arrest and DNA-damage-inducible 153, GADD153) activated downstream of ATF4. CHOP

seems to induce apoptosis by favoring translation recovery via GADD34, a component of the GADD34–PP1 eIF2α phosphatase which links to ROS production and that permits the synthesis of proteins needed to kill the cell (Han et al, 2013). Moreover, BCL-2 family induction (i.e. BIM, PUMA, etc.)(Puthalakath et al, 2007; Reimertz et al, 2003) and down-regulation (BCL-2) (McCullough et al, 2001) were reported upon CHOP activation. Of outstanding notice and although a large amount of data suggests that PERK and CHOP are crucial components of the ER stress-induced cell death pathway, PERK or CHOP-deficient cells still undergo apoptosis indicating the existence of other checkpoints and signaling events mediating this process (Urra et al, 2013; Zinszner et al, 1998).

Translation recovery is also mediated by the HSP40 family member $P58^{IPK}$, a down-stream factor of the IRE1-XBP1 axis of the UPR. $P58^{IPK}$ binds and inhibits PERK and it seems its induction only occurs several hours after phosphorylation of eIF2α. IRE1 also contributes to apoptosis in a more direct way. It has been shown that it recruits the adaptor molecule TNF-receptor-associated factor 2 (TRAF2). The IRE1–TRAF2 complex formed during ER stress can recruit the apoptosis-signal-regulating kinase (ASK1) that has been shown to relay various stress signals to the downstream effectors, most notably the pro-apoptotic c-Jun N-terminal kinase (JNK) that will impinge in the activity of both pro- and anti-apoptotic members of the BCL-2 family (Szegezdi et al, 2006; Urra et al, 2013). Moreover, the previously described protective RIDD activity of IRE1 might also function as pro-apoptotic mechanism by degrading mRNAs encoding for crucial proteins including ER chaperons such as BiP (Urra et al, 2013). BiP has been widely implicated in apoptosis inhibition and since it represents a key player during the ER stress and UPR (and is a major player in this thesis) it deserves a more detailed description (see below).

Regarding ATF6, no reports have linked it directly to ER stress-induced apoptosis, therefore, it seems that ATF6-mediated signals are purely pro-survival and aim to counteract ER stress (Szegezdi et al, 2006). However, an association can be made by considering the pro-apoptotic activities of XBP1, whose transcription is induced by ATF6 (Haze et al, 1999; Lee et al, 2002).

3.3. Sensor, controller and effector of the UPR: The BiP chaperone

Binding immunoglobulin protein, BiP, also known as 78 kDa glucose-regulated protein (GRP78) or heat shock 70 kDa protein 5 (HSPA5, official name of the gene) is a stress-inducible molecular chaperone that belongs to the heat shock protein (HSP) family. Indeed, it shares 60% amino acid homology with HSP70 and represents its homologue in the ER counting for essential functions in the process, folding and assembly of a wide range of client proteins and in the export of misfolded proteins for degradation, thus, maintaining the structural integrity and homeostasis of the ER. BiP can actively fold their substrates (foldase) or simply bind and restrict a substrate from folding or aggregating (holdase) (Lee, 2014).

3.3.1. Structure and mechanism of action and function

BiP contains two functional domains; a nucleotide-binding (NBD) and a substrate-binding domain (SBD), responsible for binding and hydrolysis of ATP and binding to the substrates, respectively (Behnke et al, 2015; Lee, 2014). NBD consists of two large globular subdomains (I and II), each further divided into two small subdomains (A and B). The subdomains are separated by a cleft where the nucleotide, one Mg^{2+} and two K^{+} ions bind and connect all four domains (IA, IB, IIA, IIB) (Wisniewska et al, 2010). SBD is divided into two subdomains: SBDβ and SBDα. SBDβ serves as a binding pocket for client proteins or peptides and SBDα serves as a helical lid to cover the binding pocket (Behnke et al, 2015). An inter-domain linker connects NBD and SBD,

favoring formation of an NBD–SBD interface. A basic representation of BiP's primary structure is shown in Figure 7A.

The ability of BiP to bind and release unfolded protein substrates is tightly regulated by a cycle of ATP binding, hydrolysis, and nucleotide exchange, which is controlled by a number of co-factors. Some of those bind directly to unfolded proteins and transfer them to BiP before stimulating its ATPase activity and two others act as nucleotide exchange factors (NEFs) that trigger the release of bound substrates (Behnke et al, 2015). In the absence of stress, BiP has been shown to be mostly ADP-ribosylated and it is thought to be phosphorylated, representing a pool of inactive oligomeric protein. In the presence of unfolded proteins, the amount of modified BiP drops quickly and it becomes monomeric, reaching a reactivated state where it is able to bind to substrates. The activity of BiP is regulated by its allosteric ATPase cycle; when it binds ATP, the status is transmitted to the SBD and leads to an opening of the substrate binding cavity by increasing SBD flexibility and SBDα lid opening but with low affinity to substrate. Upon ATP hydrolysis, ADP is bound to the NBD and the lid adoptes the closed conformation with the bound substrate. This creates a low off rate for high-affinity substrate binding and protects the bound substrate from premature folding or aggregation. Exchange of ADP for ATP results in the opening of the SBDα lid and subsequent release of the substrate, which is then free to fold and makes BiP available for another round of client binding, a cycle that must function properly for substrate maturation in the ER (Behnke et al, 2015). This cycle is summarised in Figure 7B.

In addition, BiP plays a key role as a sensor and regulator (and of course, effector) of the UPR, since in resting conditions, it binds to and inactivates all three proximal sensors (that can also be described as transducers) of ER stress: PERK, IRE1 and ATF6 (Bertolotti et al, 2000; Shen et al, 2002). When misfolded proteins accumulate in the ER, they compete for BiP binding, thereby releasing the UPR sensors and leading to the fully activation of the UPR. This activity is further supported by the observation that the UPR can be spontaneously triggered with diverse physiological consequences when BiP is depleted or inactivated in different mammalian cell lines (Lee, 2014; Li et al, 2008) and by the finding that some yeast BiP-mutant strains exhibit impairment of BiP dissociation from IRE1 and its further activation (Kimata & Kohno, 2011). Despite this, the simple competition model is unlikely to be acting isolated and therefore, it has been speculated that ER stress sensors may have intrinsic ability to release BiP from themselves upon ER stress in response to other activating mechanisms, such as direct interaction with unfolded proteins in the lumen of the ER (Kimata & Kohno, 2011).

Moreover, BiP was related to apoptosis repression in several cell lines and in mice. Indeed, BiP knock-out is already lethal before the implantation step. These embryos do not hatch from the zona pellucida *in vitro*, fail to grow in culture, and exhibit proliferation defects and a massive increase in apoptosis in the inner cell mass (ICM) (Luo et al, 2006). BiP vs apoptosis is detailed in a future section.

3.3.2. Control of BiP expression

BiP is highly conserved among eukaryotes including mammals and it is also widely-expressed at basal levels among all tissue types in human, representing an ubiquitous chaperone. On the other hand, its induction is easily detected under ER stress conditions and it is widely used as an indicator for the onset of it in many works. Increased BiP expression in stress conditions is mainly due to its condition of UPR-target gene. Indeed, the promoter of BiP contains three ER stress response elements (ERSEs) that are located upstream of the TATA element (Yoshida et al, 1998). This element is directly regulated by ATF6f (Haze et al, 1999; Yoshida et al, 1998), which binds to the ERSE through binding to NFY, associates with the constitutively expressed multi-

functional transcription factor YY1 and increases its binding to the *bip* promoter. In addition, YY1-associated chromatin modifiers were also shown to be recruited to the *bip* promoter and pro-transcription histone modifications were detected (Baumeister et al, 2005). Also, XBP1 might contribute to *bip* transcription but it seems to be not obligatory since mouse embryonic fibroblasts that were devoid of XBP1 showed only a modest effect on ER stress-mediated induction of BiP (Lee et al, 2003). Finally, ER stress induction of BiP is also partly attributed to ERSE-independent pathways mediated by ATF4 via an activating site localized upstream of the ERSEs. Therefore, the PERK/eIF2α/ATF4 axis links attenuation of protein translation with transcriptional activation of BiP (Luo et al, 2003).

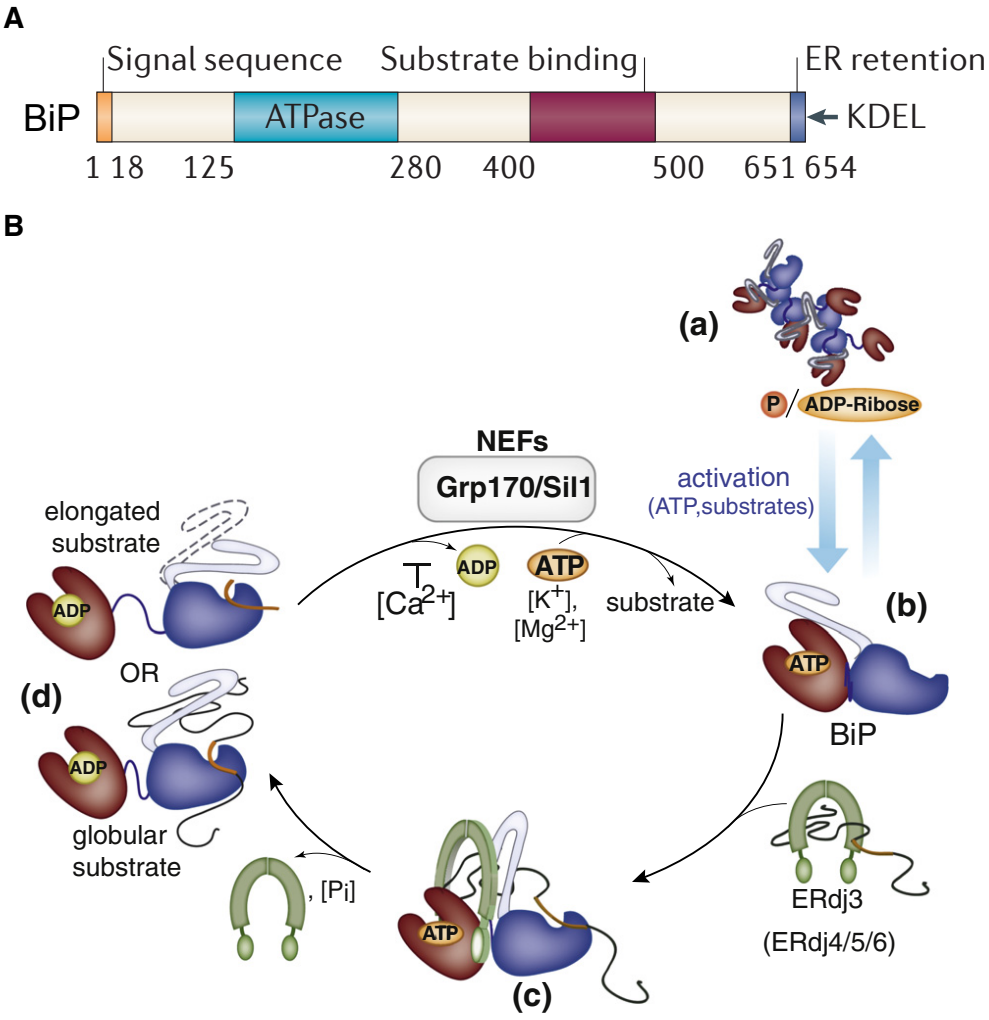

Figure 7. BiP primary structure and ATPase cycle.
A) The primary structure of BiP showing the localization of its main domains. Modified from Lee, 2014. **B)** The ATPase cycle of BiP at the ER. The oligomeric, phosphorylated and ADP-ribosylated BiP constitutes the inactive state (a). When BiP binds ATP (b), it becomes activated and binds substrates with low affinity, a step favoured by co-chaperones like ERdj3 (c). Interaction with the client protein increases BiP's ATPase activity inducing substrate locking described by low on and off rates. The SBD and NBD describe movements and the lid closes over the bound elongated or globular clients in a completely or incompletely fashion, respectively. At this point, ATP is exchanged for ADP (d) in order to release the substrate and make BiP available for a new round of substrate binding that is initiated with inclusion of new ATP molecule mediated by the Nuclear Exchange Factors (NEFs) GRP170 and Sil1. Taken from Behnke et al, 2015.

Additionally, the 5'UTR of the *bip* mRNA contains the first cellular described IRES, able to drive translation of the mRNAs containing this region in bi-cistronic constructs and in the context of

poliovirus-infected mammalian cells where the scaffolding eIF4G subunit of the eIF4F complex is cleaved by viral proteases (Macejak & Sarnow, 1991). The sequence mediating an efficient internal initiation was later on mapped to nucleotides spanning from 129 to 220 of the 5'UTR, i.e. the 3′ end of the *bip*'s 5′UTR, and was shown to establish complexes with a then-called p60 and p95 cellular proteins that we still do not know their identities (Yang & Sarnow, 1997). Later on, some other ITAFs were described to control the activity of the IRES on *bip* mRNA, such as polypyrimidine tract-binding protein PTB and autoantigen La proteins and NSAP1, a heterogeneous nuclear ribonucleoprotein (hnRNP) implicated in mRNA processing mechanisms (Cho et al, 2007).

Moreover, the 5'UTR of *bip* was recently shown to contain uORFs initiated at non-AUG codons suggesting that a non-canonical initiation mechanism may function. Indeed, Stack et al, developed an exquisite and sensitive detection system called tracing translation by T cells (3T) where presentation of tracer peptides by the MHC-I complex in the cell surface and recognition by corresponding T-cells leads to their activation and expression of a reporter gene driven by the promoter of IL-2 and detected by a colorimetric assay (Starck et al, 2016). By doing this, they described a –190 UUG Leu codon–initiated and a –61 Leu CUG-initiated uORFs, both of them expressed in normal and $NaAsO_2$-treated cells, the latter being an inducer of oxidative stress which rapidly triggers eIF2α phosphorylation by activated cytosolic HRI (Jackson et al, 2010; Starck et al, 2016). Expression of -190 uORF both in normal and stress scenarios was shown to be dependent on the alternative initiation factor eIF2A, a monomeric protein structurally and functionally distinct from the trimeric eIF2, that coordinates non-canonical Leu (CUG) initiation and expression of a CUG Leu–initiated phosphatase and tensin homolog deleted on chromosome 10 isoform, PTENα, but that is still able to mediate initiation at the AUG start codons. Interestingly, level of eIF2A was induced by subtilase cytotoxin (SubAB), a bacterial AB toxin that is endocytosed by cells and retro-transported to the ER lumen where it destroys BiP by proteolysis. Increase of eIF2A expression during ER stress favours the recruitment of eIF2A-related initiation complexes to the -190 uORF of BiP and could substitute for eIF2 during initiation at the BiP AUG start codon, then, mediating its expression when the canonical eIF2α-dependent initiation mechanism is compromised (Starck et al, 2016). Of outstanding notice, the proposed mode of action of BiP uORFs argues against the currently-accepted inhibitory effect of uORFs located in the 5'UTR of ATF4, a model that to me, results poor convincing.

Interestingly, the ORF of BiP also harbours regulatory elements able to control its own translation. It has been shown that mouse BiP inserted into the genome of HeLa cells and controlled by the tetracycline-sensitive (tet-off) expression system gradually displaced the human BiP by newly synthesized mouse BiP when the repression was removed, but importantly, total levels of BiP remained un-changed. This implies the cells count with a control mechanism maintaining BiP at a constant level in unstressed cells, highlighting the role of BiP as stress sensor in the UPR and the possibility that even subtle changes in BiP concentrations might have deleterious effects on the cells (Gulow et al, 2002). On the other hand, under tunicamycin-induced ER stress, the mRNA of human (endogenous) BiP increased while the exogenous mouse did not, but synthesis of both proteins was induced to a similar extent, suggesting that the translational restraint acting in unstressed cells is alleviated under UPR. Thus, up-regulation of BiP expression during UPR can be described as a two step process: (i) alleviation of the translational restraint present in unstressed cells resulting in increased translation efficiency of *bip* mRNA and (ii) increased transcription of *bip* from the well known classic UPR response. Importantly, it was also shown that translational response was faster than transcription in time-lapse experiments, suggesting that cells might adapt to small perturbations without inducing the transcriptional response (Gulow et al, 2002).

3.3.3. The dark side of BiP: proliferation inducer and apoptosis inhibitor

The protective role of BiP is easily understandable when considering its chaperone and repairing capacities in the ER. In addition, it has been suggested that it might help to maintain ER stress sensors and ER-associated pro-apoptotic machineries in their inactive state. Several mice models with either heterozygous or homozygous deletions of BiP and cell lines with reduced levels of BiP expression showed impaired tumor growth and proliferation rate, respectively, linking its expression with a protective and pro-survival activity. In addition, BiP overexpression is widely reported in cancer cell lines and is associated with aggressive growth and invasive properties (Lee, 2014).

BiP-dependent proliferative phenotype has been also associated to some of the its clients proteins in the ER, representing a variety of cell surface receptors and secretory proteins that are crucial for the ability of cancer cells to respond to extrinsic proliferative signals (Lee, 2014). In particular, an indirect role of BiP in favouring tumour growth was shown to be its part in WNT processing and stabilization in the ER, a feature that was lost under hypoxic conditions and that led to WNT proteasomal degradation and to reduced secretion, therefore affecting WNT–β-catenin proliferative signalling. Thus, the hipoxia would first act as a protective mechanism but would later be a selective pressure that kills the sensitive clonogens forcing the tumour to find new ways to proliferate, being one of them β-catenin mutation that renders it independent of WNT, leading to more aggressive clinical outcome (Verras et al, 2008).

A more direct and striking way for BiP to promote proliferation is by translocation into the cellular membrane, a feature seen in cancer cells and that was speculated to mediate several and diverse functions by partnering with several cell surface receptors (Gonzalez-Gronow et al, 2009). Localization of BiP at the cell surface was reported to be a preferential characteristic of tumor cells compared to their normal counterparts. For example, in prostate cancer cells, cell surface BiP functions as a receptor for the activated form of the plasma proteinase inhibitor α2-macroglobulin (α2M), thereby triggering ERK and AKT activation and promoting proliferation and inhibiting apoptosis (Misra & Pizzo, 2012). In addition, an interaction of BiP and PI3K was detected by co-immunoprecipitation and was correlated with increased phosphatidylinositol-(3,4,5)-trisphosphate PIP3 production and was shown to be dependent on the N-terminal region of BiP (Zhang et al, 2013). This was further supported by bi-allelic conditional knock-out mouse model of *Bip* and *Pten* (a plasma membrane lipid phosphatase that antagonizes the PI3K signalling pathway and major tumour suppressor gene) in the bone marrow. This showed that heterozygous knock-down of BiP in PTEN-null mice is sufficient to restore the hematopoietic stem cell population back to the normal percentage and suppress leukemic blast cell expansion. The effect was shown to depend on suppression of the PI3K/AKT/mTOR pathway by BiP knock-down in leukemia cell lines (Wey et al, 2012).

BiP was also reported to control apoptosis. In cultured cells challenged with topoisomerase II inhibitors, etoposide, doxorubicin, topoisomerase I inhibitor and camptothecin DNA-damaging drugs, specific overexpression of BiP resulted in reduced apoptosis, a phenomenon that was dependent on a putative interaction with caspase-7 and on BiP's ATP binding domain (Reddy et al, 2003). Inhibition of apoptosis by BiP was also related to complexes formation between BiP and caspase-7 and -12 at the ER membrane, that prevents release of caspase-12 from there (Rao et al, 2002). In line with this, BiP knock-out mice are not viable and die before implantation due to massive increase in apoptosis in the inner cell mass (ICM) (Luo et al, 2006). Also, several works carried out in cell lines demonstrate that BiP interferes with the interaction between the anti-apoptotic BCL-2 and the BH3-only pro-apoptotic BIK (see "Apoptosis" section and below).

Some estrogen-dependent human breast cancers cells show an essential requirement for estrogen for proliferation and survival. Indeed, estrogen starvation, or treatment with anti-estrogens drugs, trigger apoptosis. In human breast cancer cell line MCF-7/BUS treated with fulvestrant (anti-estrogen) or exposed to estrogen starvation, induction of apoptosis correlated with a strong induction of both BIK mRNA and protein. The BIK-dependent apoptosis triggered was blocked by pancaspase inhibitor zVAD-fmk and marginally changes were seen for other members of the BCL-2 family (Hur et al, 2004). An independent study looking for apoptosis-related BIK binders, found that BIP and BIK are able to interact at the ER membrane (Fu et al, 2007), a statement supported by the main localization of this pro-death factor (see future sections) (Germain et al, 2002; Mathai et al, 2002). Whereas BiP overexpression inhibits BIK and estrogen starvation–induced BAX activation and apoptosis of MCF-7/BUS cells, suppression of endogenous BiP by siRNA sensitizes them to the treatment-induced apoptosis (Fu et al, 2007). Moreover, a clonal human breast cancer cell line (MCF- 7/BUS-10) resistant to long-term estrogen deprivation exhibits elevated level of BiP, which protects the cells from estrogen starvation-induced apoptosis (Zhou et al, 2011). In these cells, BIK was shown to act cooperatively with NOXA to promote apoptosis that was suppressed by BiP overexpression. The molecular mechanism of this inhibition correlates with the BiP/BiK interaction that was shown not to depend on BIK's BH3 domain (but maps to a region preceding it), which had been implicated in some previous BIK apoptotic-related protein interactions. Furthermore, BiP was also shown to compete with BCL-2 for binding to BIK (this time involving BIK's BH3 domain), establishing that BCL-2/BIK and BiP/BIK complexes are mutually exclusive, and thus, offers an interesting model for BiP to promote the BCL-2-dependent inhibition of the apoptotic response, as shown in Figure 8 (Zhou et al, 2011).

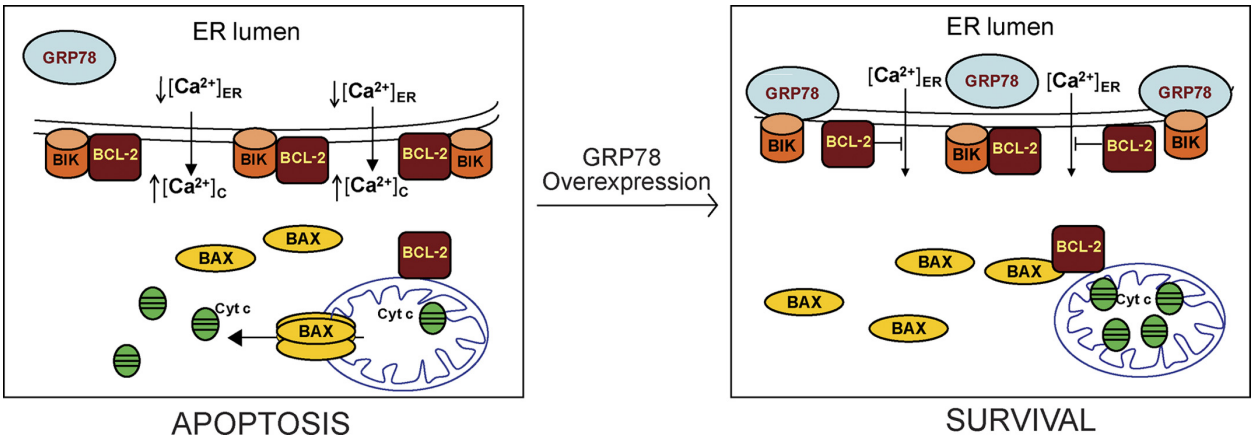

Figure 8. Model of BiP-dependent control of BIK activity in BiP-overexpressing tumours.
During severe ER stress conditions, pro-apoptotic BIK binds BCL-2 and favours the engagement of pro-apoptotic BAX to mediate the permeabilization of mitochondrial membrane leading to apoptosis. However, overexpression of BiP results in a sequestration of BIK and a free BCL-2 able to bind and inhibit the activity of BAX, resulting in survival. This model can also be applied to the sequential events occurring during the UPR, where an initial induction of BiP would counteract BIK activity and favour the recovery pathway. On the other hand, severe stress would induce apoptosis induction by lowering the levels and/or modifying the characteristics of BiP, rendering BIK free and able to prevent BCL-2-dependent inhibition of BAX. Taken from Zhou et al, 2011. GRP78 stands for Glucose Regulated protein 78 kDa, or BiP. See also section "Apoptosis".

This model requires a portion of BiP to be in the cytoplasm or localized in the ER membrane. This was indeed suggested by independent studies, where treatment of cells with ER stress inducers causes BiP to redistribute from the ER lumen to form subpopulations existing in the

cytosol and as an ER transmembrane protein with its carboxyl portion located inside the ER lumen and its N-terminal domain exposed to the cytosol (Rao et al, 2002). In line with that report, pull-down assays using full-length GST-BiP and GST-BiP/P45 (the 400 aa-long N-terminal half of BiP, including the NBD and expanding until some aa before the SBD) and extracts from BIK-overexpressing 293T cell lysates, demonstrated that the N-half of BiP is sufficient to interact with BIK, a result further probed to be direct by using both purified and tagged proteins (Zhou et al, 2011). Despite all these data, the primary amino acid sequence of BiP does not predict a traditional transmembrane configuration under normal physiological conditions. Thus, the interaction between BiP and cytosolic proteins might be mediated either by an unconventional form of BiP that spans the ER membrane or by luminal BiP in a complex with other ER transmembrane proteins, an issue that remains largely unsolved (Lee, 2014).

More recently, a novel isoform of BiP generated by alternative splicing (retention of intron 1) and alternative translation initiation was reported (called GRP78va). Bioinformatic and biochemical analyses revealed that expression of this GRP78va is enhanced by ER stress, is notably elevated in human leukemic cells and leukemia patients and is devoid of the ER signaling peptide, therefore, it is cytosolic. Knock-down of endogenous GRP78va by siRNA without affecting canonical GRP78 showed that GRP78va also promotes cell survival under ER stress by regulating PERK signaling through a direct interaction and antagonization of PERK inhibitor $P58^{IPK}$ (see “The killing face of the UPR” section) (Ni et al, 2009).

To further support the anti-apoptotic role of BiP, a conditional knock-out mice for BiP in Purkinje cells (PC) developed PC degeneration by 4.5 weeks, associating with severe motor coordination defect by week 5 and cerebellar atrophy by week 13 due to a massive apoptotic phenotype (Wang et al, 2010). Last but not least, BiP activity has also been associated with angiogenesis, invasion and metastasis through a plethora of mechanisms and interactions with many different factors and it was nicely reviewed elsewhere (Lee, 2014).

3.3.4. Target in cancer treatment

It has been suggested that BiP promotes tumour progression, cell survival, metastasis and resistance to chemotherapy and it has even been proposed as a biomarker of cancer progression. Due to these important activities, BiP overexpression was described as an adaptive response to stress that is induced by cancer treatments, including chemotoxic drugs, anti-hormonal agents, DNA-damaging agents, anti-angiogenesis drugs and chromatin-modifying drugs, as well as radiation therapy (Lee, 2014). Importantly, the cell surface expression of BiP mostly present in malignant cells but not in normal cells *in vivo*, offers the opportunity to target it without harming the normal organs and therefore standing as an interesting target to combat cancer progression and recurrence (Lee, 2014). Another advantage is that a half reduction of BiP expression, as seen by heterozygous knock-down mice models, does not hamper normal tissues but on the other hand, it impedes the growth of the tumour cells that highly depend on BiP expression (Wey et al, 2012).

There are several ways to take advantage of BiP expression in tumours. The first one is by delivering suicide genes, immunosuppressors and tumour suppressors with their expression driven by the highly active promoter of *bip* in the cancer cells. This approach has been tested with the herpes simplex kinase suicide gene in a retroviral system that resulted in the eradication of sizable tumours both in mice and human xenografts in immunodeficient mice (Dong et al, 2004). A direct effect on the BiP protein has also been tested by systemic delivery of an engineered fusion protein that combined epidermal growth factor (EGF) and the bacterial SubAb toxin that resulted toxic to EGF receptor (EGFR)-expressing cancer cells *in vitro* and caused a delay in the growth of human breast and prostate xenografts in mice (Backer et al, 2009). Also, inactivating the activity of BiP’s

domains presents promising results, as showed by direct binding of (-)epigallocatechin gallate (EGCG), honokiol and aspirin to the NBD and inhibition of its ATPase activity by competing with ATP or by binding of a BAG1 co-chaperone-derived peptide that binds SBD of BiP and inhibits its protein folding capacity. These treatments were able to sensitize cancer cells to chemotoxic agents (Ermakova et al, 2006) and reduce growth and increase apoptosis of prostate cancer cells in xenograft models (Maddalo et al, 2012), respectively.

3.4. ER stress and cancer

According to the profound alterations it can generate, ER stress has been implicated in diabetes, cardio-vascular diseases, viral infection, cancer, the immune response, aging, cerebral ischemia, neurodegenerative diseases, tyrosinemia, inclusion body myositis, among others. However, it is not clear whether the ER stress observed in some of these conditions is a primary cause of the disease or only a secondary pathological phenomenon (Zhao & Ackerman, 2006). In particular, the ER stress-induced UPR has been postulated to highly contribute to different phases of tumor growth (Ma & Hendershot, 2004).

The growth conditions in a tumour microenvironment are wide different to those encountered in normal tissues. In addition, tumours are complex tissues composed of multiple distinct cell types that participate in heterotypic interactions with one another and where an un-regulated growth and proliferation pattern must be sustained. In order to success on that, cells must adapt to the environmental constraints by reprograming their signaling pathways (Hanahan & Weinberg, 2011). The high rate of proliferation constitutes by itself an ER stressor since the cellular demand for proteins is highly incremented, thus, pushing the ER folding capacity to a new level. Moreover, the low concentration of Oxygen in solid tumours due to poor perfusion establishes a general context of hipoxia that is also a common characteristic of tumors that can impinge on ER homeostasis. One way cells can alleviate the stress is by taking advantage of the cytoprotective effects of the UPR, an idea supported by the maintenance of cellular homeostasis in specialized secretory cells, including plasmocytes, hepatocytes or pancreatic cells through UPR activation (Manie et al, 2014). Several studies have reported evidence of activation of the UPR in various tumours. XBP1 and ATF6 were found activated along with targets like CHOP, and chaperones BiP, glucose-regulated protein 94 (GRP94) and GRP170 were up-regulated in studies of primary cells from breast tumours, hepatocellular carcinomas, gastric tumours, and oesophageal adenocarcinomas, as reviewed in Ma & Hendershot, 2004 (Ma & Hendershot, 2004).

However, the final output associated with UPR activation in tumour is highly contradictory and might be both beneficial and deleterious for cancer progression. For instance, it might promote dormancy, aid tumour growth or protect the host by inducing apoptosis. It is presently unclear where the balance lies and how the fate of a tumour cell is eventually decided, although the part of the UPR pathway that up-regulates ER chaperones seems to have a protective role during tumour development, as is the case of BiP. The particular mechanism to promote or counteract tumour progression by members of the UPR escapes to the scope of this thesis and were extensively reviewed elsewhere (Hetz et al, 2013; Lee, 2014; Ma & Hendershot, 2004; Manie et al, 2014).

4. Apoptosis

4.1. General overview

Apoptosis is one of the cell death programs and the best studied so far. Its mechanism of activation was first elucidated by genetics studies in the nematode *Caenorhabditis elegans* and later on proved to be present in mammals though it presents a higher complexity and number of factors involved (Degterev & Yuan, 2008). Miss regulation of apoptosis may cause various human diseases, such as cancer and autoimmunity and on the other hand, its inappropriate activation also leads to health problems since it provoques tissue injury and functional decline in acute diseases such as stroke, myocardial infarction and brain trauma and chronic diseases such as diabetes and neurodegeneration (Degterev & Yuan, 2008).

There are two basic manners for inducing apoptosis in higher organisms; the external and internal pathways. The former, also known as death receptor pathway, relies in the interaction of the so called “death ligands” with their corresponding cellular receptors and its engagement to translocate the signal into the cell. The best studied pairs of death ligands/receptors are FasL/FasR and TNF-α/TNFR1. In both cases, upon ligand binding, cytoplasmic adapter cellular proteins FADD and TRADD, respectively, are recruited and then an association with procaspase-8 is stablished.

Caspases are a family of Cysteine proteases that cleave their substrates after Asp residues. Their activation involves the proteolytic cleavage of zymogens, the removal of the pro domains regions or allosteric conformational changes.

After procaspase-8 recruitment, a death-inducing signaling complex (DISC) is formed, resulting in the auto-catalytic activation of procaspase-8, which will in turn activate the effector caspase-3 and caspase-7 (Degterev & Yuan, 2008; Elmore, 2007). In addition, caspase-8 can also cleave the BH3-only pro-apoptotic protein BID, which in turn amplifies the cell death signal by causing mitochondrial damage (see below) (Degterev & Yuan, 2008). This external, cytokine-mediated induction of apoptosis that is present only in higher multicellular organisms allows for a coordinated mechanism of regulation of cell numbers in physiological and pathological conditions at a systemic level.

On the other hand, as its name claims, in the internal, mitochondrial or BCL-2-regulated pathway, the death signal originates inside the cell and relies on the destabilization of the mitochondria and release of cytochrome c into the cytoplasm. This event promotes the formation of an heptameric complex baptised “apoptosome” primarily composed of the apoptotic protease-activating factor 1, APAF1, and caspase-9. Interestingly, the translation of *apaf1* mRNA is driven by an IRES that supports low levels of translation to maintain sufficient levels of APAF1 protein during apoptosis to propagate caspase-9 activation (Holcik & Sonenberg, 2005). This leads to the conformational change and activation of caspase-9 which will in turn cleave and activate downstream caspase-3, 6 and 7 that act as effectors (Degterev & Yuan, 2008). The initiation of the endogenous apoptotic program highly depends on the expression, activation and balance between anti and pro-apoptotic proteins of the BCL-2 (B-cell lymphoma protein-2) family (Bhola & Letai, 2016). This intricate interplay triggers as final step of the cascade, the oligomerization of BCL-2 members BAX and BAK which will insert into and destabilize the mitochondrial membrane (Degterev & Yuan, 2008; Elmore, 2007; Segawa & Nagata, 2015) (see below).

In both exogenous and endogenous pathways, the events following activation of caspase-3 are very similar. They converge on the same terminal or execution mechanism that is initiated by the cleavage of caspase-3 and results in DNA fragmentation by Ca^{2+}- and Mg^{2+}-dependent endonucleases resulting in DNA fragments of 180 to 200 bp, degradation of cytoskeletal and

nuclear proteins, cleavage of members of the eukaryotic initiation factor-4G (eIF4G) family to reduce the protein load, cross-linking of proteins achieved through the expression and activation of tissue transglutaminase, formation of apoptotic bodies, expression of ligands for phagocytic cell receptors and finally uptake by phagocytic cells (Elmore, 2007; Holcik & Sonenberg, 2005).

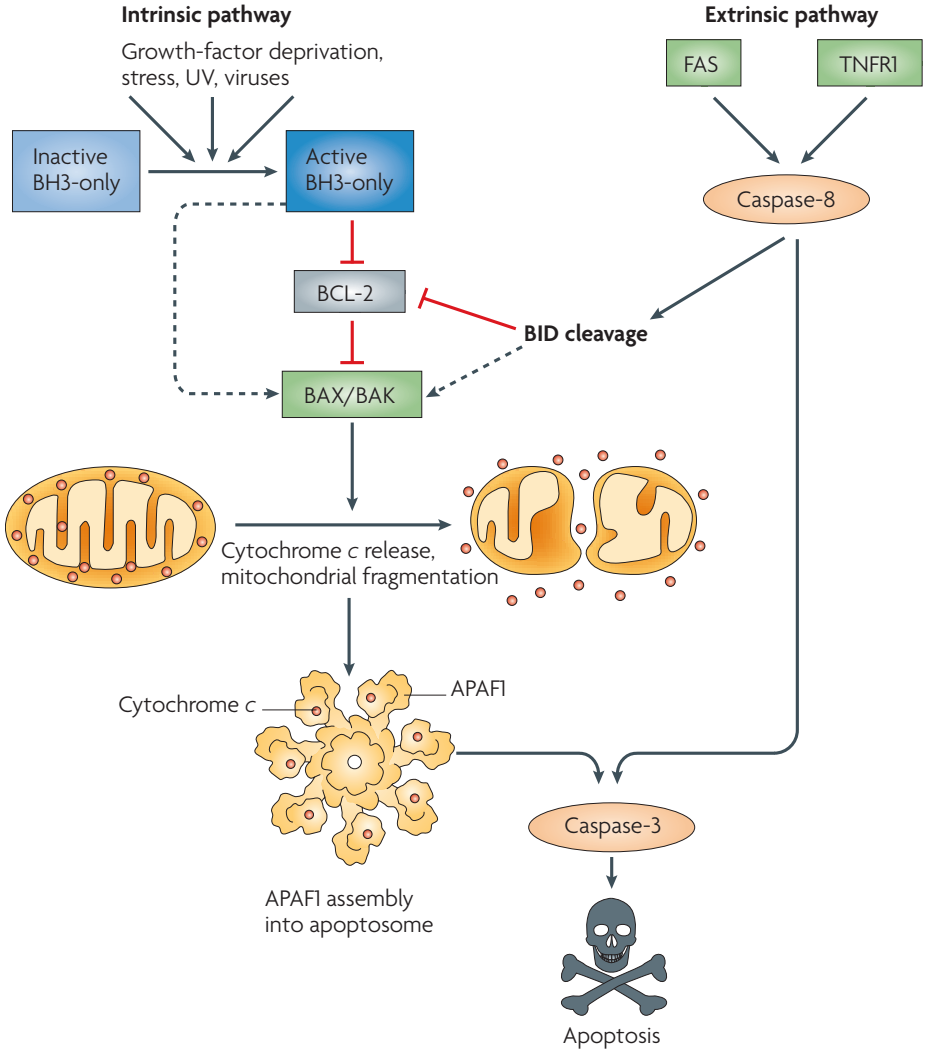

Figure 9. Apoptotic pathways.

Cartoon showing the principal events leading to extrinsic/death-receptor-mediated (in this case FasL and TNF) and intrinsic/BCL-2-mediated pathways. Upon binding of FasL to the FasR or TNF-α to TNFR1, the adapter cellular proteins FADD or TRADD are respectively recruited and then an association with procaspase-8 is stablished to finally lead to caspase-8 activation. Mitochondrial pathway relies on activation of BH3-only pro-apoptotic and inhibition of anti-apoptotic BCL-2 members to finally activate the pro-apoptotic BAX and BAK. This results in permeabilization of mitochondrial membrane, escape of cytochrome c (among others), its association with APAF1 and pro-caspase-9 to form the apoptosome. This leads to activation of caspase-9. All the pathways converge on activation of effector caspase-3 and -7 that lead to cell death as described in the text. Taken from Youle & Strasser, 2008.

The expression of cell surface markers that result in phagocytic recognition of apoptotic cells but not adjacent healthy cells, allows quick phagocytosis by macrophages and immature dendritic cells as well as epithelial and mesenchymal cells with minimal compromise to the surrounding tissue. Different signals have been proposed, including the phospholipid phosphatidylserine (PtdSer), carbohydrates (amino sugars or mannose), intercellular adhesion molecule-3 (ICAM3),

and calreticulin, though the first one is the most studied and the most likely ‘eat me’ signal candidate (Segawa & Nagata, 2015). PtdSer is normally localized in the inner leaflet of the plasma membrane along with amine-containing lipids, an asymmetric distribution that is maintained by an ATP-dependent transporting mechanism mediated by some proposed aminophospholipid translocase(s) or flippase(s), such as P4-type ATPases. These candidate proteins have other functions in cellular homeostasis and the particular mechanism of flipase activity is currently under investigation. On the other hand, scramblases antagonize the effect of flipases activity by transporting (or scrambling) nonspecifically and bidirectionally phospholipids in the plasma membrane leading to a slowly disruption of the PtdSer asymmetry by passive diffusion and to presentation of PtdSer at the cell surface. Both flipases and scramblases such as ATP11C and Xkr, respectively, posses caspase-3 cleavage sites in their lumen domains. During apoptosis, these sites become cleaved, simultaneously leading to inactivation of ATP11C flipase and activation of Xkr8 scramblase, which in turns leeds to PtdSer exposure on the cell surface (Elmore, 2007; Segawa & Nagata, 2015).

Once the apoptosis arrives to the irreversible phase, effector caspases also cleave other cellular targets, being one of them the poly (ADP-ribose) polymerase-1 (PARP-1), an enzyme that performs central roles in the repair of damaged DNA during physiological and pathological circumstances. PARP-1 participates in initiating base excision repair (BER), nucleotide excision repair, single strand base repair and contributes to double strand base (DSB) repair (Benjamin & Gill, 1980; Chaitanya et al, 2010). Caspase-3 cleaves PARP-1 after Asp (Glu-Val-Asp-Gly) *in vitro* to yield an 85-kD PARP-1 fragment. In addition, PARP-1 is also cleaved *in vivo* by caspase-3 and 7 giving the formation of two specific fragments: an 89-kD catalytic fragment and a 24-kD DBD. (Chaitanya et al, 2010) The former is excluded to the cytoplasm and the later remains bound to nicked DNA where it acts as a trans-dominant inhibitor of active PARP-1 and other repairing enzymes, thus, attenuating DNA repair. In addition, cleavage of PARP-1 by caspases also contributes to preserve cellular ATP pools and prevents the arising of passive necrotic cell death by “energy collapse” by preventing NAD+ consumption by PARP-1 (Chaitanya et al, 2010; Degterev & Yuan, 2008). All these effects act together to favour apoptosis death over other inflammation-prone mechanisms.

Both presence of PtdSer in the cell surface and the described pattern of PARP-1 cleavage are hallmarks of apoptosis induction and were used in this work to measure the level of apoptosis in our cell culture systems.

Another target of effector caspases are anti-apoptotic members of the BCL-2 family, such as BCL-2 itself. This action not only renders BCL-2 inactive in terms of apoptosis inhibition but also produces a carboxyl-terminal BCL-2 cleavage product exhibiting pro-apoptotic, rather than anti-apoptotic, activity (Cheng et al, 1997)(see below).

4.2. BCL-2 family of proteins

The B-cell lymphoma protein-2 or BCL-2 family of proteins has essential roles in the mouse from early embryogenesis through to adult tissue homeostasis, as shown by the diverse and severe effect (in some cases including death) of knocking-down/out BCL-2 protein members in mice models. In mammals, it contains the BCL-2 itself and proteins that have either three-dimensional structural similarity or a predicted secondary structure that is similar to it (Bhola & Letai, 2016; Youle & Strasser, 2008). The core members are those proteins containing the BH1, BH2 and BH3 domains, in addition to the BH4 present only in some anti-apoptotic ones such as BCL-2, BCL-XL and BCL-W. The other members, the so-called BH3-only proteins, share homology with each other and the rest of the BCL-2 proteins only through the BH3 motif. Apart from BID,

which is a BH3-only protein that shares structural homology with the core members and belongs to this group, the rest of the BH3-only factors are structurally unrelated and appear to lack a close evolutionary relationship to the core members of the BCL-2 family. It is postulated that they probably acquired BH3 motifs by convergent evolution (Youle & Strasser, 2008). Despite these differences, all the BH3-only proteins are able to interact with some other BCL-2 proteins and to regulate them in terms of apoptosis promotion (Bhola & Letai, 2016; Youle & Strasser, 2008). Based on these properties, it is possible to define three sub-clases of proteins, namely anti-apoptotic (including BCL-2, BCL-XL, MCL1, BCL-W and A1, all of them multi domain), multi domain pro-apoptotic (BAX and BAK) and BH3-only (such as BIK, BID, BIM, BAD, NOXA and PUMA) (Degterev & Yuan, 2008; Youle & Strasser, 2008). In addition to the BH domains, some members of the three sub-classes also contain a transmembrane domain that allows them to be inserted into the membrane of cellular organelles, most notably mitochondria, though they may also be present at the ER and nuclear envelope (Bhola & Letai, 2016).

Although there is controversy about the biochemical characteristics and the way anti-apoptotic proteins control it, the pro-apoptotic BAX and BAK are critical for inducing mitochondrial outer membrane permeabilization (MOMP) and the subsequent release of apoptogenic molecules that leads to caspase activation. This is supported by the fact that double BAK and BAX knock-out mice are not viable because they have reduced levels of mitochondrial-mediated apoptosis (Lindsten et al, 2000) and that they were required for apoptosis induction in response to several insults (Bhola & Letai, 2016). The proposed mechanism for such importance is based on the ability of BAX and BAK to form pores in the outer mitochondrial membrane (OMM) through which mitochondrial proteins can escape to the cytoplasm. Unfortunately, and although many insights have been gained by different biochemical and structural approaches, the postulated pore structure has been difficult to pin down due to the membrane environment of the BAX/BAK oligomer and to the fact that it does not have reproducible symmetric features suited for structural techniques (Bhola & Letai, 2016).

The pro-death activity of BAK and BAX is contrasted by the anti-apoptotic members such as BCL-2 and BCL-XL, whose activity is counteracted by BH3-only pro-apoptotic proteins, leading to a de-repression of BAX and BAK. The pro-apoptotic activation of BAK and BAX might be done in two different ways and leads to classification of pro-apoptotic BH3-only proteins in activators and sensitizers. The formers have been shown to establish a direct interaction and activation of BAK and BAX, as it was shown for BIM, tBID (a cleavage product of BID originated by the actions of caspases, see before), PUMA and, more weakly, by NOXA (Bhola & Letai, 2016; Youle & Strasser, 2008), while the sensitizers, such as BAD, BIK and NOXA, will act mostly as competitors for the BH3-binding site in anti-apoptotic proteins, displacing or preventing the binding of activators or effectors (see Figure 8 for an example) (Bhola & Letai, 2016). This classification correlates with the binding pattern of pro-apoptotic proteins towards the anti-apoptotic ones, as it has been assessed by different approaches. They have shown that activator BH3-only proteins, such as BIM and PUMA, are bound by all anti-apoptotic BCL-2 family members, whereas sensitizers, such as BAD and NOXA, interact only with certain anti-apoptotic BCL-2 family members, namely BCL-2, BCL-XL and BCL-W for the former and A1 and MCL-1 for the later (Youle & Strasser, 2008). More importantly, the classification also correlates with phenotypic abnormalities observed in the mice lacking different group of BH3-only proteins. Indeed, lack of sensitizers BAD, BIK or NOXA have mild effects, as it is shown by knock-out mice that are essentially normal in appearance and are normally fertile (Youle & Strasser, 2008). This might be related to the final effect of the interaction among the opposing factors, while the seemingly promiscuous binding pattern of anti-apoptotic proteins by the activators might result in the inhibition of the later, the more specific interactome of

sensitizers, on the other hand, may be related to the inhibitory effect that they exert on the anti-apoptotic members of the family.

Table I. Members of the BCL-2 family.
Proteins were classified according to its effect on apoptosis as shown in the column Category, where BH3-only members are further classified into direct activators or sensitizers (see the text). The BH domains present in each protein are shown as well as the presence or not of a trans-membrane motif. Based on Bhola & Letai, 2016; Youle & Strasser, 2008.

Protein	Category	BH domains	Trans-membrane domain
BCL-2	Anti-apoptotic	BH1, BH2, BH3, BH4	Yes
BCL-XL	Anti-apoptotic	BH1, BH2, BH3, BH4	Yes
BCL-W	Anti-apoptotic	BH1, BH2, BH3, BH4	Yes
MCL-1	Anti-apoptotic	BH1, BH2, BH3	Yes
BFL1/A1	Anti-apoptotic	BH1, BH2	No
BID	Pro-apoptotic, activator	BH3	No
PUMA	Pro-apoptotic, activator	BH3	No
BIM	Pro-apoptotic, activator	BH3	No
NOXA	Pro-apoptotic, activator/sensitizer	BH3	No
BAD	Pro-apoptotic, sensitizer	BH3	No
BIK	Pro-apoptotic, sensitizer	BH3	Yes
HRK	Pro-apoptotic, sensitizer	BH3	Yes
BNIP3	Pro-apoptotic, sensitizer	BH3	Yes
BMF	Pro-apoptotic, sensitizer	BH3	No
BAX	Pro-apoptotic, effector	BH1, BH2, BH3	Yes
BAK	Pro-apoptotic, effector	BH1, BH2, BH3	Yes
BOK	Pro-apoptotic, effector	BH3	Yes

All these regulatory events are mediated by direct interaction of BCL-2 proteins, notably via the BH3 motif, a 20 amino acid amphipathic alpha-helix possessed by all BCL-2 family proteins that is poorly conserved with the exception of the LeuXXXXAsp sequence that appears consistently near the C-terminus. This motif heterodimerize with an hydrophobic cleft formed by the BH1, BH2 and BH3 domains of multi domain members. Interestingly, this pocket of at least BAX, BCL-W and MCL1 that is able to bind to peptides of the BH3-domain sequences of BAK, BAD and BIM, it also has the capacity to sequester their own C-terminal transmembrane anchor (Hsu et al, 1997; Youle & Strasser, 2008). This suggest that interaction with other members of the family might regulate the exposure of the transmembrane domain, and therefore, the sub-cellular localization and protein activity (Youle & Strasser, 2008). These interactions take place in different cellular compartments, a feature that is influenced by the translocation of some BCL-2 protein from their normal localization in healthy cells to the one promoting the apoptotic response. The main pro-

apoptotic BCL-2 effectors show different localization patterns under normal conditions. While BAX is mostly cytosolic and sequesters its hydrophobic C-terminal membrane anchor in its BH3-binding pocket, though a small fraction might be bound to the outer mitochondrial membrane (Hsu et al, 1997), BAK usually resides at the outer mitochondrial membrane where it was shown to be bound to anti-apoptotic MCL-1 and BCL-XL (Willis et al, 2005; Youle & Strasser, 2008). During apoptosis induction, BAX translocates specifically to mitochondria (Hsu et al, 1997) and the interaction of anti-apoptotic proteins with BAK is disrupted either by degradation and blockade of protein synthesis as it occurs with MCL-1 in ultraviolet (UV) irradiation-induced apoptosis in HeLa cells (Nijhawan et al, 2003) and/or by disruption of MCL1–BAK and BCL-XL–BAK interactions by other members such as NOXA, BIM and BIK (Shimazu et al, 2007; Willis et al, 2005; Youle & Strasser, 2008). This leads to oligomerization of BAX and BAK at the OMM. Additionally, a third effector protein with homology to BAX and BAK, termed BOK, has more recently been described. In addition, the other members of the family also shift among nuclear envelope, ER or mitochondria membrane and cytoplasm which will impinge on the apoptotic status of the cell.

4.3. Regulation of expression of BCL-2 proteins

Expression of pro-apoptotic BH3-only members can be regulated at different steps. The first one involves the participation of transcription factors that induce mRNA synthesis, as in the case of NOXA (Oda et al, 2000) and PUMA (Nakano & Vousden, 2001) that are up-regulated upon activation of p53 during the DNA damage response and ER stress (although the later is still not confirmed) (Li et al, 2006) and BIK that is induced by p53 in adenovirus E1A-expressing cells (Mathai et al, 2002), as well as BIM whose expression is favoured by FOXO3A (Youle & Strasser, 2008) or CHOP (Puthalakath et al, 2007) in response to growth-factor deprivation or ER stress, respectively, among others. Also, post-translational mechanism positively control the expression level of BAD by loss of phosphorylation in response to growth-factor deprivation, BID is activated by caspase-8-mediated proteolysis (Youle & Strasser, 2008), BIM is stabilized by protein phosphatase 2A-mediated dephosphorylation which prevents its ubiquitination and proteasomal degradation under ER stress (Puthalakath et al, 2007) and BIK is activated by an unknown mechanism in response to inhibition of protein synthesis (Shimazu et al, 2007).

In the case of core anti-apoptotic proteins, control of transcription was also shown for example for BCL-XL, whose mRNA synthesis is induced by JAK–sTAT pathway by growth factors to inhibit apoptosis and therefore promote cell survival (Youle & Strasser, 2008) and, on the other hand, transcription of BCL-2 is repressed by direct binding of p53 to its promoter (Miyashita et al, 1994a; Miyashita et al, 1994b). In addition, post-transcriptional regulation was also shown for this group of proteins, as exemplified by the control of MCL-1 expression by increased ubiquitination and proteasome degradation in response to cytokine deprivation or UV radiation (Nijhawan et al, 2003; Youle & Strasser, 2008).

The pro-apoptotic effectors BAX and BAK also present different mechanism for controlling their expression. The former has been shown to be induced by p53 at the transcription level (Miyashita et al, 1994b; Miyashita & Reed, 1995), while the later was shown to be activated by the tumour suppressor WT1 via direct binding to the *bak* promoter, an activity blocked by mutation of WT1 which has been associated to 10% to 15% of Wilms' tumour neprhoblastoma (Morrison et al, 2005).

In all the three groups of proteins, post-translational modifications have been reported to control their activity. Particularly important is the role of phosphorylations, as in the case of BCL-XL phosphorylation in cardiomyocytes at Ser14, which antagonizes its binding to BAX, NOXA phosphorylation at Ser13, which prevents its pro-apoptotic activity, extensive phosphorylation of

BIM being some events pro- and others anti-death and BAX and BAK are also subjected to phosphorylation that may inhibit their pro-apoptotic effect (Bhola & Letai, 2016).

All these data show that expression of BCL-2 proteins is tightly controlled at several steps of gene expression and that the final apoptotic response is influenced by a sophisticated and complicated network of interaction among many different factors activated by several insults. This area is the focus of intense research not only for basic aspects but also for its putative role as clinical target in human diseases such as cancer and neurodegenerative disorders.

4.4. The founding member of the BH3-only proteins: BIK

The BCL-2 interacting killer BIK (also known as NBK) is a 160 aa protein that was first detected using a yeast 2-hybrid screen with BCL-2 as bait and it was also shown to establish interactions with BCL-XL and the viral survival-promoting proteins Epstein-Barr virus BHRF1 and adenovirus E1B-19 kDa in yeast and *in vitro* and their co-localization was shown in human cells (Boyd et al, 1995; Han et al, 1996). In addition, transfected BIK was able to induce cell death, an activity that was counteracted by co-expression of BCL-2, BCL-XL, EBV-BHRF1 and E1B-19 kDa proteins (Boyd et al, 1995; Han et al, 1996).

BIK has been reported to be modified in some cancers, such as peripheral B-cell lymphomas, gliomas, head and neck and colorectal cancers (Chinnadurai et al, 2008; Forbes et al, 2015; Mathai et al, 2005). Also, *bik* gene was reported to be inactivated by LOH and by epigenetic promoter silencing in renal cell carcinomas. Moreover, BIK protein was shown to be actively targeted by the proteasome in cancer cells that constitutively express *bik* mRNA. Although all this data does not corrobore a direct relationship between BIK lose and tumour appearance or progression, it suggests that BIK might act as a tumour suppressor protein (Chinnadurai et al, 2008). Targeting BIK offers a new interventional approach to treat cancers, as it was shown by the cell death induction observed upon activation of BIK in estrogen-responsive breast cancer cell lines (Hur et al, 2006; Hur et al, 2004).

Albeit the pro-apoptotic role of BIK, its homozygous deletion in mice did not result in overt developmental disorders (Coultas et al, 2004), arguing that its effect might be redundant with that of other BH3-only member. This notion is supported by the spermatogenesis arrest reported in mice harboring deletion of both *bik* and *bim* genes (Coultas et al, 2005).

4.4.1. Structure and localization

Although BIK does not show overt homology to the BH1 and BH2 conserved domains characteristic of the BCL-2 family, it possesses a BH3 domain shared with BAX and BAK that was proved for the first time to be linked (and crucial) to its death activity, establishing BIK as the first described member of the BH3-only pro-apoptotic proteins (Boyd et al, 1995). The BH3 domain was also crucial to establish the interaction with BCL-2 but not with E1B-19 kDa (Han et al, 1996).

In addition, a trans-membrane domain localized in its C-terminal region and shared with other BCL-2 proteins was also shown to be present in BIK (Boyd et al, 1995) and renders it mostly attached to the ER membrane, as shown by co-localization of Flag-tagged BIK and ER marker calnexin (but not mitochondrial marker TOM20) assessed by immunofluorescence confocal microscopy in KB epithelial cells (Germain et al, 2002; Mathai et al, 2002). Interestingly, membrane-associated BIK (from a ER membrane-containing, but not mitochondria, sample) was resistant to alkaline-insoluble membrane protein extraction but sensitive to proteinase K digestion, suggesting that BIK is integrated in the ER membrane leaving the bulk of the protein facing the cytoplasm, a pattern shared with transmembrane calnexin but not with the luminal chaperone BiP that was resistant to protease treatment and that was extracted by alkali treatment (Germain et al,

2002). Interestingly, a Flag-BIK protein in which its C-terminal transmembrane domain was replaced by the C-terminal transmembrane segment of cytochrome b_5, a sequence shown to selectively target fusion proteins to the ER, was still able to induce cytochrome c release and apoptosis. This suggest that BIK can function as pro-apoptotic protein from its location in the ER (Germain et al, 2005; Germain et al, 2002; Mathai et al, 2005). However, localization of BIK at the mitochondria could not be ruled out in this set of experiments nor in another work where human BIK co-localized with E1B-19 kDa exclusively in cytoplasmic and nuclear membranes of 19K1 cell line (an E1A-plus-*ts*p53(Val-135)-transformed primary baby rat kidney, BRK, cell line that stably expresses the E1B-19 KDa protein and is thereby substantially resistant to apoptosis) as detected by immunofluorescence (Han et al, 1996). In addition, mouse BIK fused to GFP showed a co-localization with the mitochondrial marker MitoTracker in transfected MCF-7 analyzed by confocal microscopy (Hegde et al, 1998).

4.4.2. Regulation of BiK expression

The pattern of BIK expression in human tissues has not been addressed so far. However, it has been shown that *bik* mRNA levels were higher in kidney and pancreas compared to other organs as seen by northern blot analysis, but no correlation with protein levels was specified (Chinnadurai et al, 2008). However, the regulation of its gene expression was analysed in some culture systems.

Transcription of the human *bik* gene and its related increase in protein expression were shown to be induced by transfection of p53 in p53-null H1299 cells after 16 h, a fact that preceded the p53-dependent induction of apoptosis as measured by exclusion of trypan blue, a phenotype partially rescued by the caspase inhibitor, zVAD-fmk (Germain et al, 2002; Mathai et al, 2002). However, they did not report a direct stimulation of the *bik* promoter by p53 though they identified several elements that exhibit homology to potential but degenerate p53 REs lying upstream of the start site of transcription and within the long 13-kb intron 1 of the *Bik* gene (Hur et al, 2006; Mathai et al, 2002). In addition, *Bik* gene was activated by genotoxic stimuli in p53-null environments, suggesting that p53-dependent and -independent mechanism might be playing a role (Chinnadurai et al, 2008). Moreover, fulvestrant-induced apoptosis of estrogen-dependent MCF-7 breast cancer cell line was also linked to BIK induction at the transcription step and this was dependent on p53 as shown by siRNA treatment and overexpression of p53-dominant negative mutant (deletion of p53 DBD). Surprisingly, the effect did not depend on the DNA-binding capacity of p53 towards a *bik* promoter-containing probe in EMSA experiments (Hur et al, 2006).

On the other hand, the direct effect of E2F1 transcription factor on inducing *bik* mRNA synthesis was demonstrated by the detection of a E2F-binding site on human *bik* promoter and by reporter assays, ChIP and EMSA during doxorubicin-induced genotoxic stress and adenovirus infection, indicating that BIK is a pro-apoptotic effector of E2F signalling pathway, instead linking BIK-induced apoptosis with the retinoblastoma tumour suppressor pathway which is an important regulator of E2F1 activity (Chinnadurai et al, 2008). In line with this, treatment of B-lymphoma cell lines with TGF-β resulted in an increase of *bik* mRNA trough recruitment of Smad. The *bik* promoter also harbors binding sites for Smad, an observation confirmed by ChiP and EMSA assays (Chinnadurai et al, 2008). Induction of BIK expression was also demonstrated for INFγ treatment of murine epithelial cells via the STAT1 transcription factor. Despite *bik* promoter has several STAT1-binding sites, no response was detected in reporter-based promoter assays under INFγ treatment (Chinnadurai et al, 2008).

4.4.3. Induction of cell death by BIK

Overexpression of BIK was shown to induce apoptosis via activation of the classical caspase-9, both in mouse and humans cell lines. In the first case, the BIK-dependent induction of apoptosis was counteracted by dominant-negative mutant of caspase-9 (Chinnadurai et al, 2008; Germain et al, 2002). The mitochondrial apoptotic pathway is initiated by BIK mainly located at the ER (Figure 10). In the context of the "direct" model, it has been shown that both endogenous and over expressed BIK are able to recruit and induce oligomerisation of BAK and BAX at the ER membrane as analysed by cellular fractionation of H1299 cells, and that this leads to ER Ca^{2+} depletion (Mathai et al, 2005). As expected, Ca^{2+} depletion was not seen in BAX/BAK double knockout mouse kidney cells (Mathai et al, 2005). The BIK-induced release of Ca^{2+} from the ER results in the activation of the mitochondrial fission protein DRP1 and its recruitment to tubular mitochondria, causing fragmentation of the organelle and sensitization to stimuli that cause release of cytochrome c to the cytosol (Germain et al, 2005). However, expression of a BH3 mutant BIK was unable to open the mitochondria cristae and this is in line with the fact that overexpression of dominant-negative DRP1 mutant also inhibits BIK-dependent mitochondria remodeling (Germain et al, 2005; Mathai et al, 2005).

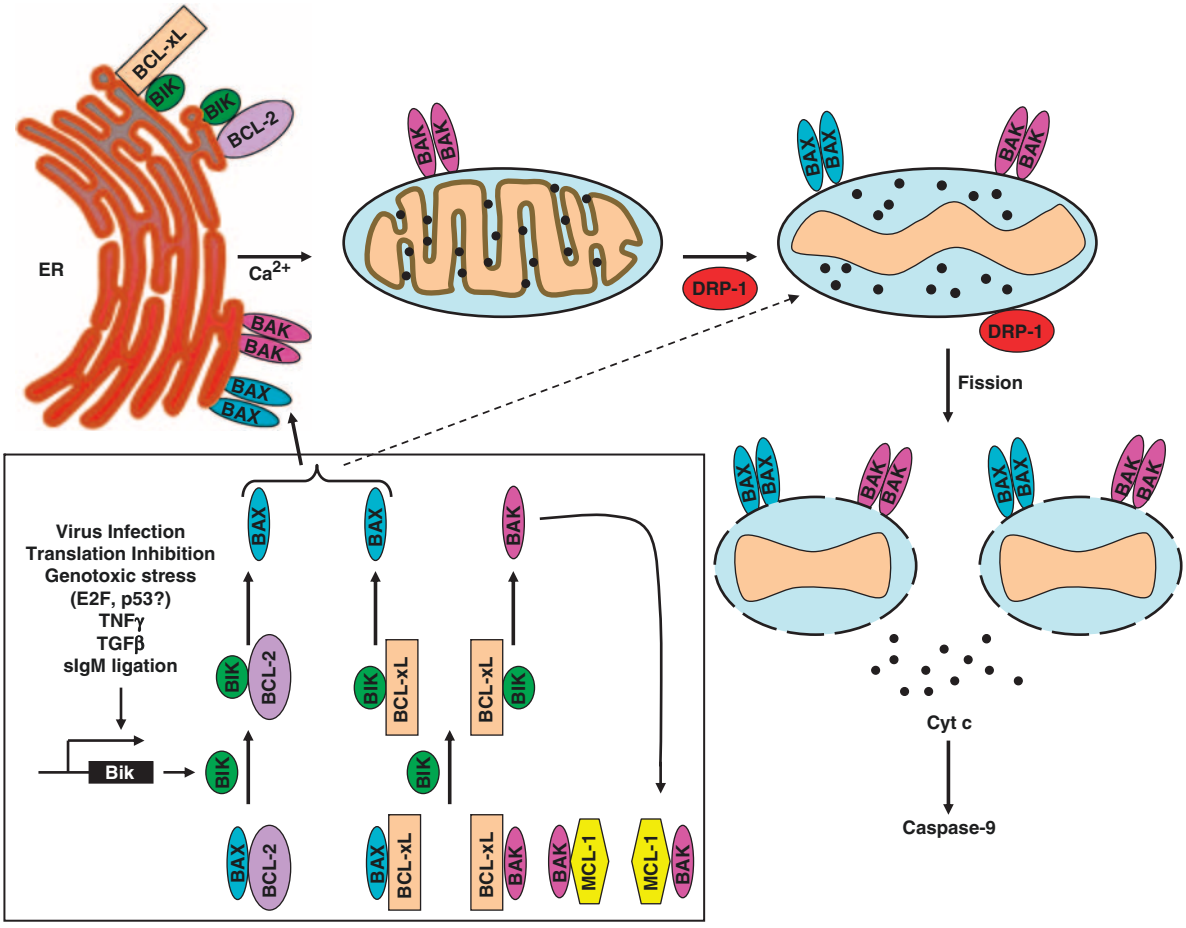

Figure 10. Induction of apoptosis by BIK.

Expression of BIK is increased by several types of stress at the transcription step. BIK may promote apoptosis as a sensitizer by competing with effector BAX for binding to negative BCL-2 and BCL-XL, leading to BAX activation. BAK activation is not reached upon BIK activity because BAK/MCL-1 interaction is not counteracted by BIK. Active effectors initiate apoptosis from the ER and mitochondria as explained above. Although not summarized in the figure, BIK was

also suggested to act as a pro-apoptotic activator by directly enhancing the activity of BAX. Taken from Chinnadurai et al, 2009.

In addition, the “indirect” model of apoptosis induction by BIK constitutes an important feature. In several human epithelial cancer cell lines, the pro-apoptotic activity of BIK was shown to be dependent only on BAX, which was shown to be sequestered by pro-survival BCL-2 and BCL-XL (Chinnadurai et al, 2008). Since BIK was shown to efficiently interact with BCL-2 and BCL-XL (Boyd et al, 1995; Han et al, 1996) and not with MCL-1 that usually binds to and inhibits BAK (Gillissen et al, 2007), it is likely that BIK might displace BAX, but not BAK, from the anti-apoptotic regulators to allow the apoptotic pathway to proceed defining, thus, the dependency on the former pro-apoptotic BCL-2 protein (Chinnadurai et al, 2008; Gillissen et al, 2007). In addition, expression of a HA-tagged BIK induced insertion of BAX into the OMM and the consequent cytochrome c escape and apoptosis in H1299 and although the presence of zVAD-fmk restored the cell viability, it was not able to restore the cytoplasmic distribution of BAX, as assessed by cellular fractionation and alkali extraction on mitochondria-enriched fractions. This shows that insertion of BAX into the OMM but not the down-stream events depends on BIK activity (Germain et al, 2002). It is worth to point out that the “direct and indirect” modes of action of BIK to induce apoptosis are not exclusive and therefore, they certainly contribute to magnify the final output in cells expressing activated BIK.

5. The integrative and concluding chapter: p53 and ER stress

The role of p53 during ER stress has been popping up during the previous sections and is, as expected, tightly related to its expression. However, the expression pattern of p53 during ER stress is still not fully understood. Indeed, there are some works contradicting each other regarding the level of p53 in this scenario and its actual role. Synthesis of p53 is, as expected, affected by the general inhibition of cap-dependent translation taking place during ER stress by PERK-mediated phosphorylation of eIF2α (Bourougaa et al, 2010; Candeias et al, 2006). Moreover, destabilization of p53 protein and prevention of p53-dependent apoptosis were reported during ER stress in human diploid WI-38 and human fibrosarcoma HT1080 cells. This effect was reported to be at least in part mediated through the increased cytoplasmic localization of p53 as a response to direct interaction with and phosphorylation by the ER stress- and eIF2α kinases PERK and PKR-induced glycogen synthase-3 (GSK-3) kinase (Baltzis et al, 2007; Qu et al, 2004). The induction of the cytoplasmic translocation and degradation of p53 by ER stress was also related to MDM2, functioning after the phosphorylation of p53 by GSK-3 marked it as a target for degradation. Significantly, the cooperative action of GSK-3 and MDM2 also occurs in unstressed cells, but it seems it is enhanced in cells subjected to ER stress (Pluquet et al, 2005). In line with this, Synoviolin (also called HRD1) an E3-ubiquitin ligase implicated in ERAD and actually proposed to be part of the ERAD complex charged of dislocation and ubiquitination of proteins (Morito & Nagata, 2015), was also reported to sequester p53 to a perinuclear/ER region and target it for degradation in the cytoplasm, in addition to negatively regulate its cellular level and biological functions, including transcription, cell cycle regulation and apoptosis. This was shown to be dependent on a direct interaction between p53 and Synoviolin (Yamasaki et al, 2007). Down-regulation of Synoviolin by siRNA in the p53wt human colon cancer cell line RKO, resulted in increased p53 protein level and nuclear accumulation as well as triggering of UPR, as judged by PERK activation and probably due to accumulation of unfolded proteins inside the ER (Yamasaki et al, 2007). Thus, Synoviolin may also control p53 levels when induced as a target of the UPR upon ER stress.

However, stabilization of p53 was also reported upon treatment of stablished cell lines with tunicamycin or glucose deprivation in a PERK-dependent manner (Zhang et al, 2006). Because ribosome biogenesis is the major biosynthetic and energy-consuming activity of eukaryotic cells, it is sensitive to the availability of nutrients, growth factors and to the stress in the ER due to reduction of active polysomes through eIF2α phosphorylation. In this context, activation of p53 was reported to be associated to reduced MDM2-mediated p53 ubiquitination and degradation because MDM2 described a stronger association with the ribosomal proteins (rpL5, rpL11, and rpL23) (Zhang et al, 2006). Therefore, UPR activation induces ribosomal stress, which directly contributes to p53 stabilization. p53 protein activity was also shown to be increased during ER stress using as model MEFs with their corresponding p53-null cells as control (Li et al, 2006). Induction of NOXA and PUMA, two well-described pro-apoptotic BCL-2 proteins, by ER stress in MEFs was largely dependent on p53 and both proteins contribute to ER stress-induced apoptosis. Opposite to what it was described before concerning localization of p53 upon ER stress, in these MEFs p53 was detected primarily nuclear, and its level was elevated after treatment. Further, ER stress-induced apoptosis is partially suppressed in *p53*$^{-/-}$ MEFs, a response that can be attributed to other pathways. For instance, the induction of CHOP remains intact suggesting both p53-dependent and -independent pathways may trigger apoptosis in this set of experiments (Li et al, 2006).

All the data presented before gives more questions than answers. Conciliate the effects described in one work with another seems to be quite difficult, although different cell lines,

stressors, concentrations and most importantly, time of incubations were used and may be lying under the differences observed. In addition, one important factor that was not take into account was the specific and regulated induction of p53ΔN40.

5.1. The short p53ΔN40 isoform raises

The N-truncated isoform p53ΔN40 that can be originated both from alternative splicing or from a second in-frame AUG (see "p53 isoforms" section), relies on the latter to be specifically induced during ER stress via its cap-independent translation capacity from the constitutive and normal-spliced *p53* mRNA (Candeias et al, 2006). As described before, the region up-stream of the 40th codon which is the AUG initiation codon of p53ΔN40, lies within the *p53fl* coding region and was shown to contain at least one IRES (Candeias et al, 2006; Ray et al, 2006; Yang et al, 2006) that is able to mediate translation of p53ΔN40 when the cap-dependent mechanism is compromised in response to serum deprivation, cell-cycle progression, or ER stress (Bourougaa et al, 2010; Candeias et al, 2006). p53ΔN40 retains the DNA-binding and oligomerization capacity and induces and represses a stress-dependent set of target genes that differs from the full-length p53 (p53FL) (Powell et al, 2008).

Our group has previously shown that cells facing ER stress in culture respond with a p53-dependent G2 arrest. In particular, this effect was caused by p53ΔN40 and not by p53FL which was involved in controlling the G1 phase of the cell cycle, a result that was well correlated with *p21*^{CDKN1A} mRNA expression levels. On the other hand, p53ΔN40 induced a 7-fold induction of *14-3-3σ* mRNA levels and a further 2-fold increase when cells were treated with thapsigargin while the p53FL gave a non-significant up-regulation. In addition, this correlated with an increased binding of *14-3-3σ*'s promoter by p53ΔN40 and with the arrest in G2 phase (Bourougaa et al, 2010). 14-3-3σ was previously shown to promote G2 arrest by sequestering the Cdc25 phosphatase in the cytosol and thereby preventing dephosphorylation of Cdc2 and activation of the cyclinB/Cdc2 complex (Peng et al, 1997). Moreover, co-expression of the dominant-negative PERKΔC suppressed thapsigargin-induced p53ΔN40 expression independently of the 5'UTR of p53 but required the 120 nt of the mRNA expanding from the initiation site of p53FL and the one of p53ΔN40. This indicates that ER stress-induced G2 arrest is dependent on the capacity of PERK to control IRES-mediated translation of p53ΔN40 and the subsequent induction of *14-3-3σ* (Bourougaa et al, 2010). In addition, ER stress was suggested to promote the specific formation of p53ΔN40 homo-oligomers, therefore, magnifying the unique properties of p53ΔN40 that are not carried by p53FL and rendering them dominant, even in the presence of higher levels of p53FL because the ratio of p53FL:p53ΔN40 has important consequences for p53 activity (Bourougaa et al, 2010; Powell et al, 2008). Interestingly, p53ΔN40 was also able to promote apoptosis during ER stress and this was magnified by down-regulation of 14-3-3σ by siRNA transfection. This strongly indicates that a pro-apoptotic response is more likely if the cell fails to arrest in G2 and restore the ER homeostasis, as it occurs with the p53FL-dependent G1 arrest followed by apoptosis during the DNA-damage response. Taken together, these results illustrate how cells can differentiate p53 activity in response to a defined cellular stress pathway in order to trigger a specific and suitable cell-biological outcome (Bourougaa et al, 2010).

Later on, our group also showed that p53 actively suppresses expression of p21^{CDKN1A} in order to avoid promotion of COP-1-mediated degradation of 14-3-3σ, finally leading to stabilization of 14-3-3σ and G2 arrest. Additionally, by doing this, p53 lowered the apoptotic threshold upon DNA damage (Mlynarczyk & Fahraeus, 2014). Inhibition of p21^{CDKN1A} expression was achieved by blocking two different steps. First, the ER-stress mediated specific induction of p53ΔN40 and suppression of p53FL prevented the ability of p53FL to drive transcription of *p21*^{CDKN1A}, in

agreement with previous reports (Ghosh et al, 2004; Mlynarczyk & Fahraeus, 2014; Yin et al, 2002). This deficiency probably relies on the inability of p53ΔN40 to promote expression of those target genes that depend on the TAD I of p53. Second, both p53FL and p53ΔN40 isoforms suppressed synthesis of p21^{CDKN1A} protein in a way that depends on two different regions in the *p21^{CDKN1A}* mRNA restricted to the ORF, as shown in Figure 11 (Mlynarczyk & Fahraeus, 2014).

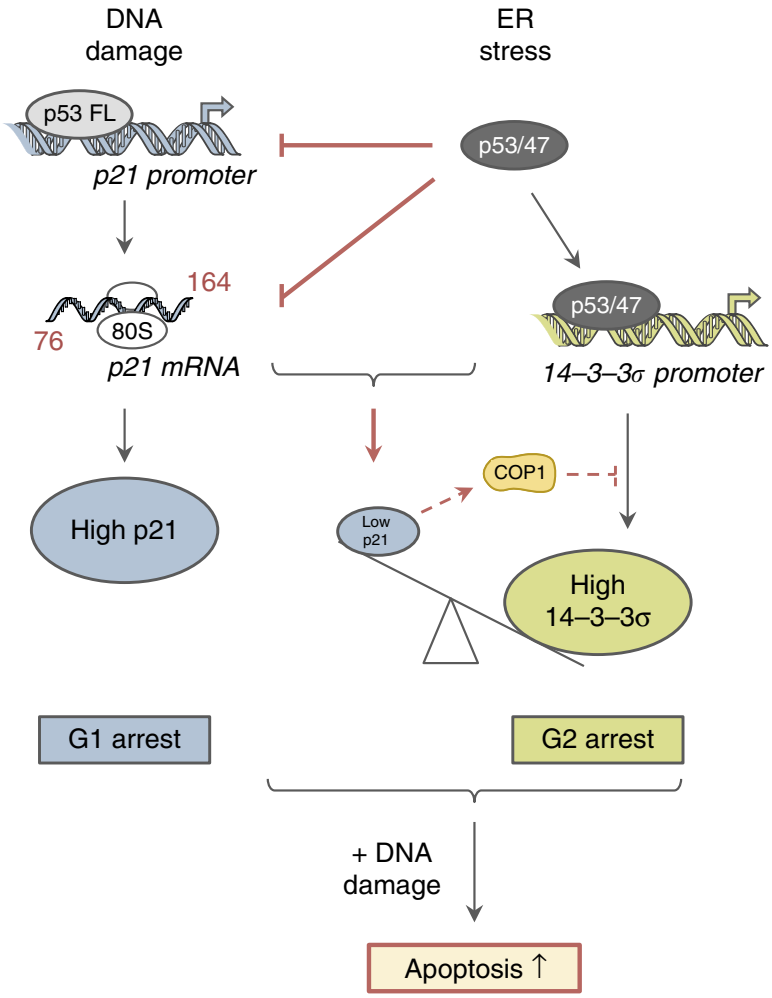

Figure 11. p53ΔN40 suppresses the expression of p21^{CDKN1A} during ER stress.
p53FL and p53ΔN40 (referred to as p53/47) mediate the p53-mediated cellular response under DNA damage and ER stress, respectively. While p53FL activation induces transcription of p21^{CDKN1A}, p53ΔN40 promotes transcription of 14-3-3σ and favours its further stabilization by preventing p21^{CDKN1A}-dependent induction of the E3-ubiquitin ligase COP1. Suppression of p21^{CDKN1A} by p53ΔN40 relies on inhibition of p53FL-dependent trans-activation of transcription activity and on direct inhibition of mRNA translation via part of the coding sequence of *p21^{CDKN1A}*. ER stress sensitizes cells to apoptosis after treatment with DNA-damaging agents. Taken from Mlynarczyk & Fåhraeus, 2014.

These results reinforce the existence of a dialogue in the p53 pathway based on the control of p21^{CDKN1A} expression and an intrinsic balance between two p53-downstream G1 and G2 regulatory factors (Mlynarczyk & Fahraeus, 2014). In addition, it adds to the idea that controlling mRNA translation is an important feature of the p53 response pathway during the UPR.

Here, we further questioned the capacity of p53 to control the translation of two key regulators, such as MDM2 and MDMX and the one of the sensor of UPR; BiP. In addition, we studied the link between p53-mediated control of BiP expression and induction of apoptosis.

Experimental work

The activity of p53 as a regulator of mRNA translation was shown to occur under normal conditions and in cells facing ER stress. Previous reports show that p53 has the capacity to directly bind to and to inhibit the translation of a small set of mRNAs. In this work, I present new evidence highlighting the role of p53 as a repressor of translation of three new mRNAs. Therefore, this section will be presented based on the manuscripts that are either published, under revision or being prepared to be submitted.

The first, which is the main focus of my thesis, is the mRNA of the ER chaperone BiP. BiP has essential functions in controlling the triggering and in mediating several responses of the UPR. The former refers to its capacity to bind and inhibit the UPR proximal sensors PERK, IRE1 and ATF6 and to sense the amount of misfolded or unfolded proteins in the ER lumen (Bertolotti et al, 2000; Shen et al, 2002). BiP has also principal roles as effector of the UPR in the folding, transport and degradation of proteins (Behnke et al, 2015; Morito & Nagata, 2015). In addition, it plays a role in signalling pathways related to apoptosis and proliferation in chemo-resistant tumours and it was actually proposed to be involved in tumour progression (Lee, 2014; Ma & Hendershot, 2004). Thus, control of BiP expression is of vital importance for proper cellular output. Herein, I uncover a new role of p53 as regulator of BiP synthesis. Indeed, I present data showing that p53 is able to bind *bip* mRNA to the very N-terminal region (346 nt) of the coding sequence and this correlates with down-regulation of *bip* mRNA translation. This capacity relies on a 7-aa domain of p53 protein located right after the p53ΔN40 initiation site, thus, it is present in the ER stress-induced p53 isoform. Interestingly, this capacity of p53 seems to be constitutive since it is also detected in normal conditions. However, the effect of controlling BiP expression becomes crucial during the UPR due to its relationship with apoptosis induction. The data presented also supports the idea that induction of apoptosis by p53 under ER stress correlates, at least in part, with activation of the BCL-2 interacting killer and pro-apoptotic BIK. This is mediated by induction of *bik* transcription by both p53 isoforms and by lowering the amount (and somehow changing the characteristics) of the inhibitory interaction BiP/BIK, as a result of BiP down-regulation. This offers a plausible molecular mechanism that might be important in the switch between the protective and killing phases of the UPR.

The other two mRNAs are those of MDM2 and MDMX. They share a high grade of homology at the level of protein primary structure, they both bind p53 and block its trans-activation capacity and their expression was found to be increased in some types of cancer and, interestingly, this was detected more often in tumours harboring wild-type p53 (Marine et al, 2007; Wade et al, 2013). However, they present very important differences; although they both contain a C-terminal RING domain, only MDM2 posses E3-ubiquitin ligase activity towards p53 (Fang et al, 2000; Wade et al, 2013). In addition, and despite both of them were shown to switch to act as positive regulators of p53 during the DNA damage response, MDMX was shown to act as an RNA chaperone that mediates folding of *p53* mRNA to generate the platform to which MDM2 binds, a requisite for inducing p53 protein synthesis. Another difference was also found in their p53-dependent expression pattern. While induction of *mdm2* transcription is one of the earliest events upon p53 activation during the DNA damage response, transcription of *mdmx*, although reported, seems to be less extended and remains controversial (Phillips et al, 2010). This shows that the effect p53 exerts on the expression of the MDM proteins is different, a fact that goes in line with the results presented here. Indeed, we have found that p53 controls the mRNA translation of both MDM2 and MDMX but with differences in the mechanisms and in the associated cellular context. Inhibition of MDM2 expression by p53 associates with a particular cellular condition; ER stress. In this case, I

show that p53FL-dependent induction of *mdm2* transcription is inhibited by increasing levels of p53ΔN40 isoform (p53/47) as a result of ER stress and I propose that both isoforms might down-regulate the translation of *mdm2* mRNA. Thus, the ER stress-dependent suppression of p53FL-induced *mdm2* transcription opens a window to detect the trans-suppression capacity of p53 towards MDM2. Interestingly, the region involved in the suppression of MDM2 expression is restricted to its own coding sequence and it shows similarities with the control that p53 exerts over $p21^{CDKN1A}$ expression during ER stress (Mlynarczyk & Fahraeus, 2014). On the other hand, the observations made for MDM2 differ to the mechanism controlling MDMX expression. In collaboration with a former PhD student in the lab, Anne-Sophie Tournillon, we have found that p53 binds the 5'UTR of *mdmx* in order to inhibit its translation. Induction of *mdmx* transcription was suggested to be important in the attenuation phase of p53 (Phillips et al, 2010), and thus, inhibition of MDMX synthesis might be an early event during the DNA damage response to favour p53 activation. Intriguingly, we could not see any difference in the capacity of p53 to inhibit MDMX expression when we compared normal and DNA damage conditions, suggesting that this activity would be constitutive.

The data presented below highlights the capacity of p53 to bind RNA in a specific manner and adds more support to its role as a controller or mRNA translation in resting conditions and in particular, during the UPR. In addition, it points out the opposing ways by which p53 regulates gene expression during the UPR as compared to the transcription-dependent gene regulation taking place during the DNA damage response. Finally, this work proposes for the first time a particular physiological response associated to RNA-binding and mRNA translation control by p53.

1. p53-mediated suppression of BiP triggers BIK-induced apoptosis during the Unfolded Protein Response

p53-mediated suppression of BiP triggers BIK-induced apoptosis during the Unfolded Protein Response

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Key words: p53, p53ΔN40 (p53/47), mRNA translation, ER stress, BiP, BIK, apoptosis

This work was supported by la Ligue Contre le Cancer, the Inserm and the project MEYS-NPS I-L01413. I. L. was supported by AXA Research Fund and Fondation pour la Recherche Médicale FRM (FDT20150532276). A-S. T. was supported by PACRI. The authors claim no conflict of interest.

NOTE: This article is currently under preparation.

Abstract

Physiological and pathological conditions that affect the folding capacity of the Endoplasmic Reticulum (ER) provoke ER stress and trigger the Unfolded Protein Response (UPR). The UPR aims to either restore the balance between newly-synthesized and misfolded proteins or if the damage is severe, to trigger cell death. The ER-resident chaperone BiP governs the UPR but the molecular events underlying the switch between reversible and irreversible cellular outcomes of the UPR are not well understood. Activation of the UPR promotes G2 cell cycle arrest and repair by inducing the alternative translated isoform, p53ΔN40 (p53/47), which via suppression of $p21^{CDKN1A}$ activates 14-3-3σ. Here we show that prolonged expression of p53ΔN40 promotes apoptosis by inhibiting the expression of BiP which leads to the activation of the pro-apoptotic BH3-only BIK. Suppression of BiP requires the specific binding to the first 346-nt of the *bip* mRNA and on a p53 domain located within residues 40-47 that together prevent *bip* mRNA translation. This work shows how p53 targets BiP to promote apoptosis during severe ER stress and further illustrates how regulation of mRNA translation plays a key role in p53-mediated regulation of gene expression during the UPR.

Introduction

Endoplasmic Reticulum (ER) stress constitutes a cellular state where the folding capacity of the ER is overwhelmed due to both physiological and pathological conditions. ER stress was shown to be linked to glucose deprivation, underglycosylation of glycoproteins, elevated protein synthesis and secretion and failure of protein folding, among others. All these phenomena lead to accumulation and/or aggregation of misfolded or unfolded proteins inside the ER and results in overwhelming of the ER protein quality control or ERQC (Behnke et al, 2015; Morito & Nagata, 2015). The ERQC possess three axes; the protein folding machinery itself, the Unfolded Protein Response or UPR and the ER-associated degradation of proteins or ERAD, a clearance system for misfolded or misassembled proteins. Interestingly, most ERAD components are targets of the UPR (Morito & Nagata, 2015).

The UPR first execute an adaptive pathway that aims at restoring the balance between newly synthesized and mature proteins. In mammals, the canonical UPR pathway counts on three transmembrane proximal sensors: inositol-requiring enzyme 1 (IRE1; α and β), protein kinase RNA-like ER kinase (PERK) and activating transcription factor 6 (ATF6; α and β) (Hetz et al, 2013; Zhao & Ackerman, 2006). Activation of IRE1 and PERK involves oligomerization and trans-autophosphorylation followed by formation of large clusters (Bertolotti et al, 2000). In addition, IRE1α describes a conformational change that activates its RNase domain and splices out a 26-nucleotide intron from the *xbp-1* mRNA via an unconventional mRNA splicing mechanism. This leads to production of the stable transcription factor XBP-1s involved in expression of several UPR target genes (Calfon et al, 2002; Lee et al, 2002; Yoshida et al, 2001). Moreover, IRE1α's RNase activity also degrades a subset of mRNAs through a process known as regulated IRE1-dependent decay (RIDD) of mRNA (Hollien & Weissman, 2006). Activated PERK mediates phosphorylation of translation initiation factor eIF2α resulting in general inhibition of cap-dependent translation (Jackson et al, 2010). However, this opens for the translation of mRNAs containing upstream open reading frames or uORFs in their 5′-UTRs, such as activating transcription factor 4 (ATF4) (Harding et al, 2000; Vattem & Wek, 2004). ATF4 up-regulates expression of genes related to REDOX proceses, amino acid metabolism, ER chaperones and foldases (Hetz et al, 2013). Finally, ATF6 translocates to the Golgi apparatus where it is processed by Site 1 and Site 2 proteases (S1P and

S2P) to release the cytosolic N-terminal fragment ATF6f; a transcription factor that migrates to the nucleus (Haze et al, 1999) and regulates the expression of genes with an ER stress response element (ERSE) in their promoters, such as those of the ER-associated degradation (ERAD) pathway, chaperones and notably XBP-1 (Haze et al, 1999; Lee et al, 2002; Yoshida et al, 1998). All this promotes the transduction of signals to the cytosol and nucleus in order to, at first, restore protein folding capacity by clearing the ER trough down-regulation of general protein synthesis and induction of protein quality control mechanisms and to restore the cellular homeostasis via antioxidant responses, amino acid metabolism, autophagy, and organelle biogenesis.

However, when damage is un-repairable, cells describe a mitochondria-dependent apoptotic pathway. ER stress-induced apoptosis is mainly attributed to the activity of CHOP (C/EBP homologous protein, also named growth arrest and DNA-damage-inducible 153, GADD153) which is downstream of ATF4 (Hetz et al, 2013). Although not clear, CHOP can induce apoptosis via GADD34, a component of the eIF2α phosphatase GADD34–PP1 that favours translation recovery, a requirement to develop death pathways. Moreover, BCL-2 family induction (BIM and PUMA) and down-regulation (BCL-2) has been reported upon CHOP activation (Urra et al, 2013). Interestingly, PERK or CHOP-deficient cells still undergo apoptosis indicating the existence of other checkpoints and signaling events mediating cell death (Urra et al, 2013; Zinszner et al, 1998).

The ER-resident chaperone Binding immunoglobulin protein BIP (also known as GRP-78 and HSPA5) is a stress-inducible molecular chaperone that belongs to the heat shock protein family (Lee, 2014). In addition to its activity as chaperone, BiP plays a key role as a sensor and regulator of the UPR and binds to and keep the three proximal sensors of ER stress PERK, IRE1 and ATF6 in an inactive form (Bertolotti et al, 2000; Shen et al, 2002). When misfolded proteins accumulate in the ER, they compete for BiP binding thereby releasing and activating the UPR sensors (Lee, 2014). Interestingly, BiP was also related to apoptosis repression in several cell lines and in mice (Lee, 2014; Luo et al, 2006) and this might be related with direct and repressive interaction of BiP with caspase-7 and -12 (Rao et al, 2002) and with the BH3-only pro-apoptotic member of the BCL-2 family, BIK (Fu et al, 2007; Zhou et al, 2011). Due to these important activities (and others), BiP overexpression was described as an adaptive response to stress that is

induced by cancer treatments, including chemotoxic drugs and anti-hormonal and DNA-damaging agents, etc. (Lee, 2014). This goes in line with the early suggestion that UPR highly contributes to different phases of tumor growth (Ma & Hendershot, 2004). UPR-driven induction of BiP was shown to be mediated at two different steps. Transcription from *bip* gene is induced upon activation and binding of ATF6f to *bip*'s ERSE-containing promoter (Yoshida et al, 1998). In addition, up-regulation of BiP also relies on sustained translation that depends on regulatory elements present in both BiP's 5'UTR and ORF (Gulow et al, 2002; Macejak & Sarnow, 1991; Starck et al, 2016).

BIK is the founding member of the BH3-only proteins and it exists as a free cytoplasmic and ER membrane-bound protein (Chinnadurai et al, 2008). BIK was reported to induce apoptosis through both direct and indirect mechanisms. The former refers to its ability to recruit and induce oligomerisation of BAK and BAX at the ER membrane that leads to ER Ca^{2+} depletion (Mathai et al, 2005) while the latter depends on displacement of BAX, but not BAK, from the anti-apoptotic regulators BCL-2 and BCL-XL to allow the apoptotic pathway to proceed (Chinnadurai et al, 2008; Gillissen et al, 2007). Interestingly, transcription of *Bik* gene was shown to be controlled, in part, by p53 (Germain et al, 2002; Mathai et al, 2002).

The tumour suppressor protein p53 is mainly known by its role as a transcription factor that both positively and negatively regulates the expression of a diverse multitude of genes. It becomes activated upon a plenitude of different insults, such as DNA damage, nutrient deprivation, viral infection or oncogene activation, among many others (Vousden & Lane, 2007). p53 activation can either result in cell cycle arrest and repair or irreversible responses such as apoptosis or senescence. In response to DNA damage, p53 triggers G1 cell cycle arrest via induction of $p21^{CDKN1A}$ transcription or, if the damage is severe, apoptosis via the induction of pro-apoptotic factors such as *Bax* or *Puma*. In response to ER stress and activation of PERK, the translation initiation of the p53 mRNA switches from the full length protein (p53FL) to the p53ΔN40 (p53/47) isoform that is initiated at the second in frame AUG located 40 codon downstream of the first AUG (Bourougaa et al, 2010; Candeias et al, 2006). Expression of p53ΔN40 is driven by an IRES element located between the above-mentioned two AUGs (Candeias et al, 2006; Ray et al, 2006). In addition, p53FL is de-stabilized by the cooperative action of PERK- and PKR-induced glycogen synthase-3 (GSK-3) kinase and MDM2 (Baltzis et al, 2007; Pluquet et al, 2005; Qu et al, 2004).

The p53ΔN40 lacks the MDM2-binding site and the first of p53's two trans-activation domains (TAD I). It actively suppresses the expression of p21^{CDKN1A} during ER stress via preventing p53FL-mediated induction of *p21^{CDKN1A}* transcription and by suppressing *p21^{CDKN1A}* mRNA translation, resulting in the induction and stabilization of 14-3-3σ and G2 cell cycle arrest (Bourougaa et al, 2010; Mlynarczyk & Fahraeus, 2014). p53-mediated suppression of mRNA translation during ER stress was also suggested for the *mdm2* mRNA (Lopez et al, 2015). This adds to previous studies implicating p53 in translation control of *mdmx*, *fgf-2*, *cdk4* and its own mRNAs, even though the physiological implications of p53's role is yet relatively unknown (Galy et al, 2001a; Galy et al, 2001b; Miller et al, 2000; Mosner et al, 1995; Tournillon et al).

Here we set out to better understand how the cells utilize the p53 pathway to switch between cell cycle arrest and repair vs. apoptosis in the UPR pathway. We show that p53-dependent apoptosis during the UPR requires a direct interaction between p53 and a small region of the 5' end of the coding sequence (CDS) of the *bip* mRNA. A short 7-aa region containing a helical domain of p53 within the trans-activation domain II (TAD II) mediates suppression of *bip* mRNA translation, which release BIK from BiP and triggers apoptosis. These results further emphasises the important role of p53-mediated mRNA translation control during the UPR.

Methods

Cell culture. p53-positive HCT116 (colon carcinoma) and A549 (lung carcinoma) or p53-null H1299 (non-small cell lung carcinoma) and Saos-2 (osteosarcoma) human cell lines were used. HCT116 cells were kindly provided by Professor B. Vogelstein (Johns Hopkins University, Baltimore, MD, USA). Other cell lines were purchased from American Type Culture Collection. H1299 and Saos-2 cells were cultured in RPMI 1640 medium (no glutamine), HCT116 cells in McCoy's 5A medium (modified, GlutaMAX) and A549 cells in Dulbecco's modified Eagle's medium medium (high glucose, no glutamine). All media (Gibco) were supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 $U.mL^{-1}$ penicillin and 100 $mg.mL^{-1}$ streptomycin (Gibco) except for McCoy's 5A where L-glutamine was not added. Cell lines were maintained at 37°C in an humidified 5% CO_2 incubator. All experiments were performed using exponentially growing cells and cell counts were carried out using a Malassez hemocytometer.

Cell transfection and treatment. 24 hours before transfection, $1.75x10^5$ cells were seeded in each well of a six-well plate for most experiments, $7.5x10^5$ cells in 10-cm diameter plates for metabolic pulse labelling and $1.5x10^4$ cells in each well of a 24-well plate for Proximity ligation assay and immunofluorescence. cDNA transfections were made using GeneJuice reagent (EMD Chemicals) as per manufacturer's protocol and empty vector was added when needed to keep constant the total amount of transfected DNA. When indicated, after 8 hours, medium was replaced for siRNA transfection. siRNAs targeting BiP, BIK, CHOP or p53 and AllStars negative control siRNA were from Qiagen and transfected using HiPerFect reagent (Qiagen) following manufacturer's instructions. Efficiency of siRNAs was assessed by western blot analysis. Cells were further incubated for 24 hours before treatment. Drugs' stock solutions were prepared in DMSO (Euromedex) and cells were treated with 50 nM thapsigargin (THAP.), 7.5 $mg.mL^{-1}$ tunicamycin or 0.1% DMSO for 24 hours unless specified otherwise.

Expression vectors. All constructs were in pcDNA3 unless otherwise indicated. p53wt and p53ΔN40 constructs have been described previously (Bourougaa et al, 2010; Mlynarczyk & Fahraeus, 2014) and are schematically represented in Figure 4a. p53wt codes for both p53 full length (p53FL) and p53ΔN40 isoforms. Site-directed mutagenesis was performed to create

p53ΔN47 by deleting the aa 2 to 8 from p53ΔN40. The BiP construct was made by amplifying the BiP's ORF from total mRNA extracted from H1299 cells, retro-transcribed using oligo(dT)$_{12\text{-}18}$ primer and then amplified by PCR using site restriction-containing primers restricted to the ORF of BiP. The HA-BiP plasmid was obtained by PCR amplification from the above-mentioned BiP with the forward primer containing the HA-tag in it and sub-cloned. The constructs carrying *bip* mRNA segments +1 to +982, +983 to +1965, +1 to +491, +492 to +982, +1 to +246 and +1 to +346 were amplified with specific primers from the BiP construct and sub-cloned into pCDNA3 and are schematically represented in Figure 5b. *bip346*-GFP (*bip-gfp*) reporter construct was made as follows: GFP ORF was amplified from pEGFP-N1 plasmid and was inserted into pCDNA3. The first 346 nt of the above-mentioned BiP construct were amplified and cloned in front of GFP's ORF in-frame and the in-frame Met codons 1 and 9 of BiP were sequentially converted into Ala (GCG) codons by site-directed mutagenesis.

Western blotting. Cells were lysed in buffer (20 mM HEPES KOH pH 7.5, 50 mM β-glycerophosphate, 1 mM EDTA pH 8.0, 1 mM EGTA pH 8.0, 0.5 mM Na_3VO_4 100 mM KCl, 10% glycerol and 1% Triton X-100) supplemented with complete protease inhibitor cocktail (Roche). Protein concentration was determined using Bradford (Bio-Rad) and equal protein amounts were separated by NuPAGE gel electrophoresis (Life technologies). After electrophoretic transfer to BioTrace NT pure nitrocellulose blotting membrane (PALL), membranes were blocked with 5% non-fat dry milk in Tris-buffered saline pH 7.6 containing 0.1% Tween-20. Proteins were probed by overnight (ON) incubation at 4°C with the following antibodies. Anti-HA-tag mouse monoclonal antibody (mAb), as well as anti-p53 CM-1 and ACMDD rabbit polyclonal antibodies (pAbs) were kindly provided by Dr. B. Vojtesek (Masaryk Memorial Cancer Institute, Brno, Czech Republic). For the ACMDD sera raised against the N-terminus of p53/47 and used where indicated, the membranes were pre-incubated with 0.4% paraformaldehyde (PFA) at room temperature for 1 h and washed with water before blocking. Anti-cleaved PARP-1 rabbit pAb was from Cell Signaling Technology, anti-BiP rabbit pAb and anti-CHOP mouse mAb were from Abcam, anti-BIK mouse mAb and anti-PARP-1 rabbit pAb were from Santa Cruz, anti-tubulin and anti-β-actin mouse mAbs were from Sigma-Aldrich, anti-GFP mouse mAb was from Roche. Membranes were then incubated with appropriate HRP-conjugated secondary antibodies (Dako) and detection was performed using

WestDura (Thermo) and either Hyperfilm (GE), CHEMI-SMART 5000 documentation system and Chemi-Capt software (Vilbert Lourmat) or myECL Imager and myImage Analysis software (Thermo). The two latter were used for protein bands quantification by densitometry analysis performed with either Bio-PROFIL Bio 1-D software (Vilbert Lourmat) or ImageJ.

Apoptosis assay. Both floating and attached cells were collected 24 hours following DMSO or THAP. treatment for HCT116 and H1299. Cells were then simultaneously stained with Annexin V-FITC and PI using the Annexin V-FITC Apoptosis Detection Kit from Sigma-Aldrich, as per manufacturer's instructions. Annexin V binds to exposed phosphatidylserines on early apoptotic cells, whereas the non-vital dye propidium iodide (PI) stains late apoptotic and necrotic cells. Counting of cells was performed for 20,000 events using BD FACSCanto II flow cytometer and analysis was carried out with BD FACSDiva software.

RNA extraction, reverse transcription and RT–qPCR. Total RNA was extracted with RNeasy Mini Kit (Qiagen) following manufacturer's instructions. cDNA synthesis was carried out using the Moloney murine leukaemia virus reverse transcriptase and Oligo$(dT)_{12\text{-}18}$ primer (Life technologies). RT–qPCR was performed on StepOne real-time PCR system (Applied Biosystems) using Perfecta SYBR Green FastMix, ROX (Quanta Biosciences) and the following primers: BiP-F 5'GCAACCAAAGACGCTGGAACT3', BiP-R 5'CCTCCCTCTTATCCAGGCCATA3', BIK-F 5'CCTGCACCTGCTGCTCAAG3', BIK-R 5'ACCTCAGGGCAGTGGTCATG3', β-Actin-F 5'TCACCCACACTGTGCCCATCTACGA3' and β-Actin-R 5'TGAGGTAGTCAGTCAGGTCCCG3'.

Metabolic radiolabelling and immunoprecipitation. After seeding, transfection and treatment as described above, cells were kept at 37°C, 5% CO_2 in Dulbecco's modified Eagle's starvation medium (without methionine, cystine and L-glutamine, Sigma-Aldrich) supplemented with 2% dialysed fetal bovine serum, 2 mM L-glutamine, 100 $U.mL^{-1}$ penicillin and 100 $mg.mL^{-1}$ streptomycin, and DMSO or THAP. for 1.5 hours together with 25 μM of the proteasome inhibitor MG132 during the last 50 min. Cells were metabolically radiolabelled with 45 $\mu Ci.mL^{-1}$ of EasyTag Express ^{35}S-methionine Protein Labeling Mix (PerkinElmer) for the last 20 min. Cells were lysed in buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl and 1% NP-40) supplemented with complete protease inhibitor cocktail (Roche). Equal protein amounts, as determined by Bradford, were pre-cleared with rabbit or mouse serum (Dako) and Dynabeads Protein G (Life Technologies). Samples

were immunoprecipitated by overnight incubation with anti-BiP rabbit pAb (Abcam), anti-HA-tag mouse mAB (provided by Dr. B. Vojtesek) or non-specific mouse IgG antibody (Jackson ImmunoResearch Laboratories) at 4°C and beads that were added 2 hours after the Abs. Beads were washed and boiled in 2X Laemmli buffer. Proteins were resolved in NuPAGE gel electrophoresis (Life Technologies), followed by fixation in 7% methanol and 20% acetic acid, amplification in Amplify (Amersham) and drying of the gels. Detection was achieved by exposure to X-ray film. Autoradiography of input samples confirmed equal incorporation of overall ^{35}S-methionine into cellular proteins. For quantification of immunoprecipitated radiolabelled proteins, gels were exposed to phosphor imager screen, scanned using a Storm 840 phosphorimager (Molecular Dynamics) and analysed with Image-Quant software (Molecular Dynamics).

In vitro translation. N-terminal His-tagged p53 protein was expressed in BL21(*DE3*) *Escherichia coli* strain transformed with the p53 ORF cloned into pET-28a (Novagen). Protein induction was carried out for 3 hours at 30ºC with 1mM IPTG in 2XYZ medium. Lysis was performed with 25 mM Hepes pH 8.0, 100 mM NaCl, 1mM Tris, 20 mM imidazole, 10% glycerol, 10 µM ZnSO4 and supplemented with protease inhibitor cocktail EDTA-free (Roche). p53 was then purified with HisTrap HP 1 mL columns (GE Healthcare Life Biosciences) and ÄKTApurifier 10 (GE Healthcare Life Biosciences) as per manufacturer's instructions. *bip* and *gfp* mRNAs were *in vitro*-synthesized and capped with mMESSAGE mMACHINE T7 kit (Ambion) following manufacturer's instructions and using as template the linearized pCDNA3 containing either BiP or GFP or bip-GFP sequences (described above). Both *bip* and *gfp* mRNAs (400 ng of each) in the same reaction, along with 0.5 µM of partially-purified p53 protein were pre-incubated in binding buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 0.02 µg.ml^{-1} yeast tRNA (Ambion) , 0.2 mg/ml BSA (Sigma-Aldrich) for 15 minutes at 37°C. *In vitro* translation assays were performed with 41 µCi.mL^{-1} of Easytag Express Protein Methionine Mix (PerkinElmer) and Reticulocyte Lysate system (Promega) according to the manufacturer's protocol during 1.5 hours at 30ºC and boiled in 2X Laemmli buffer. *In vitro* translation of *bip-gfp* was done with 400 ng of mRNA and as described above. Proteins were resolved in NuPAGE gel electrophoresis (Life Technologies), followed by fixation, amplification and drying of the gels. Detection was achieved by exposure to X-ray film.

In vitro protein-RNA co-immunoprecipitation. p53-purified protein was the same as the one used for *in vitro* translation. In addition to *bip* FL mRNA, segments +1 to +982, +983 to +1965, +1 to +491, +492 to +982, +1 to +246 and +1 to +346 were synthesized as described before. All binding reactions were carried out for 15 min at 37°C in binding buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 0.02 $\mu g.ml^{-1}$ yeast tRNA, 0.2 $mg.ml^{-1}$ BSA. 120 ng of recombinant p53 protein and a fixed amount (0.01 pmol) of different *bip* mRNAs were used. After incubation, RNA-protein complexes were pulled-down ON at 4°C using anti-p53 DO-12 mouse mAb kindly provided by Dr. B. Vojtesek and protein G sepharose Fast Flow (Sigma-Aldrich). The unbound fraction was recovered for later analysis and the bound RNA was released from the beads using proteinase K (Sigma-Aldrich) for 30 min at 55ºC. All RNA fractions were then extracted and purified using TRIzol protocol (Life Technologies). RT-qPCR was performed using primers for the different segments as it follows: FL (+1 to +1965), +1 to +982, +492 to +982; same F and R above-described for qPCR, +983 to +1965; F 5'GTCCCACAGATTGAAGTCACC3' and R 5'CCTGTACCCTTGTCTTCAGC3', +1 to +491 and +1 to +346; F 5'CACGCCGTCCTATGTCGC3' and R 5'TGTTCTCGGGGTTGGAGG3', +1 to +246; F 5'GGCCGCGTGGAGATCATC3' and R 5'GGCGGCATCGCCAATCAG3'. The relative binding of mRNAs to proteins was expressed as the ratio between bound and total (bound+free) RNA.

Immunofluorescence (IF) and proximity ligation assay (PLA). For IF, after seeding, transfection and treatment as described above, cover-slips with cells were briefly washed with PBS and fixed with 4% PFA for 20 min at RT, washed again with PBS and blocked with PBS 3% BSA, 0.1% saponin (blocking buffer) for 30 min at RT. Primary Abs were diluted in blocking buffer, 1:500 for anti-p53 CM-1 rabbit pAb, 10 $\mu g.mL^{-1}$ for anti-BiP rabbit pAb (Abcam) and 1.5 $\mu g.mL^{-1}$ for anti-BIK mouse mAb (Santa Cruz) and incubated in wet chamber for 1.5 hours at RT. After washes with PBS, goat secondary Abs anti-mouse IgG-Alexa488 and anti-rabbit IgG-Alexa647 (Molecular Probes, Life Technologies) diluted 1:500 into blocking buffer were added and incubated in wet chamber for 45 min at RT. Finally, samples were stained with 50 $ng.mL^{-1}$ DAPI in PBS for 5 min at RT and washed with PBS at RT before mounting. For protein-protein PLA, samples were treated as for IF until primary antibody incubation. After that, DuoLink II PLA kit (Sigma-Aldrich) was used following manufacturer's instructions using custom solutions, followed by DAPI staining, washing with PBS

and mounting. For protein-protein PLA followed of IF against p53, PLA protocol was followed until rolling-circle amplification. After that, samples were washed with PLA buffer B for 5 min at RT, incubated with 1:250 dilution of anti-p53-Alexa488 mouse mAb 1801 (Abcam) in blocking buffer for 40 min in wet chamber at RT, stained with DAPI in buffer B for 5 min at RT, washed with buffer B, rinsed with 0.01X buffer B and mounted. In protein-RNA PLA, cover-slips with cells were briefly washed with PBS and fixed with 4% PFA for 20 min at RT, washed again with PBS and incubated in 70% ethanol for 6 hours at 4°C. Samples were re-hydrated with PBS for 30 min at RT, permeabilized in PBS 0.4% Triton X-100, 0.05% CHAPS for 5 min at RT, washed with PBS, incubated in hybridization buffer (2X SSC, 0.2 $mg.mL^{-1}$ *E. coli* tRNA (Roche), 0.2 $mg.mL^{-1}$ sheared salmon sperm DNA (Life Technologies), 2 $mg.mL^{-1}$ BSA (Sigma-Aldrich)) in wet chamber for 30 min at RT and hybridized with 50 ng of DNA probe coupled to digoxigenin at its 3' (previously denatured at 80ºC for 5 min) in hybridization buffer in wet chamber ON at 37ºC. Anti-bip DNA probe 5'CTGGACGGGCTTCATAGTAGAAAAA-DIG3' (Eurogentec). Samples were briefly washed with wash buffer (2X SSC, 10% formamide), further washed twice with hybridization buffer for 20 min and once with PBS for 20 min all at 37ºC. From this point, the above-described PLA protocol was followed using 1:200 dilution of anti-digoxigenin mouse mAb (Sigma-Aldrich) and 1:500 for anti-p53 CM-1 rabbit pAb in blocking buffer.

Statistical analysis. Data shown are mean±S.D. of minimum three independent experiments. Two-tailed paired and unpaired Student's t-test was performed by comparing data to the corresponding reference point or as indicated and two-way ANOVA was used when different groups of samples were compared. P values are shown. *P<0.05; **P<0.01; ***P<0.001; NS, not significant.

Results

p53 induces apoptosis under ER stress

It was previously reported that ER stress leads to p53 activation and apoptosis induction (Li et al, 2006). We could confirm this by treating cells with thapsigargin that induces ER stress by inhibiting the sarcoplasmic/endoplasmic reticulum calcium ATPase pump, thereby preventing normal Ca^{2+} uptake into the ER from the cytosol. Early and late p53-dependent apoptosis was determined using Annexin-V-FITC and propidium iodide (PI) using FACS analysis in H1299 p53-null non-small cell lung carcinoma treated with 50 nM thapsigargin for 24 hours in the presence, or absence, of p53. Thapsigargin treatment alone induced BiP expression but did not generate a significant amount of apoptotic cells. p53 expression (300 ng of cDNA/1.75x10^5 seeded cells) under ER stress conditions increased the level of apoptotic cells in 60% as compared to the empty vector (EV)-transfected cells **(Figure 1a)**.

Cell death was further analysed by detection of caspase-mediated cleavage of poly (ADP-ribose) polymerase-1 (PARP-1) using western blotting (Chaitanya et al, 2010). **Figure 1b (left panel)** shows an increase in the 89-KDa fragment of PARP-1 (CL-PARP) in H1299 cells transfected with p53. A 15-fold increase in CL-PARP detection was observed in cells treated with thapsigargin and expressing p53 when compared to the EV-transfected. In line with this, an approximate 2.3-fold p53-dependent induction of CL-PARP was observed in cells treated with 7.5 $mg.mL^{-1}$ tunicamycin for 24 hours, a drug causing ER stress by inhibiting N-linked glycosylation **(Supplementary Figure 1a)**. When we used siRNA to suppress p53 in HCT116 p53-positive colon carcinoma cells, we observed a 40% reduction in thapsigargin-induced cleavage of PARP-1 **(Figure 1b, right panel)**. Similar results were obtained using p53-positive A549 lung carcinoma cells **(Supplementary Figure 1b)**. These results point towards an important role for p53 in inducing apoptosis during ER stress.

To address the kinetics of apoptosis induction, we determined PARP-1 cleavage during 24 hours in H1299 cells treated with 50 nM thapsigargin both in the absence and presence of p53. As shown in **Figure 1c**, PARP-1 cleavage was detected in cells treated with thapsigargin after 24 hours. Expression of p53 alone was sufficient to detect PARP-1 cleavage and gave a significant

additional increase in CL-PARP in cells treated with thapsigargin for 6 hours and longer. This indicates that p53 and ER stress together potentiate the induction of apoptosis. ER stress-induced apoptosis is commonly attributed to the activity of CHOP. However, CHOP expression peak was detected at 3 hours and peaked at 6 hours to gradually decrease after that and was not detected after 24 hours. This expression pattern was not affected by p53 **(Figure 1c)**. Even though CHOP levels peaked after 6 hours following thapsigargin treatment, CL-PARP was not observed until 24 hours unless p53 was expressed. When we down-regulated CHOP by siRNA, there was only a limited induction of apoptosis following thapsigargin treatment, as estimated by CL-PARP detection. However, cells expressing p53 displayed a 3-fold higher p53- and ER stress-dependent PARP-1 cleavage in the absence of CHOP, compared to cells transfected with control siRNA and treated with DMSO (**Figure 1d**). These results show that p53 can promote ER stress-induced apoptosis in a CHOP-independent fashion. This is in line with previous reports showing that CHOP-deficient cells undergo apoptosis upon ER stress induction (Urra et al, 2013; Zinszner et al, 1998).

BiP prevents ER stress- and p53-induced apoptosis

We have previously, and in this study, not been able to observe an induction of the pro-apoptotic genes during ER stress that are linked to p53 in response to DNA damage (**data not shown**). This could be due to the general suppression of protein synthesis during the UPR but we did, however, observe that the presence of p53 was associated with a reduced induction of BiP protein following thapsigargin treatment. As BiP has previously been suggested to have an anti-apoptotic effect in both cell lines and in mice (Lee, 2014; Luo et al, 2006) we set out to test if suppression of BiP can help explain p53's capacity to enhance ER stress-induced apoptosis in a CHOP-independent fashion. We first knocked-down BiP using siRNA and estimated apoptosis directly using FACS analysis and indirectly by detection of CL-PARP in cells treated with 50 nM thapsigargin for 24 hours. This resulted in an increase in apoptosis of approximately 40 % and 70 %, in both p53-negative (H1299) and p53-positive (HCT116) cell lines, respectively (**Figure 2a and Supplementary Figure 2a and b**). BiP knock-down in DMSO-treated cells did not result in any change in the rate of apoptosis and only a faint induction of cleaved PARP-1. Similarly, down-

regulation of BiP resulted in almost 7 and 2 fold induction of PARP-1 cleavage when compared to the cells transfected with control siRNA in H1299 and HCT116, respectively (**Figures 2a and b**).

We then asked whether the p53-dependent induction of apoptosis during ER stress is dependent on the levels of BiP expression by overexpressing p53 and/or BiP and estimated the change in apoptotic cells. As shown in **Figure 2c and Supplementary Figure 2c**, BiP transfection alone did not change the level of apoptosis under normal conditions or during ER stress. However, the p53- and ER stress-dependent apoptosis was counteracted by over 50 % following BiP overexpression. Importantly, p53-dependent apoptosis induction in the absence of ER stress was not affected by overexpression of BiP. PARP-1 cleavage analysis confirmed these data (**Figure 2d**). These data suggest that BiP levels play an important role in p53-mediated induction of apoptosis during ER stress.

p53 controls synthesis of BiP

In order to understand how BiP controls p53-mediated apoptosis during ER stress, we first wanted to know what lies behind the observed inverse correlation between p53 and BiP expression (**see Figures 1b, 2d**). When we introduced increasing amounts of exogenous p53 into H1299 cells treated with 50 nM thapsigargin for 24 hours we observed a dose-dependent down-regulation of endogenous BiP (**Figure 3a**). In addition, when we introduced an exogenous HA-tagged BiP construct consisting of the CDS of BiP and driven by the CMV promoter, we observed a p53 dose-dependent suppression of HA-BiP expression (**Figure 3a**). Using RT-qPCR we could confirm that endogenous *bip* mRNA levels were not affected by the presence of p53 under normal or ER stress conditions (**Figure 3b**). This suggests that p53 controls BiP's expression on a post-transcriptional level.

We then analysed the effect of p53 on the rate of BiP protein synthesis using metabolic pulse labelling. After a 20 min pulse with ^{35}S-Met in the presence of 25 μM of the proteasome inhibitor MG132, BiP was immunoprecipitated, separated by SDS-PAGE and the amount of newly synthesized protein was estimated by autoradiography. **Figure 3c** shows that the amount of BiP synthesized inversely correlates with the quantity of transfected p53, obtaining up to 45% down-regulation of BiP synthesis. Together with the observation that *bip* mRNA levels are not affected by

p53, this shows that the translation of the *bip* mRNA is compromised in the presence of p53 and that this effect is mediated via the CDS of the *bip* mRNA. To confirm this, we also evaluated the rate of BiP protein synthesis in an *in vitro* system. A His-tagged p53 protein was expressed in *Escherichia coli* and purified by affinity chromatography using Nickel columns (**Supplementary Figure 3**). In parallel, capped *bip* and control *gfp* mRNAs were transcribed *in vitro* and a mixture containing the same amount of mRNAs were pre-incubated with or without p53 protein for 15 min at 37ºC before the *in vitro* translation was performed using rabbit reticulocyte extracts. **Figure 3d** shows that GFP synthesis was not modified by the presence of p53 but the expression of BiP was reduced by 70 %. These data suggest that p53's negative effect on BiP synthesis during ER stress is mediated by a suppression of *bip* mRNA translation. In addition, the *in vitro* experiment indicates that p53's capacity to control translation of BiP does not depend on post-translational modifications by UPR pathways.

p53 full length and p53ΔN40 control BiP expression

To better understand the suppression of BiP synthesis and to gain insight into the mechanism of action, we tested different p53 isoforms. p53ΔN40 is a natural isoform of p53 that lacks the first 39 aa including the trans-activation domain I (TAD I), and the binding site for MDM2 as well as several important post-translational modifications sites. We used this isoform along with different N-terminal deletion mutants in order to identify the p53 domain affecting BIP expression (**Figures 4a, b and Supplementary Figure 4a**). p53ΔN40 and p53wt both down-regulated endogenous and HA-tagged BiP during ER stress. The effect on HA-BiP was detected in normal and stressed conditions, adding to the argument that the capacity to control BiP expression does not require ER stress-dependent activation of p53 (**Figure 4b and Supplementary Figure 4a and b**). Deletion of the 7 aa containing a helical domain (p53ΔN47) located adjacent to the initiation site for p53ΔN40 abolished the suppression of BiP expression (**Figure 4b**). RT-qPCR confirmed that endogenous *bip* mRNA levels were not affected (**Figure 4c**). This argues that the 7-aa region expanding from the initiation site of p53ΔN40 and the aa 47 on the full length protein is required for controlling expression of BiP.

We then analysed the effect of these three p53 constructs on apoptosis induction by looking at cleavage of PARP-1. While transfection of p53ΔN47 in H1299 did not modify the level of PARP-1 cleavage in cells treated with DMSO or with 50 nM thapsigargin for 24 hours, p53wt and p53ΔN40 induced CL-PARP both in normal and stress conditions (**Figure 4d**). These data show that the helical-containing short domain consisting of seven residues and located just C-terminal of the initiation of p53ΔN40 is required for the suppression of BIP synthesis and for part of p53-induced apoptosis.

p53 binds to the coding sequence of bip mRNA

We and others have shown that p53 binds to a selected set of mRNAs to control their translation (Galy et al, 2001a; Galy et al, 2001b; Miller et al, 2000; Mosner et al, 1995; Tournillon et al) and we have previously observed that p53 mediates control of the *p21*^{CDKN1A} mRNA via its CDS (Mlynarczyk & Fahraeus, 2014). In order to test if p53 binds the endogenous *bip* mRNA *in cellulo* we used the proximity ligation assay (PLA) assay. A 25 nt. DNA oligo corresponding to nt +1010 to +1029 of the *bip* mRNA coupled to digoxigenin in its 3' end was hybridized to fixed cells. We used an anti-digoxigenin mouse monoclonal antibody together with the CM-1 rabbit anti-p53 sera for the PLA reaction between endogenous *bip* mRNA and p53wt, p53ΔN40 and p53ΔN47 following treatment with thapsigargin (50 nM for 24 hours). The CM-1 polyclonal sera detected all three p53 constructs predominantly in the nucleus when analysed by immunofluorescence (IF) (**Figure 5a, left panels)**. However, the RNA-protein PLA signal was detected in the nuclear and the cytoplasmic compartments. Cells expressing EV where negative both for the IF and PLA (**Figure 5a, right panels and Supplementary Figure 5a**).

Having shown the proximity of p53 with the *bip* mRNA *in cellulo,* we next tested if this interaction was direct and to which region of the *bip* mRNA p53 binds. Recombinant purified p53 protein (**Supplementary Figure 3**) was incubated together with *in vitro*-synthesized *bip* mRNA for 15 minutes at 37ºC before anti-p53 DO1 mAb was added. Following immunoprecipitation, the *bip* mRNA bound to p53 and the unbound was quantified by RT-qPCR and the ratio bound:unbound was calculated. This revealed that p53 bound to the full length CDS of the *bip* mRNA (+1 to +1965) and more specifically to the first 491 nts of the CDS (+1 to +491)(**Figure 5b**). However, the +1 to +

246 sequence did not interact with p53 (data not shown), whereas the +1 to +346 did, suggesting that the +246 to +346 sequence is essential for the interaction (**Figure 5b**). None of the mRNAs covering the +346 to +1965 positions (without the first 346 nt) were bound to p53. The predicted secondary structure of the +1 to +346 sequence is shown in **Supplementary figure 5b**.

In order to test if this RNA sequence is sufficient to mediate p53-dependent suppression of protein expression we replaced the +1 AUG and the second in frame AUG codons with alanine (GCG) and fused this sequence to the GFP reporter (bip-GFP). This was expressed in H1299 cells in the presence of different p53 constructs and revealed an approximately 80% suppression of GFP expression using the p53ΔN40 and less than 20% with p53ΔN47. The effect on GFP expression alone using the p53ΔN40 and the p53ΔN47 was on average 20 % and 10 %, respectively (**Figure 5c**).

The p53ΔN40 and the p53ΔN47 constructs retain p53 DNA binding activity and in order to ensure that the effect on BIP expression was not related to p53 DNA binding activity we carried out *in vitro* mRNA translation assays. Recombinant p53 (**Supplementary Figure 3**) was pre-incubated with in vitro transcribed capped *bip-gfp* and *gfp* mRNAs for 15 minutes at 37ºC. Rabbit reticulocyte lysates where then added and the rate of produced GFP was estimated. This revealed that p53 suppressed the translation of the *bip-gfp* mRNA but had limited, or not, effect on the translation of the GFP message (**Figure 5d**).

These results show that the first 346 nt of the *bip* mRNA binds to p53 and that this interaction is sufficient to prevent BIP expression. Together with previous data showing that p53 does not affect *bip* mRNA levels and the metabolic pulse labelling in the presence of proteasome inhibitors, these data support the notion that p53 controls *bip* mRNA translation via a direct interaction.

p53 induces BIK levels and prevents its interaction with BIP

Having established that p53 suppresses BIP levels by inhibiting its synthesis and that this is sufficient to trigger apoptosis, we next set out to determine the mechanism whereby reduced BiP levels induced cell death. As our previous results have indicated that this is not via CHOP, we instead turned the attention to the BIP binding pro-apoptotic BCL2 interacting killer (BIK). The BiP/

BIK interaction takes place at the ER membrane, preventing BIK from activating BAX (Fu et al, 2007; Gillissen et al, 2007; Mathai et al, 2005; Zhou et al, 2011). We first observed that p53wt was able to induce *bik* mRNA levels both under normal and ER stress conditions (**Figure 6a**)(Germain et al, 2002; Mathai et al, 2002). Interestingly, the p53ΔN40 isoform up-regulated *bik* transcription to the same level as the p53wt, a capacity that has not been described so far. This is opposite to what occurs for other genes like $p21^{CDKN1A}$, since p53ΔN40 prevents its induction. However, p53ΔN47 did not affect *bik* levels. The *bik* mRNA expression pattern was mirrored by the BIK protein levels, although a bit diminished under ER stress (**Figure 6b**)**.**

We next studied the effect of BiP/BIK protein interaction using PLA under normal and ER stress conditions (50 nM of thapsigargin for 24 hours). Immunofluorescence shows that BIK (in green) (**Figure 6c, upper panel**) expression and cytoplasmic distribution was not greatly affected by ER stress. As expected, the levels of BiP (in red), on the other hand, increased dramatically following ER stress. The interaction between BIK and BIP was then assessed by PLA in the presence, or absence, of p53. In order to distinguish cell expressing p53 and at the same time carrying out PLA on the endogenous BIK/BiP interaction, we used a p53 antibody (1801) coupled to Alexa Fluor 488. This antibody was applied after the PLA reaction and allowed us at the same time to determine the red PLA interactions and green p53 staining (**Figure 6c, lower panel**). The BiP/BIK interaction occurs in the cytoplasm and is, interestingly, not affected by ER stress. It should be kept in mind that these cells grow fast and have a high protein production level and might therefore have a weak constitutive ER stress. But, nevertheless, it suggests that the basal levels of BiP are sufficient to sequester BIK under non-ER stress conditions. However, when we expressed p53 we observed a sharp drop in BiP/BIK interactions in cells treated with thapsigargin. p53 had no effect on the BiP/BIK interaction under normal conditions. Taken together with the observation that p53wt, as well as p53ΔN40, induce BIK levels, and that overexpression of BiP prevents p53-dependent apoptosis during ER stress, these observations offer a plausible molecular explanation for p53-induced apoptosis in cells enduring prolonged or severe ER stress.

Discussion

We have studied the role of p53 in inducing apoptosis after prolonged ER stress. Our data suggest a p53 pathway during the UPR that favours BIK-depending apoptosis acting at two different steps. First, induction of *bik* transcription by p53wt and p53ΔN40 correlates with increased BIK protein levels. Secondly, p53-mediated down-regulation of BiP expression leads to the release and activation of free BIK. Control of BiP expression by p53 occurs at the mRNA translation level and depends on the direct interaction between p53 and a short region of the CDS of BiP's mRNA and on a 7 aa trans-suppression domain of p53 located right after the initiation site of p53ΔN40.

The environmental conditions found in solid tumours are different compared to the ones observed in normal tissues. The low concentration of nutrients and/or oxygen and the high rate of protein production contribute to the generation of ER stress and therefore, impose a selective scenario. Induction of UPR constitutes an adaptive response that aims at restoring the balance between newly synthesized and mature proteins. This is mediated by promoting the expression of chaperones and ERAD components trough IRE1/XBP-1 and ATF6 together with a suppression of protein synthesis by PERK-mediated suppression of cap-dependent translation and by degrading specific mRNAs via RIDD IRE1 (Hetz et al, 2013; Zhao & Ackerman, 2006). However, translation of some mRNAs coding for proteins required for ER repair is favoured in a PERK-dependent way, as it is the case of p53ΔN40 (also called p53/47) (Bourougaa et al, 2010; Candeias et al, 2006). We have previously demonstrated that p53ΔN40 induces *14-3-3σ* transcription and this leads to G2 cell cycle arrest where protein synthesis is lower, adding a third mechanism to ER stress-mediated control of protein synthesis (Bourougaa et al, 2010). The stabilization of 14-3-3σ requires the suppression of $p21^{CDKN1A}$ which is mediated by inhibition of its mRNA translation by the two p53 isoforms and by suppression of p53FL-dependent transcription of $p21^{CDKN1A}$ due to the increased expression of p53ΔN40 (Mlynarczyk & Fahraeus, 2014). However, in the presence of severe stress, the UPR switches to a pro-death signaling pathway that ultimately ends in mitochondrial-dependent apoptosis (Hetz et al, 2013; Urra et al, 2013).

ER stress-induced apoptosis is commonly attributed to the activity of CHOP. However, CHOP-deficient cells still undergo apoptosis indicating the existence of other checkpoints and signaling events mediating cell death (Urra et al, 2013; Zinszner et al, 1998). We now show that p53-induced apoptosis during prolonged ER stress is also mediated via a p53-dependent suppression of protein synthesis. In this case, however, the target is the *bip* mRNA. BiP is a key component and regulator of the UPR that has been previously reported to counteract cell death induction (Lee, 2014; Luo et al, 2006).

Our results show that a direct binding between p53 and the 5' of the coding sequence of the *bip* mRNA is required to control BiP synthesis. Fusion of this RNA sequence to a GFP reporter mRNA results in 80% p53-dependent reduction in GFP expression. Control of mRNA translation by p53 has previously been reported for some other mRNAs although these were not linked to ER stress (Galy et al, 2001a; Galy et al, 2001b; Miller et al, 2000; Mosner et al, 1995; Tournillon et al). The lack of apparent sequence homology among the mRNAs implicated as targets for p53 trans-suppression activity add more evidence to the idea that p53 binding to RNA depends on RNA structure rather than sequence, as suggested before (Riley et al, 2006; Riley & Maher, 2007). In line with this, 2D prediction of mRNA folding revealed a well structured and stable motif within the first 346 nt of CDS of the *bip* mRNA. The fact that it is the coding sequence that mediates the p53 response is in line the suppression of $p21^{CDKN1A}$ mRNA translation that is also dependent on the CDS (Mlynarczyk & Fahraeus, 2014). Similar observations of translation suppression via the CDS were also made for mdm2 (Lopez et al, 2015). As *mdm2* and $p21^{CDKN1A}$ are induced by p53 on a transcriptional level during the DNA damage response, these observations highlight the difference in p53 activity during these two stress responses both in terms of mechanisms of gene expression control but also in terms of the down stream effectors.

The suppression of BiP expression also requires a 7-aa region of p53 containing an amphipathic α-helical domain located right after the initiation site of p53ΔN40 that was reported to bind factors like replication protein A (RPA) (Bochkareva et al, 2005) and the p62 and Tfb1 subunits of human and yeast TFIIH (Di Lello et al, 2006). It is plausible that the trans-suppressive activity of p53 depends on a yet unknown factor binding this domain. The activity towards *bip* mRNA translation does not require ER stress, indicating that p53 does not require specific

modifications to suppress mRNA translation. This notion is further supported by observations that recombinant p53 effectively suppresses BiP synthesis *in vitro*.

The capacity of p53ΔN40 to induce transcription of the BCL-2 interacting killer *bik* further adds to the notion that the TAD I (aa 1 to 40) and TAD II (aa 40 to 60) domains of p53 have specific cell biological down-stream targets. The TAD I is associated to control of cell cycle arrest and repair, as shown by p53 derivatives lacking TAD I but retaining TAD II that are unable to trans-activate, for example, $p21^{CDKN1A}$ (Ghosh et al, 2004; Mlynarczyk & Fahraeus, 2014; Phang et al, 2015). On the other hand, the TAD II will impinge on apoptosis-related genes as supported by the induction of *Bax* (Yin et al, 2002) and here on *bik*. The capacity of p53ΔN40 to induce apoptosis upon exposure to a variety of stress signals also includes genes such as *Fas*, *Dr5*, *Api1* and *Pig3* (Phang et al, 2015). In addition, the inability to activate $p21^{CDKN1A}$-like genes was shown to depend on lack of acetylation on K382 that requires the presence of N-terminal domain of p53 (Phang et al, 2015). Under these conditions of active ER stress we could not observe the induction of pro-apoptotic genes related to the p53 response during DNA damage and ER stress such as PUMA and NOXA (Li et al, 2006).

The suppression of BiP induction during the UPR liberates BIK from its interaction with BiP. BiP/BIK interaction occurs at the ER membrane (Fu et al, 2007) and BiP competes with BCL-2 for binding to BIK, suggesting a model in which BCL-2/BIK and BiP/BIK complexes are mutually exclusive, offering an interesting model for how suppression of BiP results in BIK-dependent induction of the apoptotic response (Fu et al, 2007; Zhou et al, 2011). Some, but not all, of the steps that lead to BiP/BIK dissociation are ER stress-dependent and while the down-regulation of BiP by p53 occurs under normal and stress conditions, the release of BiK from BiP depends on both p53 and ER stress (as shown in Figure 6c). It is possible that the latter reflects the competition between BiP binding to its downstream effectors of the UPR pathway and misfolded proteins. Hence, only when the levels of BiP becomes decisive for the cellular response does the suppression of BiP levels play a determining role. It should be pointed out that it is not known if the affinity of BiP to the different mediators of the UPR is similar, or if the accumulation of misfolded proteins leads to a successive induction of the different UPR axis.

BiP overexpression is an adaptive response to stresses that are induced by cancer treatments and therapies. Thus, activating endogenous p53 in the context of BiP-overexpressing resistant tumours could offer a way to sensitize such cells to apoptosis induction.

Acknowledgements

This work was supported by la Ligue Contre le Cancer, the Inserm and the project MEYS-NPS I-L01413. I. L. was supported by AXA Research Fund and Fondation pour la Recherche Médicale FRM (FDT20150532276). A-S. T. was supported by PACRI. The authors claim no conflict of interest.

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Figure legends

Figure 1. p53 induces apoptosis during ER stress

a) p53-null non-small cell lung carcinoma H1299 cell line expressing or not p53 were treated with DMSO or 50 nM of thapsigargin (THAP.) for 24 hours and analysed for apoptosis by flow cytometry after staining with Annexin V-FITC and propidium iodide (PI). Representative dot plots show the discrimination of viable cells (FITC- PI-, Q3), early apoptotic (FITC+ PI-, Q4) and late apoptotic or necrotic cells (FITC+ PI+, Q2). Histogram shows the relative change in percentage of cells in early and late apoptosis/necrosis compared to empty vector (EV)-transfected and DMSO-treated cells, set to 1 (mean±S.D., n=5). Two-tailed paired t-test compared data as indicated, **p<0.01, ***p<0.001, ns not-significant.

b) p53-null H1299 or p53-proficient colon carcinoma HCT116 cell lines were transfected or not with p53 cDNA or siRNA against p53, respectively, and treated as described in Figure 1a. Levels of apoptotic marker cleaved PARP-1 (85-kDa fragment; CL-PARP) and p53 isoforms were detected by western blotting. ACMDD serum was used to detect p53 isoforms, full-length PARP-1 (FL-PARP) confirmed PARP-1 expression levels are not modified, BiP was used as a positive control for UPR activation and β-actin as a loading control. Numbers below the blots correspond to relative quantification by densitometry compared to the reference point set to 1.

c) H1299 cells transfected or not with p53 cDNA were incubated with 50 nM thapsigargin (THAP.) at indicated times. Levels of pro-apoptotic CHOP and apoptotic marker CL-PARP were detected by western blotting to follow apoptosis induction. FL-PARP confirmed PARP-1 expression levels are not modified, BiP expression showed induction of UPR and β-actin was used as a loading control. p53 isoforms were detected with ACMDD sera. Numbers below the blots correspond to relative quantification by densitometry compared to the reference point set to 1.

d) H1299 transfected or not with p53 cDNA and treated as described in Figure 1a were transfected with siRNA targeting CHOP or control siRNA. Down-regulation of CHOP was confirmed by western blotting and its effect on apoptosis induction was assessed by detection of CL-PARP. FL-PARP confirmed PARP-1 expression levels are not modified, BiP was analysed as readout of UPR

activation and β-actin was used as a loading control. p53 isoforms were detected with ACMDD sera. For all, blots represent n≥2.

Figure 2. BiP prevents ER stress- and p53-induced apoptosis

a) p53-null H1299 or p53-proficient HCT116 cells transfected with siRNA against BiP or control were treated and analysed by cytometry as described in Figure 1a. Histograms show the relative change in percentage of cells in early and late apoptosis/necrosis compared to control siRNA-transfected and DMSO-treated cells, set to 1 (mean±S.D., n=4). Two-tailed paired t-test compared data as indicated, *p<0.05, ns not-significant. See also Supplementary Figure 2 a and b.

b) H1299 and HCT116 cell lines were transfected or not with siRNA against BiP or control as in Figure 2a and treated as in Figure 1a and b. Levels of apoptotic marker CL-PARP and BiP were detected by western blotting. FL-PARP confirmed PARP-1 expression levels are not modified and β-actin was used as a loading control. Numbers below the blots correspond to relative quantification by densitometry compared to the reference point set to 1.

c) Apoptosis induction was analysed in H1299 transfected with BiP and/or p53, treated with thapsigargin (THAP.) and stained with FITC and PI as described in Figure 1a. Histograms show the relative change in percentage of cells in early and late apoptosis/necrosis compared to EV-transfected and DMSO-treated cells, set to 1 (mean±S.D., n=5). Two-tailed paired t-test compared data as indicated, *p<0.05, **p<0.01, ns non-significant.

d) Apoptosis induction was estimated by western blotting detection of CL-PARP in H1299 transfected and treated as in Figure 2c. The pro-apoptotic CHOP was also detected and shows no important variation. BiP was detected to monitor the UPR and siRNA transfection, FL-PARP confirmed PARP-1 expression levels are not modified and β-actin was used as a loading control. p53 isoforms were detected with ACMDD sera. Numbers below the blots correspond to relative quantification by densitometry compared to the reference point set to 1. For all, blots represent n≥2.

Figure 3. p53 controls BiP protein synthesis

a) Expression level of endogenous BiP and exogenous HA-tagged BiP containing only the coding sequence were studied in H1299 cells transfected with increasing amounts of p53 cDNA and treated with 50 nM thapsigargin (THAP.) or DMSO for 24 hours. HA-BiP expression was differentiated from the endogenous BiP by the use of an Ab against the HA tag. CM-1 sera was used to detect p53 isoforms and β-actin was used as a loading control.

b) Expression of endogenous *bip* mRNA was quantified using relative RT-qPCR in H1299 cells transfected or not with p53 cDNA and treated with 50 nM thapsigargin (THAP.) or DMSO for 24 hours. Values were normalized against actin and are presented as fold change relative to EV-transfected and DMSO-treated cells, set to 1 (mean±S.D., n=3 performed in duplicates). 2-way ANOVA compared data of the effect of treatment and transfection of p53 on *bip* mRNA expression as indicated, **$p<0.01$.

c) De novo BiP protein synthesis. H1299 transfected with increasing amounts of p53 cDNA and incubated with 50 nM thapsigargin (THAP.) for 24 hours were metabolically pulse labelled with ^{35}S-Met for 20 min in the presence of proteasome inhibitor MG132. Cell extracts were immunoprecipitated (IP) with BiP Ab and levels of IP radiolabelled protein were assessed by autoradiography. Input samples served as control for equal incorporation of ^{35}S-Met into cellular proteins. Western blotting (WB) showed increasing expression of p53 isoforms detected with CM-1 sera and β-actin was used as a loading control. Numbers below the autoradiography correspond to relative quantification compared to the reference point set to 1. Autoradiograph and western blotting shown are representative of n≥2.

d) *In vitro* translation of *bip* and *gfp* mRNAs in the presence of p53. A His-tagged p53 protein that was expressed in bacteria and partially purified using affinity chromatography was pre-incubated with a mixture of *in vitro*-synthesized *bip* and control *gfp* mRNAs for 15 min at 37ºC. Then, they were translated *in vitro* at 30ºC for 1.5 hours using rabbit reticulocyte extract in the presence of ^{35}S-Met. Level of radiolabelled proteins were assessed by autoradiography. Autoradiograph shown is representative of n≥2.

Figure 4. p53 full length and p53ΔN40 control BiP expression

a) Cartoon representing the p53 isoforms and deletion mutant used to localize the region involved in control of BiP expression. p53 wild-type (p53wt) cDNA codes for both p53 full length (FL) and p53ΔN40 isoforms. p53ΔN40 (also known as p53/47) lacks the first 39 aa of p53FL. p53ΔN47 lacks the first 7 aa of p53ΔN40. TAD; trans-activation domain I and II, PRR; Proline-rich region, DBD; DNA-binding domain, NLS; nuclear localization signal, TET; tetramerization domain, Ct; Carboxi-terminal regulatory domain. The interacting site for some important and representative factors are signalled on top of the image. p300/CBP; transcriptional co-activator CREB-binding protein, MDM2; minute-deficient mouse 2, RPA; replication protein A, TFIIH; transcription factor II H that binds through its p62 subunit.

b) p53wt and p53ΔN40 isoforms but not p53ΔN47 reduce expression of BiP during ER stress. H1299 cells were transfected with different p53 cDNA constructs depicted in Figure 4a, along with HA-tagged BiP and treated with 50 nM thapsigargin (THAP.) or DMSO. Expression level was assessed by western blotting directed to BiP and HA-tag with specific antibodies. p53 isoforms were detected with CM-1 sera and β-actin was used as a loading control.

c) Expression of endogenous *bip* mRNA was quantified using relative RT-qPCR in H1299 cells transfected with different p53 cDNA constructs depicted in Figure 4a and treated as in 4b. Values were normalized against actin and are presented as fold change relative to EV-transfected and DMSO-treated cells, set to 1 (mean±S.D., n=3 performed in duplicates). 2-way ANOVA compared data of the effect of treatment and transfection of p53 on *bip* mRNA expression as indicated, $^{**}p<0.01$.

d) Induction of apoptosis was established by analysing the amount of CL-PARP detected by western blotting in H1299 cells transfected with different p53 cDNA constructs depicted in Figure 4a. Level of apoptotic marker CL-PARP was detected. FL-PARP confirmed PARP-1 expression levels are not modified and β-actin was used as a loading control. p53 isoforms were detected with CM-1 sera. Numbers below the blots correspond to relative quantification by densitometry compared to the reference point set to 1. For all, blots represent n≥2.

Figure 5. p53 binds to a short segment of *bip* mRNA

a) Immunofluorescence (IF) of p53 protein (left panels) and proximity ligation assay (PLA) (right panels) between p53 and *bip* mRNA in H1299 transfected or not with p53 cDNAs described in Figure 4a and treated with 50 nM thapsigargin (THAP.) for 24 hours. IF and PLA were performed with CM-1 rabbit antibody anti p53 and in the PLA a mouse antibody against digoxigenin that was coupled to the 3' end of a DNA probe against *bip* mRNA was added to localize the interaction. Images are magnified 63 times and are representative of several cells obtained in two independent experiments. See also Supplementary Figure 5a for PLA controls.

b) *In vitro* protein-RNA co-immunoprecipitation shows the specific interaction between p53 and a small region of *bip* mRNA. Upper panel shows the different regions of *bip* mRNA that were cloned and used to generate mRNA *in vitro* to assess their capacity to interact with p53. Lower panel shows the quantification of different *bip* mRNAs bound to p53 as determined by RT-qPCR. Data is presented as percentage of RNA bound to p53 and represents the ratio between the RNA bound to protein and the total RNA (bound+unbound), normalized to the corresponding no protein partner, set to 100% (mean±S.E.M., n≥3 performed in triplicates). Two-tailed unpaired t-test compared data to the corresponding reference point, *p<0.05, **p<0.01, ns non-significant.

c) Control of GFP expression from reporter construct bip-GFP by p53. H1299 were co-transfected with either GFP or bip-GFP (+1 to +346 segment of *bip* mRNA without in frame Met fused to GFP CDS) and p53ΔN40 and p53ΔN47 cDNAs and GFP expression was analysed by western blotting. HSP90α+β was used as a loading control. Numbers below the blots correspond to relative quantification by densitometry compared to the reference point set to 1. Blot shown is representative of n=2.

d) *In vitro* translation of *bip-gfp* and *gfp* mRNAs in the presence of p53. A His-tagged p53 protein that was expressed in bacteria and partially purified using affinity chromatography was pre-incubated with *in vitro*-synthesized *bip-gfp* or control *gfp* mRNAs for 15 min at 37ºC. Then, they were translated *in vitro* at 30ºC for 1.5 hours using rabbit reticulocyte extract in the presence of ^{35}S-Met. Level of radiolabelled proteins were assessed by autoradiography. Autoradiograph shown is representative of n=3.

Figure 6. p53 activates BIK

a) Quantification of endogenous *bik* mRNA by relative RT-qPCR. H1299 cells were transfected with different p53 cDNAs depicted in Figure 4a and treated with 50 nM thapsigargin (THAP.) or DMSO for 24 hours. Values were normalized against actin and are presented as fold change relative to EV-transfected and DMSO-treated cells, set to 1 (mean±S.D., n=3 performed in duplicates). Two-tailed paired t-test compared data to the corresponding EV-transfected cells, *p<0.05, **p<0.01, ns non-significant.

b) BIK protein levels analysed by western blotting. Same samples as in Figure 6a were used to detect endogenous BIK protein. β-actin was used as a loading control. p53 isoforms were detected with CM-1 sera. Blots represent n≥2.

c) Immunofluorescence (IF) and proximity ligation assay (PLA) of BiP and BIK protein in normal and in 50 nM thapsigargin (THAP.) for 24 hours treated cells. p53 status was revealed by IF against p53 with an Ab labelled with Alexa Fluor 488 (1801). Images are magnified 63 times and are representative of n≥10 pair of cells analysed in two independent experiments.

Supplementary Figure 1 (relates to Figure 1b)

a) p53-null H1299 cell line was transfected or not with p53 cDNA and treated with 7.5 $mg.mL^{-1}$ tunicamycin (TUN.) for 24 hours. Levels of apoptotic marker cleaved PARP-1 (85-kDa fragment; CL-PARP) and p53 isoforms were detected by western blotting. CM-1 serum was used to detect p53 isoforms, BiP was used as a positive control for UPR activation and β-actin as a loading control. Numbers below the blots correspond to relative quantification by densitometry compared to the reference point set to 1.

b) p53-proficient A549 lung carcinoma cell line was transfected with siRNA against p53 or control and treated with 50 nM thapsigargin (THAP.) for 24 hours. Levels of apoptotic marker cleaved PARP-1 (85-kDa fragment; CL-PARP) and p53 isoforms were detected by western blotting. CM-1 serum was used to detect p53 isoforms, full-length PARP-1 (FL-PARP) confirmed PARP-1 expression levels are not modified, BiP was used as a positive control for UPR activation and β-actin as a loading control.

Supplementary Figure 2

a) and **b) (relates to Figure 2a)** p53-null H1299 or p53-proficient HCT116 cells transfected with siRNA against BiP or control were treated and analysed by cytometry as described in Figure 1a. Representative dot plots show the discrimination of viable cells (FITC- PI-, Q3), early apoptotic (FITC+ PI-, Q4) and late apoptotic or necrotic cells (FITC+ PI+, Q2) in H1299 and HCT116 cells, respectively.

c) (relates to Figure 2c) Apoptosis induction was analysed in H1299 transfected with BiP and/or p53, treated with thapsigargin (THAP.) and stained with FITC and PI as described in Figure 1a. Dot plots show the discrimination of viable cells (FITC- PI-, Q3), early apoptotic (FITC+ PI-, Q4) and late apoptotic or necrotic cells (FITC+ PI+, Q2).

Supplementary Figure 3 (relates to Figures 3d, 5b and 5d)

Coomassie staining of cell extract from E. coli BL21(DE3) expressing full length His-p53 and the His-p53 partially purified with affinity columns used for *in vitro* co-immunoprecipitation and *in vitro* translation experiments.

Supplementary Figure 4

a) (relates to Figure 4b) H1299 cells were transfected with different and increasing amounts of p53 cDNA constructs p53ΔN40, p53ΔN43, p53ΔN47 (lacking the first 39, 43 and 47 aa of p53FL, respectively) along with HA-tagged BiP and treated with 50 nM thapsigargin (THAP.) or DMSO for 24 hours. Expression level was assessed by western blotting directed to BiP and HA-tag with specific antibodies. p53 isoforms were detected with CM-1 sera and β-actin was used as a loading control.

b) (relates to Figure 4b) De novo HA-BiP protein synthesis. p53-null osteosarcoma Saos-2 were transfected with cDNAs coding for p53 isoforms described in Figure 4a along with GFP and OVA-HA cDNAs as controls, were metabolically pulse labelled with ^{35}S-Met for 20 min in the presence of proteasome inhibitor MG132. Cell extracts were immunoprecipitated (IP) with HA Ab (provided by Dr. Borek Vojtesek) and GFP Ab (Roche) and levels of IP radiolabelled proteins were assessed by autoradiography (left panels). Input samples served as control for equal incorporation of ^{35}S-Met

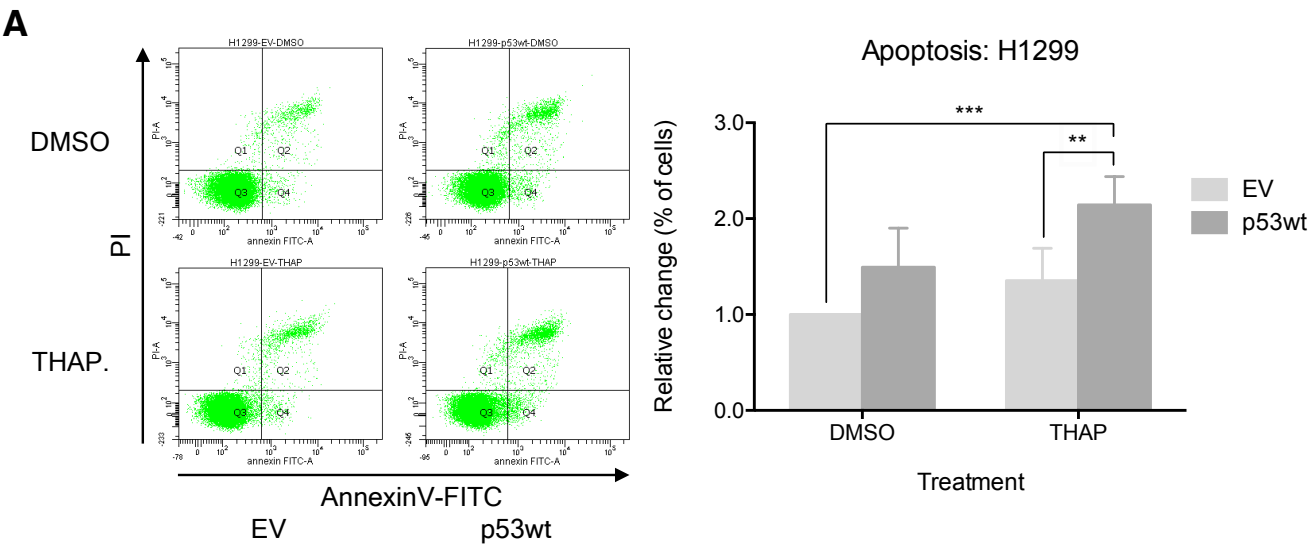
into cellular proteins. Western blotting (WB) showed expression of p53 isoforms detected with CM-1 sera and β-actin was used as a loading control. Autoradiograph and western blotting shown are representative of n=3. Histogram on the right shows the relative quantification of HA-BiP expression normalized to corresponding input and the EV-transfected cells set to 1, in three independent experiments. For quantification of immunoprecipitated radiolabelled proteins, gels were exposed to phosphor imager screen, scanned using a Storm 840 phosphorimager (Molecular Dynamics) and analysed with Image-Quant software (Molecular Dynamics). GFP and OVA-HA levels were marginally affected by p53 expression. OVA stands for chicken ovalbumin and its CDS was amplified by PCR and cloned into pCDNA-3 with the HA-tag on the C-terminal. Two-tailed paired t-test compared data to the EV-transfected cells, *$p<0.05$, ns non-significant.

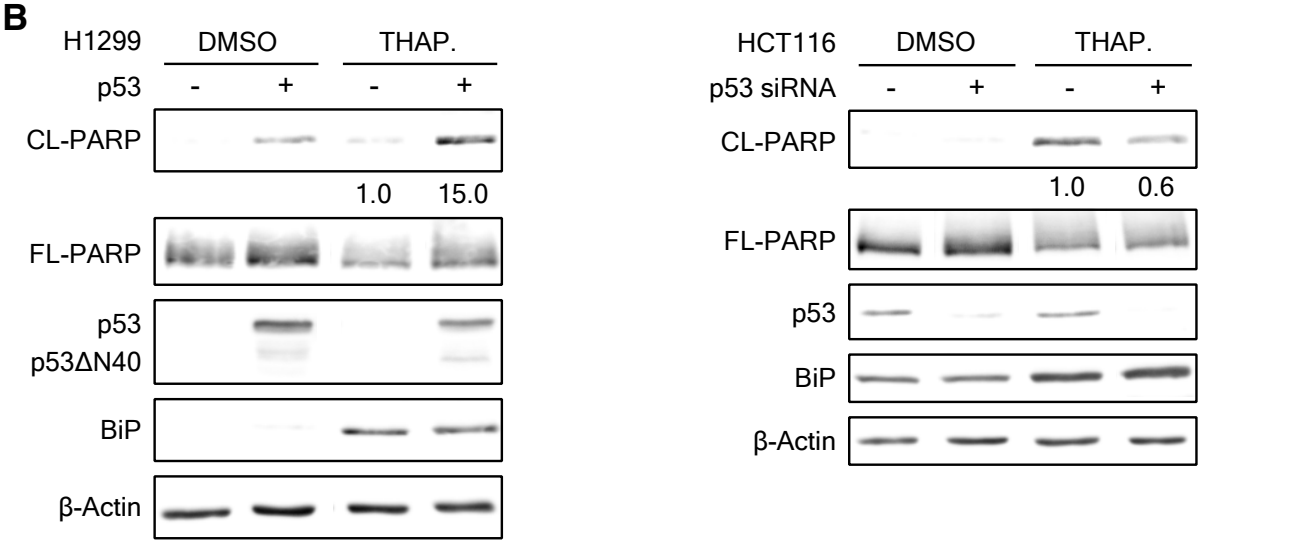
Supplementary Figure 5

a) (relates to Figure 5a) Controls of proximity ligation assay (PLA) between p53 and *bip* mRNA in H1299 transfected or not with p53 cDNAs described in Figure 4a and treated with 50 nM thapsigargin (THAP.) or DMSO for 24 hours. The figure shows the control without oligo DNA probe anti endogenous *bip* mRNA coupled to digoxigenin but with anti-digoxigenin mouse Ab (no probe) and the control without anti-digoxigenin mouse Ab in the presence of the probe (anti Dig only). PLA was performed along with CM-1 rabbit Ab anti p53. Images are magnified 63 times and are representative of n≥10 cells obtained in two independent experiments. See also Supplementary Figure 5a for PLA controls.

b) (relates to Figures 5b, c and d) Predicted mRNA secondary structure of the first 346 nt of bip mRNA (+1 to +346) that was shown to be the region p53 binds. Prediction was done with mFOLD application and shows a well structured folding. Heat code shows the probability from 0 (violet) to 1 (red) for the folding to be truth (Zuker, 2003).

Figure 1


A
DMSO
THAP.
PI
H1299-EV-DMSO
H1299-p53wt-DMSO
H1299-EV-THAP
H1299-p53wt-THAP
annexin FITC-A
PI-A
Q1
Q2
Q3
Q4
AnnexinV-FITC
EV
p53wt
Apoptosis: H1299
Relative change (% of cells)
3.0
2.0
1.0
0.0
***
**
DMSO
THAP
Treatment
EV
p53wt


B
H1299
DMSO
THAP.
p53
-
+
-
+
CL-PARP
1.0
15.0
FL-PARP
p53
p53ΔN40
BiP
β-Actin
HCT116
DMSO
THAP.
p53 siRNA
-
+
-
+
CL-PARP
1.0
0.6
FL-PARP
p53
BiP
β-Actin

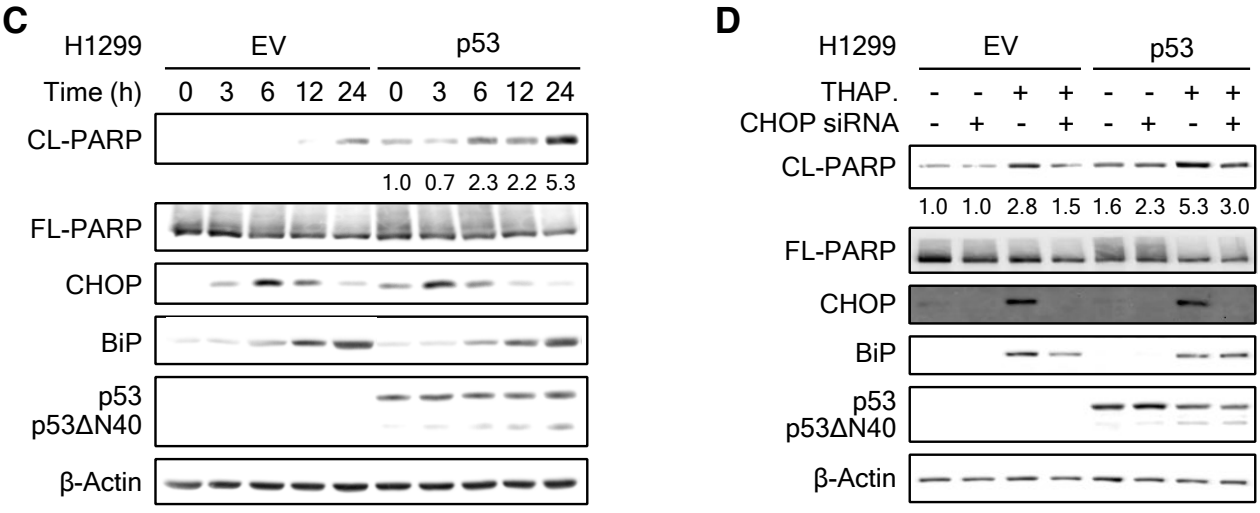

C
H1299
EV
p53
Time (h)
0 3 6 12 24 0 3 6 12 24
CL-PARP
1.0 0.7 2.3 2.2 5.3
FL-PARP
CHOP
BiP
p53
p53ΔN40
β-Actin
D
H1299
EV
p53
THAP.
- - + + - - + +
CHOP siRNA
- + - + - + - +
CL-PARP
1.0 1.0 2.8 1.5 1.6 2.3 5.3 3.0
FL-PARP
CHOP
BiP
p53
p53ΔN40
β-Actin

Figure 2

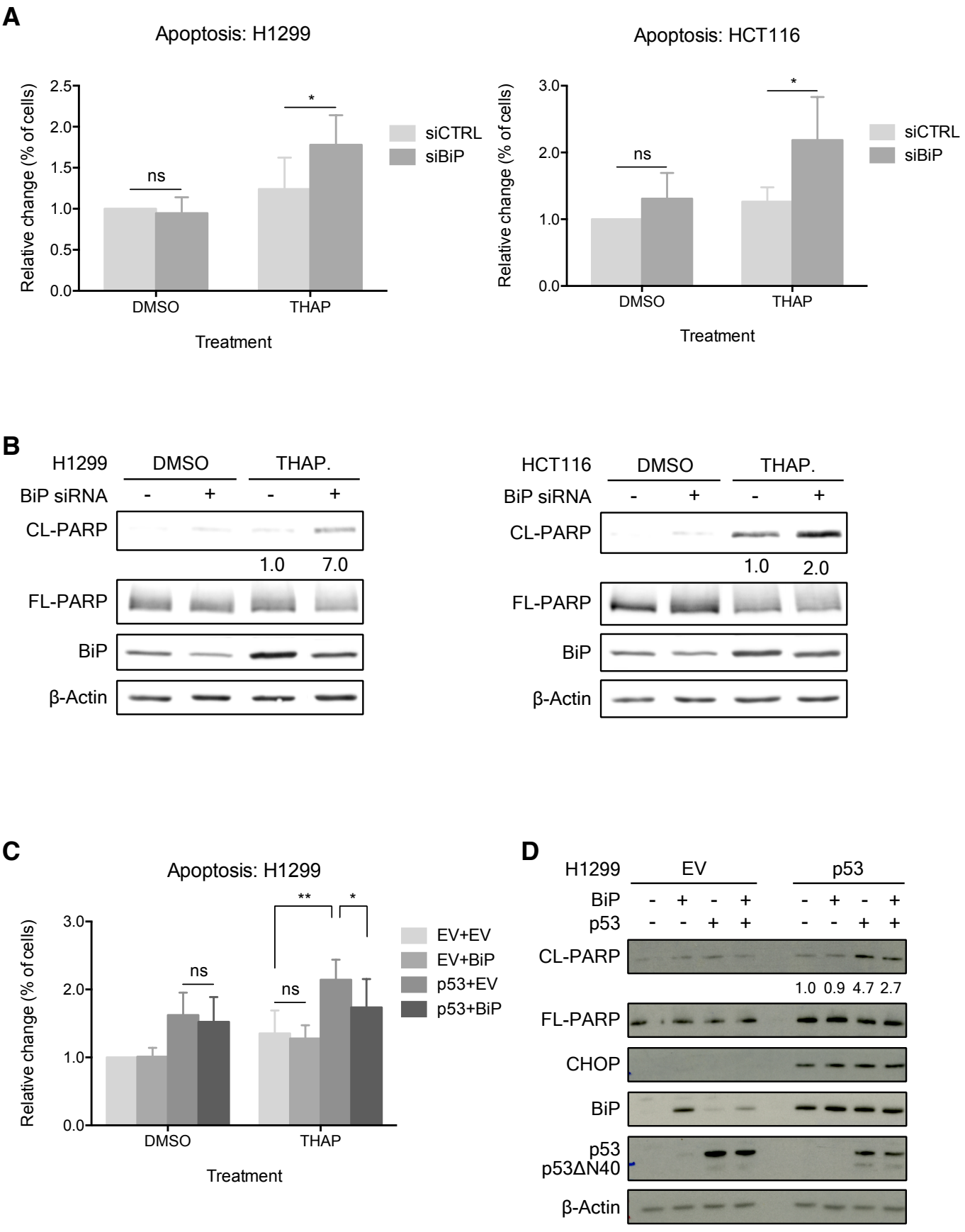

A
Apoptosis: H1299
Relative change (% of cells)
2.5
2.0
1.5
1.0
0.5
0.0
ns
*
DMSO
THAP
Treatment
siCTRL
siBiP
Apoptosis: HCT116
Relative change (% of cells)
3.0
2.0
1.0
0.0
ns
*
DMSO
THAP
Treatment
siCTRL
siBiP
B
H1299
DMSO
THAP.
BiP siRNA
- + - +
CL-PARP
1.0 7.0
FL-PARP
BiP
β-Actin
HCT116
DMSO
THAP.
BiP siRNA
- + - +
CL-PARP
1.0 2.0
FL-PARP
BiP
β-Actin
C
Apoptosis: H1299
Relative change (% of cells)
3.0
2.0
1.0
0.0
**
*
ns
ns
DMSO
THAP
Treatment
EV+EV
EV+BiP
p53+EV
p53+BiP
D
H1299
EV
p53
BiP - + - + - + - +
p53 - - + + - - + +
CL-PARP
1.0 0.9 4.7 2.7
FL-PARP
CHOP
BiP
p53
p53ΔN40
β-Actin

Figure 3

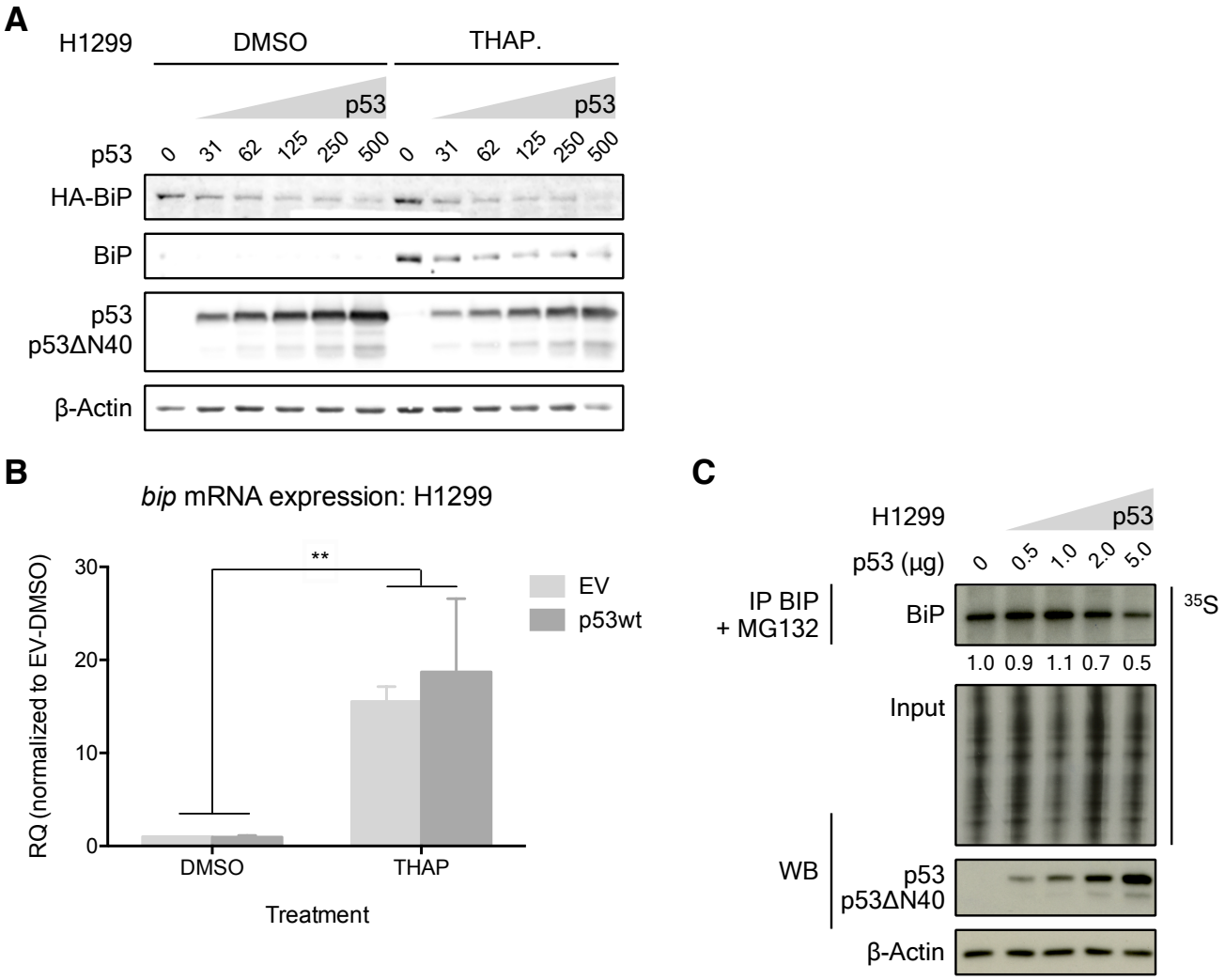
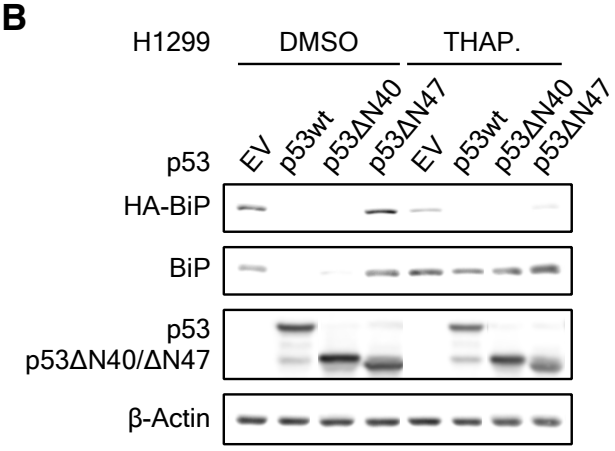

A
H1299
DMSO
THAP.
p53
p53
p53 0 31 62 125 250 500 0 31 62 125 250 500
HA-BiP
BiP
p53
p53ΔN40
β-Actin
B
bip mRNA expression: H1299
RQ (normalized to EV-DMSO)
30
20
10
0
**
EV
p53wt
DMSO
THAP
Treatment
C
H1299
p53
p53 (μg) 0 0.5 1.0 2.0 5.0
IP BIP
+ MG132
BiP
35S
1.0 0.9 1.1 0.7 0.5
Input
WB
p53
p53ΔN40
β-Actin
D
bip/gfp
p53 - +
35S
BiP
GFP

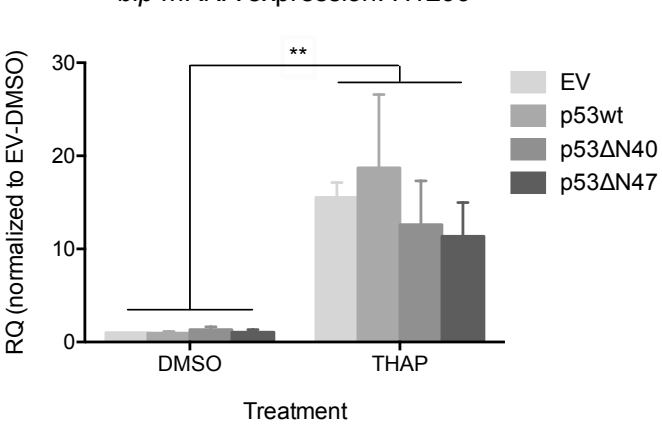
Figure 4

A

B



C



D

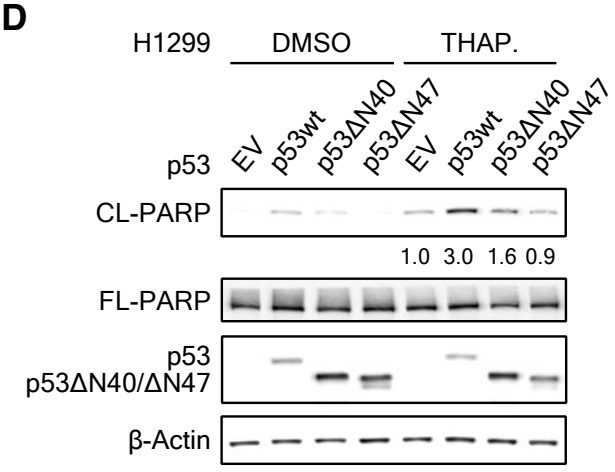


Figure 5

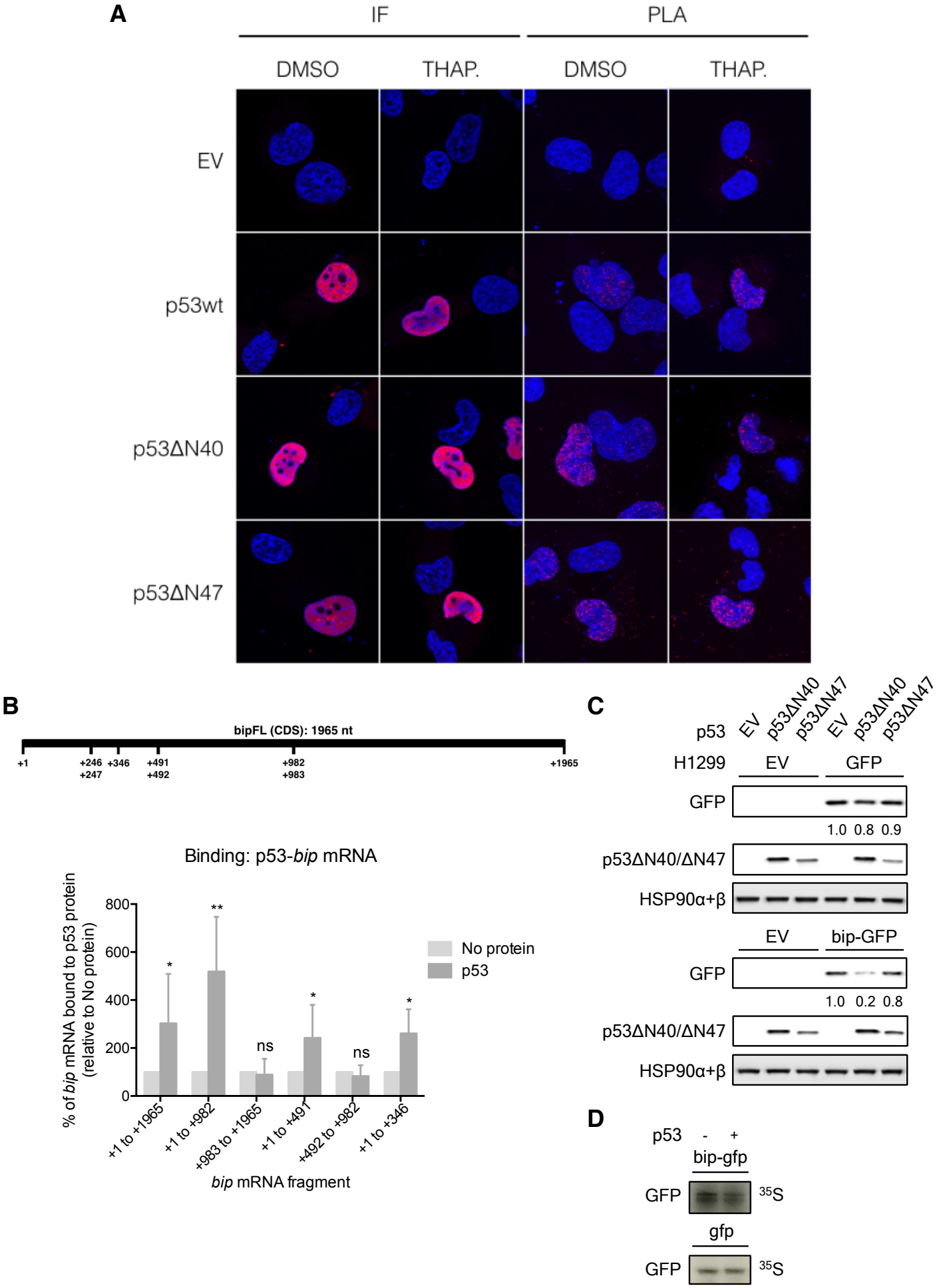
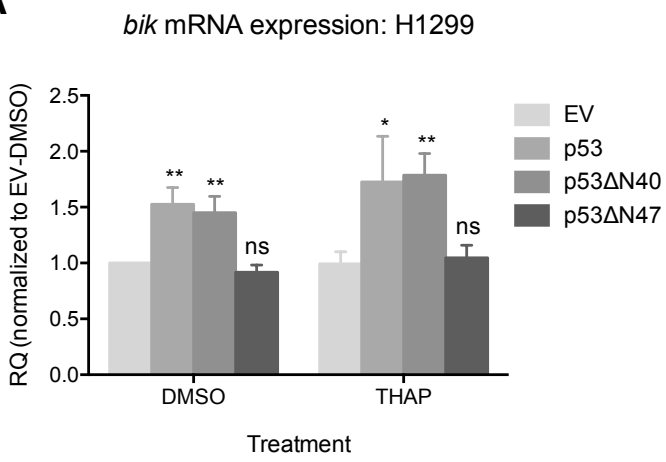
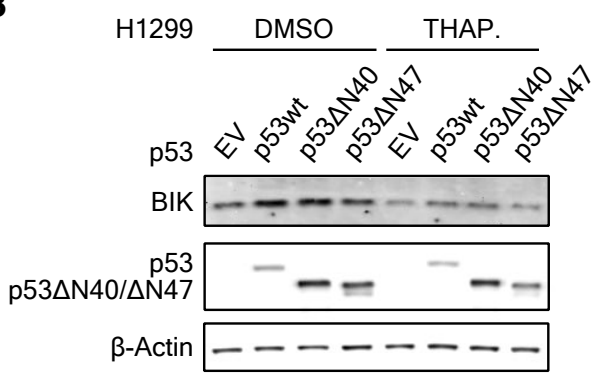

A
IF
PLA
DMSO
THAP.
DMSO
THAP.
EV
p53wt
p53ΔN40
p53ΔN47
B
bipFL (CDS): 1965 nt
+1
+246 +247
+346
+491 +492
+982 +983
+1965
Binding: p53-bip mRNA
% of bip mRNA bound to p53 protein (relative to No protein)
800
600
400
200
0
**
*
ns
*
ns
*
No protein
p53
+1 to +1965
+1 to +982
+983 to +1965
+1 to +491
+492 to +982
+1 to +346
bip mRNA fragment
C
p53
EV
p53ΔN40
p53ΔN47
EV
p53ΔN40
p53ΔN47
H1299
EV
GFP
GFP
1.0 0.8 0.9
p53ΔN40/ΔN47
HSP90α+β
EV
bip-GFP
GFP
1.0 0.2 0.8
p53ΔN40/ΔN47
HSP90α+β
D
p53
-
+
bip-gfp
GFP
35S
gfp
GFP
35S

Figure 6

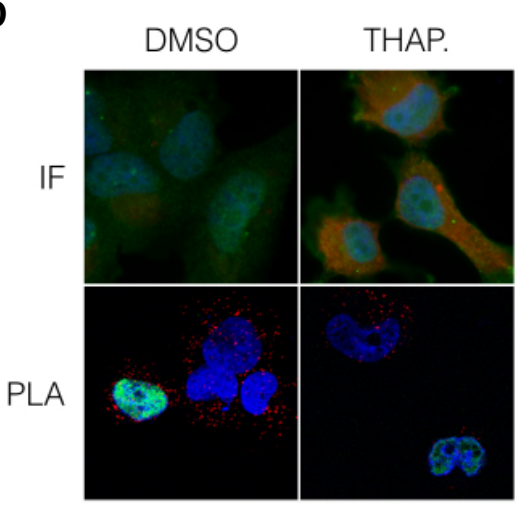
A


bik mRNA expression: H1299
RQ (normalized to EV-DMSO)
2.5
2.0
1.5
1.0
0.5
0.0
**
**
ns
*
**
ns
DMSO
THAP
Treatment
EV
p53
p53ΔN40
p53ΔN47

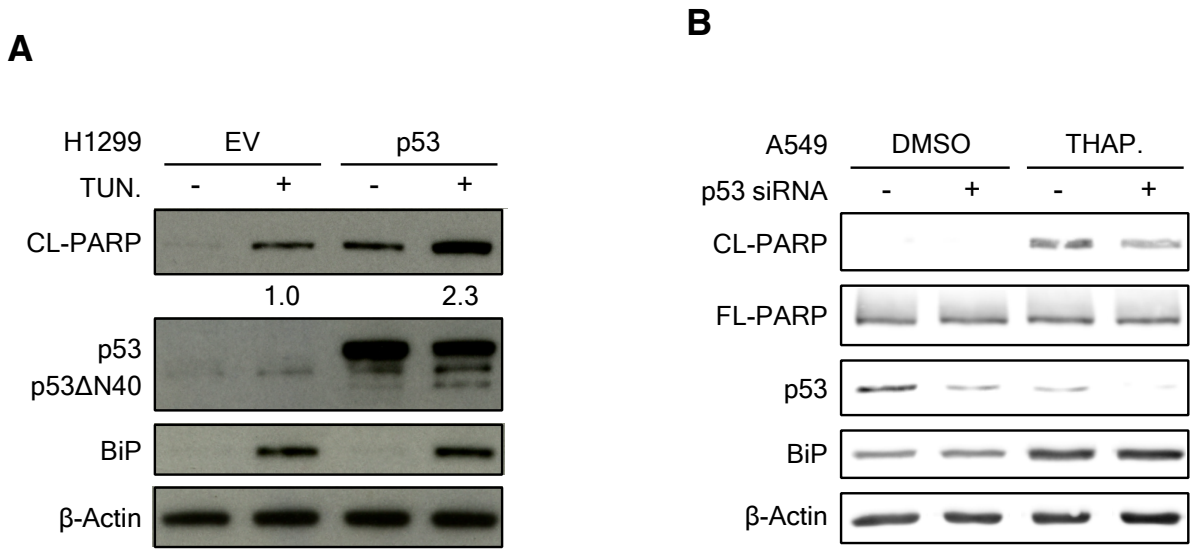
B


H1299
DMSO
THAP.
EV
p53wt
p53ΔN40
p53ΔN47
EV
p53wt
p53ΔN40
p53ΔN47
p53
BIK
p53
p53ΔN40/ΔN47
β-Actin

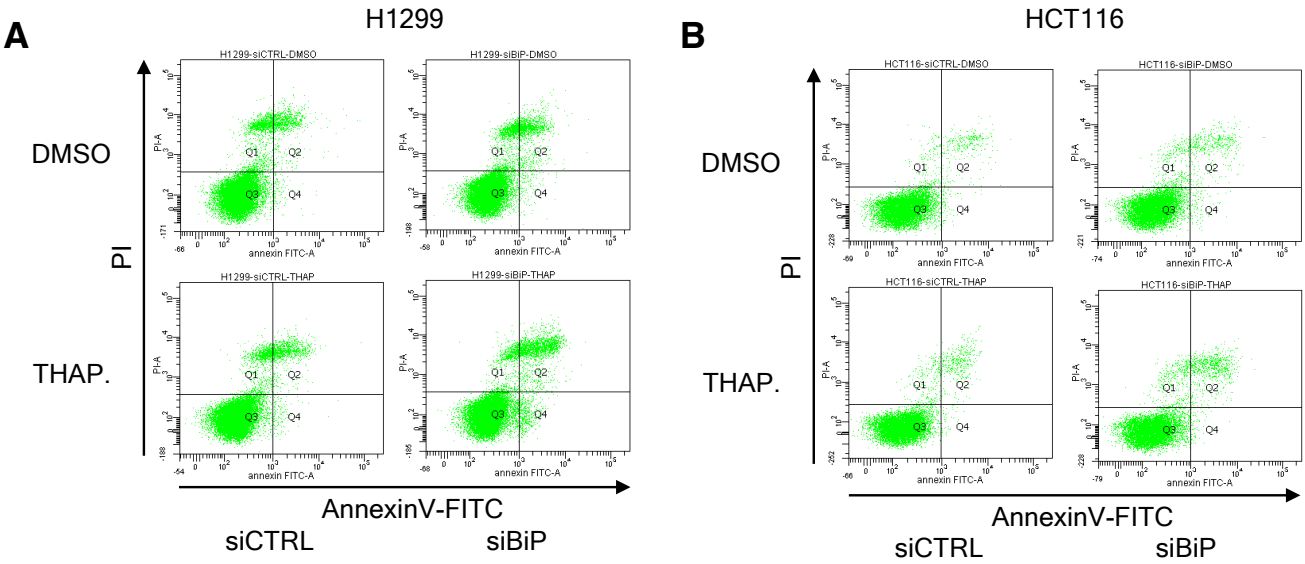
D

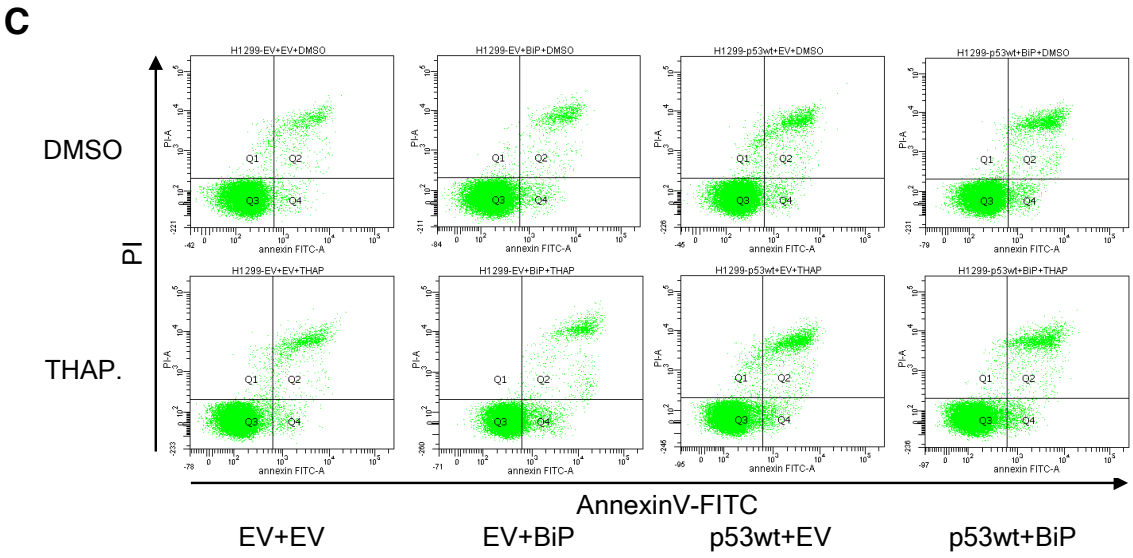

DMSO
THAP.
IF
PLA

Supplementary Figure 1

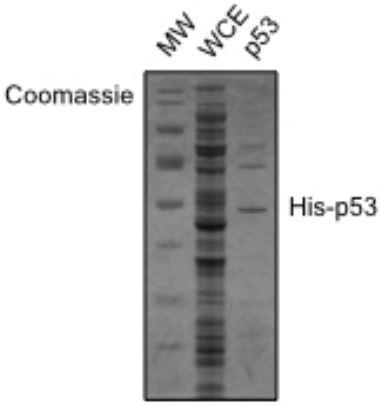


Supplementary Figure 2

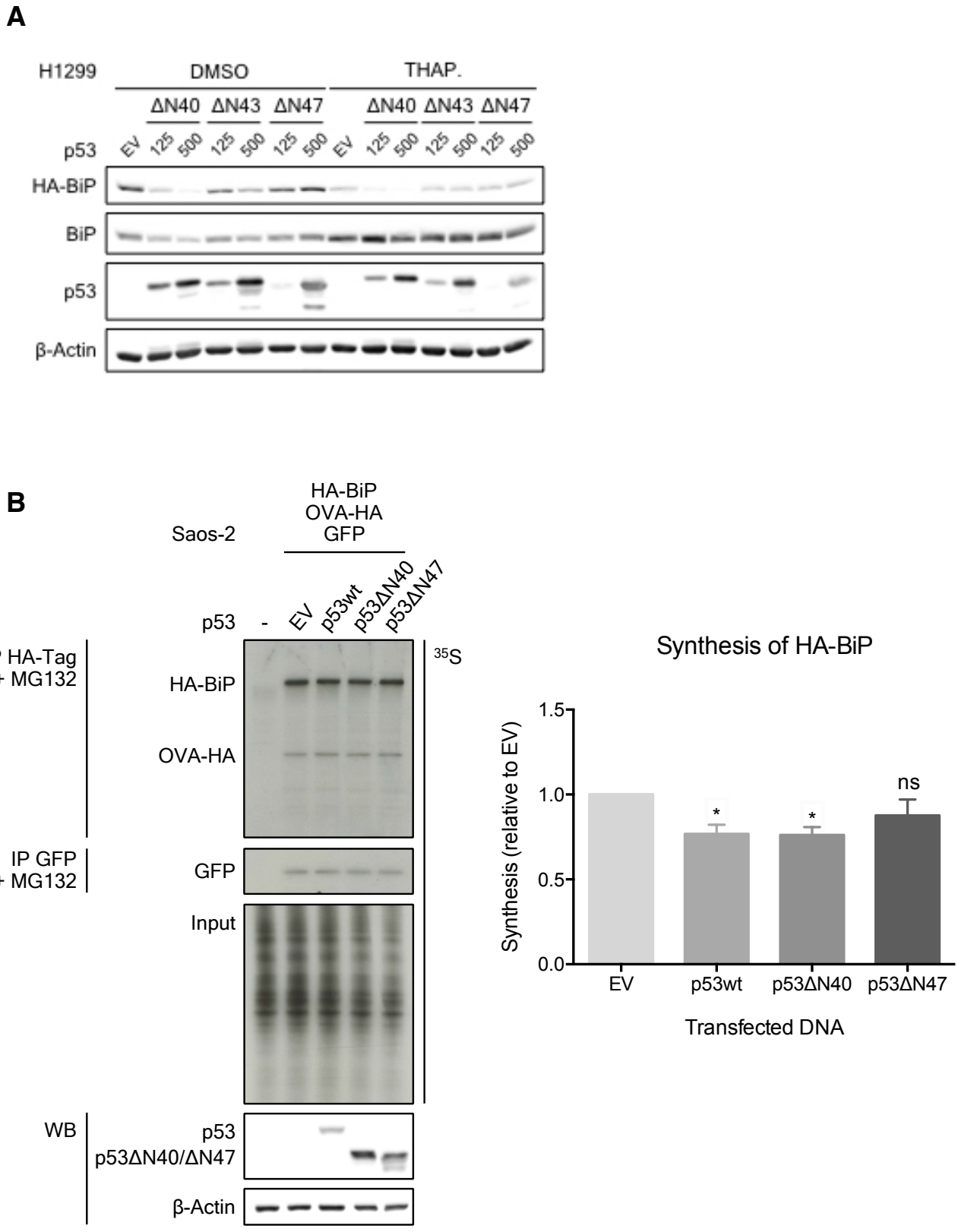




Supplementary Figure 3

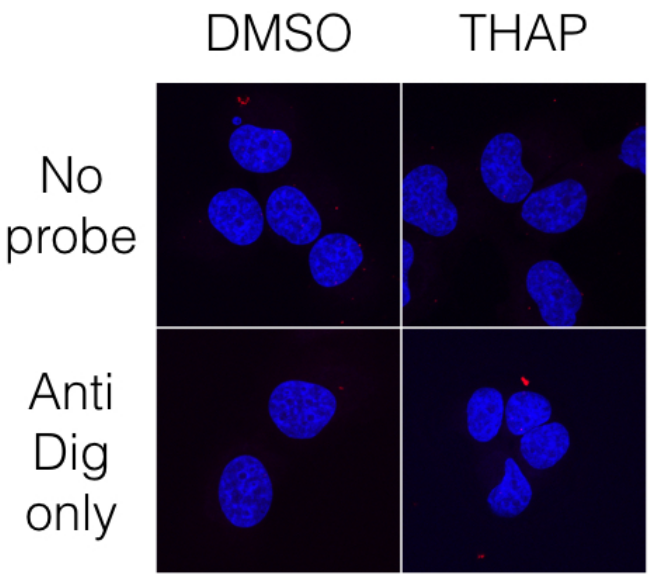


Supplementary Figure 4

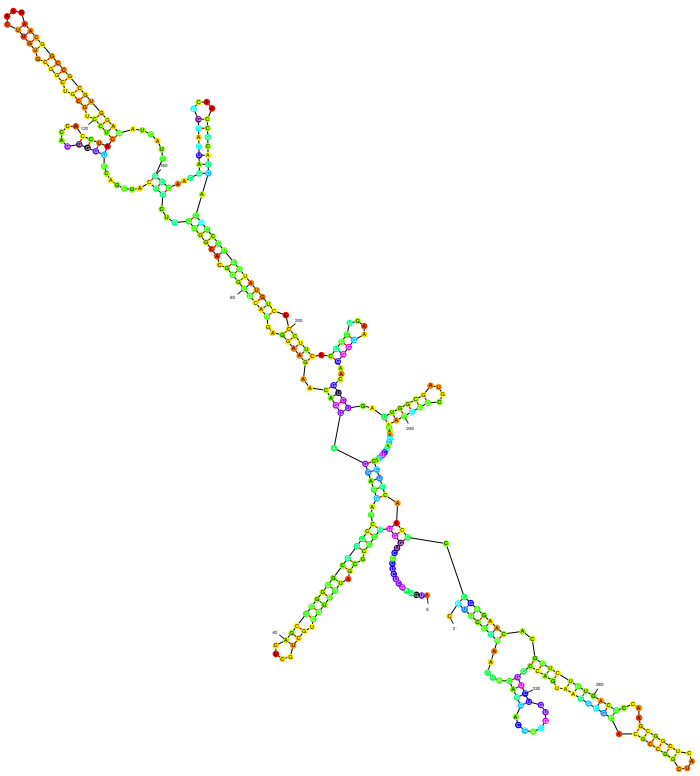


Supplementary Figure 5

A



B



2. p53-mediated control of gene expression via mRNA translation during Endoplasmic Reticulum stress

PERSPECTIVE

Cell Cycle 14:21, 3373–3378; November 1, 2015; Published with license by Taylor & Francis Group, LLC

p53-mediated control of gene expression via mRNA translation during Endoplasmic Reticulum stress

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p53 is activated by different stress and damage pathways and regulates cell biological responses including cell cycle arrest, repair pathways, apoptosis and senescence. Following DNA damage, the levels of p53 increase and via binding to target gene promoters, p53 induces expression of multiple genes including *p21*^{CDKN1A} and *mdm2*. The effects of p53 on gene expression during the DNA damage response are well mimicked by overexpressing p53 under normal conditions. However, stress to the Endoplasmic Reticulum (ER) and the consequent Unfolded Protein Response (UPR) leads to the induction of the p53/47 isoform that lacks the first 40 aa of p53 and to an active suppression of p21^{CDKN1A} transcription and mRNA translation. We now show that during ER stress p53 also suppresses MDM2 protein levels via a similar mechanism. These observations not only raise questions about the physiological role of MDM2 during ER stress but it also reveals a new facet of p53 as a repressor toward 2 of its major target genes during the UPR. As suppression of p21^{CDKN1A} and MDM2 protein synthesis is mediated via their coding sequences, it raises the possibility that p53 controls mRNA translation via a common mechanism that might play an important role in how p53 regulates gene expression during the UPR, as compared to the transcription-dependent gene regulation taking place during the DNA damage response.

Keywords: ER stress, MDM2, mRNA translation, p53, p53/47

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Submitted: 07/22/2015

Revised: 08/26/2015

Accepted: 08/29/2015

http://dx.doi.org/10.1080/15384101.2015.1090066

The tumor suppressor protein p53 becomes activated when different stresses are infringed to cells, such as DNA damage, nutrient deprivation, viral infection or oncogene activation.[1,2] Following the well-studied DNA damage response, p53 induces a multitude of downstream target genes. The induction of different sets of gene products trigger particular biological effects that match the insults and are aimed at either preventing abnormal growth of compromised cells by reversible arrest of the cell cycle in G1 or G2 to facilitate repair processes, or at inducing irreversible outcomes including apoptosis or senescence.[3-6] Due to its importance in cellular and organism maintenance, p53 is inactivated by mutations in over 50 % of human cancers while changes in downstream and upstream pathways are thought to be present in most cancer cells.

Two of the major and best-described p53-target genes following DNA-damage are *p21*^{CDKN1A} (*p21Cip1/Waf1*) and *mdm2*, whose p53-dependent mRNA induction is mirrored by an increase in protein levels. Induction of p21^{CDKN1A} constitutes an important branch of the p53-dependent cancer protection and is observed at early stages of DNA damage response via its capacity to suppress both G1 and S phase cyclin and cyclin-dependent kinase (CDKs) activity and to prolong the G1 phase in order to prevent cells from entering replication carrying damaged DNA.[3,7] p21^{CDKN1A}-deficient mice have an increase in tumor incidence later in life.[8]

The E3-ubiquitin ligase MDM2 binds the conserved BOX-I motif in the N-terminus of p53 and masks p53's transactivation domain and catalyzes the ubiquitination of p53.[9] The *mdm2* P2 promoter includes a p53 binding site offering a putative regulatory p53-MDM2 feed-back loop.[10] Although MDM2-dependent control of p53 activity is vital

during mice development to subdue p53 activity, recent studies indicate that the p53-MDM2 feed-back loop is important during the DNA damage response but is not required to suppress p53 activity during mice development.[11] This can be explained by the observations that following genotoxic stress, MDM2, helped by its homolog MDMX, becomes a positive regulator of p53 by stimulating the rate of p53 protein synthesis and increasing its half-life.[12-14]

A less attended cellular scenario regarding p53 activity constitutes the Endoplasmic Reticulum (ER) stress that is triggered by several conditions including accumulation of unfolded or misfolded proteins, nutrient deprivation, or high rate of synthesis or underglycosylation of proteins.[15] Induction of ER stress is also caused by compounds like tunicamycin and thapsigargin that affect protein glycosylation or target Ca^{2+} homeostasis, respectively. In tissues, physiological fluctuations of protein production and folding, poor perfusion and lack of nutrient supply and oxidative stress, or pathological scenarios linked with viral infection, cancer and aging are all linked with ER stress.[15] Cells respond to ER stress through the unfolded protein response (UPR) that triggers an adaptive 3-branched pathway. The UPR inhibits global cap-dependent protein synthesis via PERK, promotes induction of ER chaperons and favors the elimination of misfolded proteins via ATF6 and IRE-1.[15,16] We have previously shown that during ER stress, a selective PERK-dependent induction of the alternatively translated p53 isoform p53/47 leads to increased 14-3-3σ expression and the corresponding G2/M arrest.[17] The levels of protein synthesis at G2/M are estimated to be 30 % less and, thus, the prolongation of this face of the cell cycle offers a window to facilitate repair of the damaged ER organelle and restore the balance between newly synthesized and mature proteins.[18] More recent data show that in order to avoid COP-1-mediated degradation of 14-3-3σ, the expression of $p21^{CDKN1A}$ is suppressed by p53-dependent mechanisms.[19]

We now report that the MDM2 protein levels are inhibited in the p53-positive HCT116 and A549 and in the p53–null H1299 and Saos-2 cell lines following expression of ectopic p53 during the UPR induced by treatment with thapsigargin (THAP) (**Fig. 1A**). As the suppression of MDM2 requires p53, this cannot be explained simply by PERK-mediated phosphorylation of eIF2α and instead implicates p53 in the inhibition of MDM2 expression during the UPR. The expression of the ER-located chaperone BIP, which is a target of the UPR at the transcription level, was used to monitor proper induction of the UPR. Importantly, the *mdm2* mRNA expression pattern was induced by p53 irrespectively of the UPR status of the cells (**Fig. 1B**). Hence, similar to $p21^{CDKN1A}$, p53 also suppresses the expression of MDM2 during the UPR.

Expression of 31 nanograms (ng) of *p53wt* cDNA in H1299 resulted in the down-regulation of an exogenous HA-tagged MDM2 (HA-MDM2) protein expression, as revealed using anti-HA antibodies (**Fig. 1C, upper right**). On the other hand, the levels of the endogenous MDM2 still increased when p53 was overexpressed due to the strong induction of *mdm2* mRNA levels as determined using RT-qPCR (**Fig. 1C, upper and lower left**). However, plotting the ratio of *mdm2* mRNA levels *vs*. MDM2 protein levels at 31 ng of p53 transfection shows that the mRNA expression increased over 2-fold as compared to MDM2 protein levels (**Fig. 1C, lower right**).

The suppression of $p21^{CDKN1A}$ mRNA translation by p53 is mediated via its coding sequence and, thus, not via the more commonly described mechanisms of mRNA translation initiation control that act via the untranslated regions (UTRs).[19] This appears to also be the case for MDM2 as the exogenous HA-tagged MDM2 is actively suppressed. In addition, treatment with the proteasome inhibitor MG132 failed to prevent p53-mediated suppression of HA-MDM2 expression in thapsigargin-treated cells (**Fig. 1C, upper right**), supporting the notion that p53 does not affect the turnover rate of MDM2 but instead targets its rate of synthesis. Hence, similar to $p21^{CDKN1A}$, p53 also suppresses the expression of MDM2 by a mechanism that involves the inhibition of *mdm2* mRNA translation.

The suppression of HA-MDM2 by p53 occurred both under normal conditions and thapsigargin treatment indicating this capacity of p53 is ubiquitous. However, as with $p21^{CDKN1A}$ mRNA and protein levels, an increasing amount of p53/47 prevented full-length p53 (p53FL)-mediated induction of *mdm2* mRNA levels and induced a decrease in MDM2 protein levels (**Fig. 1D**). The p53/47 isoform is initiated 40 codons downstream of the first AUG and lacks the first of p53's 2 transacting domains, including the conserved BOX-I motif that includes the MDM2 interaction site.[20] Hence, p53/47 has different activity and stability as compared to p53FL. It retains the DNA binding and oligomerisation domains and has the capacity to affect p53 related activities either as homo-tetramer, which binds the same promoter sequences as the p53FL isoform, or as hetero-oligomer with p53FL. *In vitro* and *in cellulo* data support the idea that p53/47 due to the lack of the N-terminus forms oligomers more easily as compared to p53FL so that relatively low levels of expression give a dominant cellular phenotype.[17] It is conceivable that the induction of p53/47 during the UPR prevents p53-mediated transcription of the $p21^{CDKN1A}$ and *mdm2* mRNAs which then allows p53's translation suppressor activity of these 2 mRNAs to become prominent and physiologically important. The suppression of $p21^{CDKN1A}$ and MDM2 during the UPR can thus, be attributed to the induction of p53/47.

Animal models have indicated that p53/47 alters the activity of p53 and transgenic mice overexpressing p53/47 show a dramatic pre-mature aging phenotype and cells from such mice have altered stem cell pluripotency.[21,22] Furthermore, the presence of p53/47 in glioblastomas has indicated a role for this isoform in this type of cancer [23] and it will be important to test to which extent these different phenotypes reflects the suppression of gene expression in a similar fashion as with MDM2 and $p21^{CDKN1A}$.

Repression of gene expression by p53 via competing with activators for binding sites on gene promoters (*IGF-1R, POLD1*) or by interfering with transcriptional machinery (*cyclin B, Cox-2*) or by

recruiting chromatin remodelers (*Map4, surviving*), has been reported.[24] Also, p53 is able to repress translation of its own, *cdk4* and *fgf-2* mRNAs via binding to respective 5′UTR.[25] But it is surprising that the expression of 2 of the main p53 target genes during the DNA damage response are in fact actively suppressed by p53 during the UPR. We do not yet know the molecular mechanism of action of p53-mediated suppression of translation during the UPR, but in both cases it involves the coding sequences and not the UTRs. As different point mutations in p53 that suppress its DNA binding activity were shown to prevent suppression of $p21^{CDKN1A}$ mRNA translation, it is possible that the effect of p53 on the

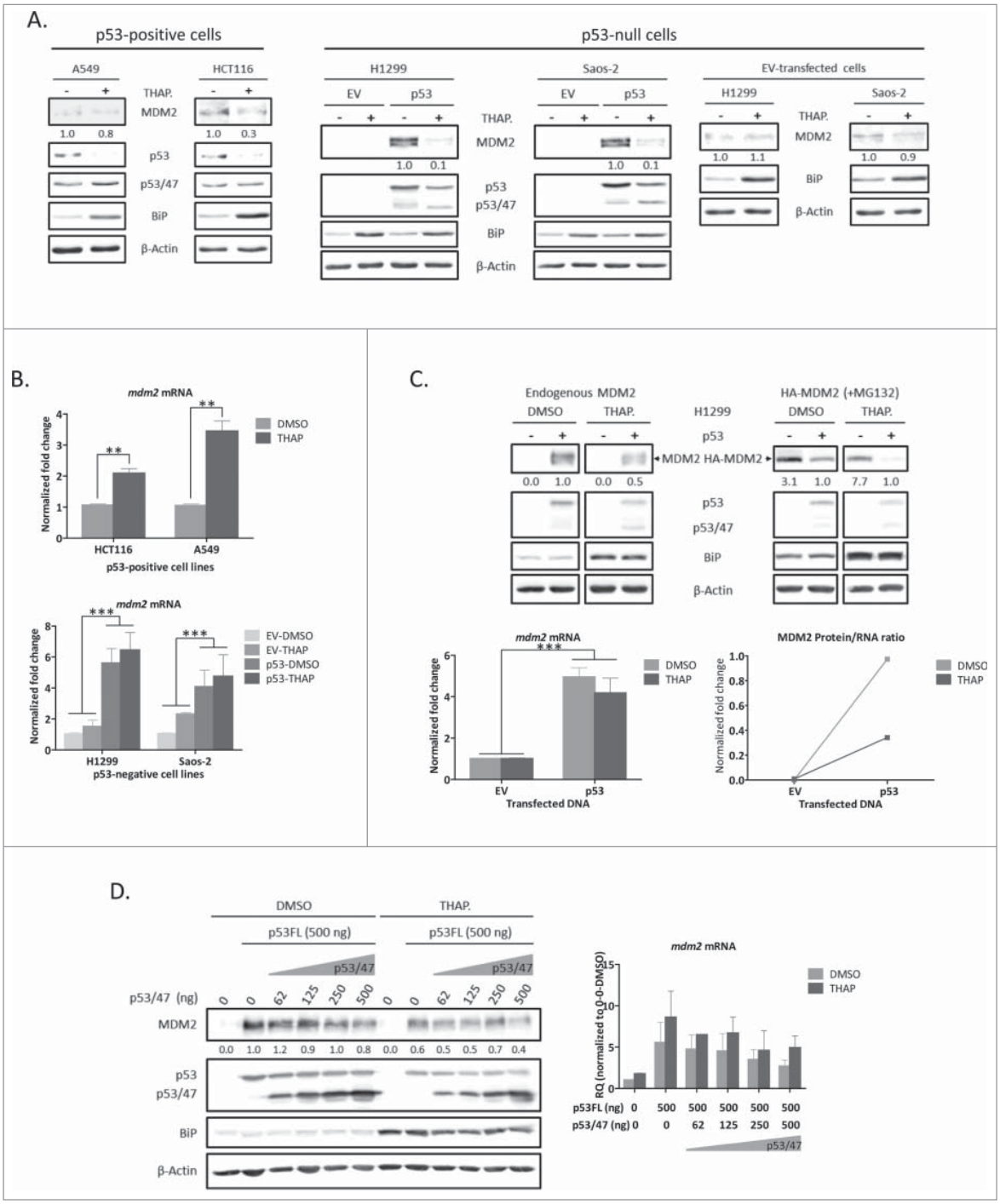

Figure 1. For figure legend, see next page.

p21^{CDKN1A} mRNA is indirect via a hitherto unknown p53 gene target.[19] It is also possible that it is direct and that p53 binds a class of mRNAs via the DNA-binding domain and, hence, mutations in p53 that affect DNA binding also affect RNA binding. A direct interaction with mRNAs has been proposed for *p53, cdk4* and *fgf-2* and similar observations have been done regarding the *mdmx* mRNA (unpublished data). Hence, the possibility of 2 different pathways to control mRNA translation by p53 cannot be excluded.

Nevertheless, it is clear that p53 has an mRNA translation suppressor activity and this raises some important questions: **i**) which RNAs are targeted by p53 and what mediates the specificity to some mRNAs and not others? **ii**) is the effect of p53 direct or indirect? **iii**) what are the underlying molecular mechanisms? **iv**) and, finally, what are the physiological implications? We have started to address some of these questions and a deletion series of the *p21*^{CDKN1A} mRNA indicated that 2 separate sequences are required for translation control, which makes interference by ncRNAs less likely mediators and instead points toward a structured region in the RNA as being critical.[19] This can form a platform to which RNA structure-sensitive protein/s can bind and this would help to explain why there is no apparent sequence homology between the *mdm2* and *p21*^{CDKN1A} mRNAs, or the other mRNAs implicated as targets for p53 trans-suppression activity. If so, this makes predictions of which RNAs that are p53 targets more difficult. In terms of how p53 mediates translation suppression it is important to keep in mind that more than one mechanism might be in place and it is safer to investigate each mRNA suppressed by p53 separately before any general conclusions are drawn. Protein-RNA binding assays together with *in vitro* translation should help to address if p53 affect translation directly or via downstream targets. However, *in vitro* translation systems are not the same as cell-based assays and if folding of RNA structures plays a role, then this might have significant consequences using *in vitro* transcribed mRNAs. Another important aspect is to differentiate p53 activities toward DNAs and RNAs in order to generate tools required to investigate the cell biological role of mRNA translation control. This could be achieved either by identifying differences in the RNA binding vs DNA binding capacity or by separating the trans-activation from the trans-suppression. The specificity of trans-suppression will depend on different cellular factors linking p53, or its downstream target factor, with the translation machinery and a major step forward will be to identify the target/s in the RNA translation process. Hence, the characterization of mRNAs affected by p53, as well as identification of the targets within the translation machinery, will play equal important roles in elucidating the underlying molecular and physiological role of p53 trans-suppressor activity.

As a perspective, it is worth considering the possibility that different aspects of p53 are prominent during different cellular conditions and the fact that p53 has opposite effects on p21^{CDKN1A} and MDM2 expression during the DNA damage vs the UPR justifies this notion. The DNA damage response and over expression of p53 gives to a large extend a similar cell phenotype in *in cellulo* conditions via the induction of mRNA levels but this is not the case for ER stress. Why is this? The suppression of protein synthesis by inactivation of eIF2α during the UPR relates to the need of the cell to slow down synthesis

Figure 1 (See previous page). p53 down-regulates MDM2 expression under Endoplasmic Reticulum stress. (**A**) Western blots of cell lysates extracted from p53-positive A549 and HCT116 (left) and Empty Vector (EV)- (right) or p53-transfected (center) p53-null H1299 and Saos-2 cell lines show that MDM2 expression is down-regulated on a p53 and ER stress dependent manner. Expression of MDM2 was estimated from densitometry analysis performed with Bio-PROFIL Bio 1-D software (Vilbert Lourmat) on chemiluminescence images acquired using CHEMI-SMART 5000 documentation system and Chemi-Capt software (Vilbert Lourmat). Values of MDM2 were normalized against their correspondent actin value and then against DMSO-treated cells in the case of A549 and HCT116 and EV- or p53-transfected and DMSO-treated cells in the case of H1299 and Saos-2. 500 ng of DNA were used in transfection. (**B**) Samples from A were analyzed in parallel for the effect of p53 expression on endogenous *mdm2* mRNA levels using relative RT-qPCR for p53-positive (top) and EV- of p53-transfected p53-negative (below) cell lines. Values were normalized against actin and are presented as fold change relative to DMSO-treated cells in the case of HCT116 and A549 and relative to EV-transfected and DMSO-treated cells for H1299 and Saos-2, set to 1 (mean ± s.d., n = 3 performed in duplicates). (**C**) Western blots show expression of endogenous MDM2 (upper left) or exogenous HA-tagged MDM2 carrying only the coding sequence (HA-MDM2) (upper right) in the presence or absence of a small amount (31 ng) of transfected p53 in H1299 cells. In the case of exogenous HA-MDM2, cells were also treated with proteasome inhibitor MG132 (25 μM, 2 h) to minimize effects related to protein stability. Values of MDM2 and HA-MDM2 protein expression were obtained as in A and normalized against their correspondent actin value and then against p53-transfected and DMSO-treated cells in the case of MDM2 and against p53-transfected and DMSO or THAP-treated cells for HA-MDM2. Relative RT-qPCR on endogenous *mdm2* was carried out in parallel (lower left). Values were normalized against actin and are presented as fold change relative to EV-transfected and DMSO-treated cells, set to 1 (mean ± s.d., n=3 performed in duplicates). Ratio of protein/RNA for endogenous MDM2 expression were calculated and presented as fold change compared to p53-transfected and DMSO-treated cells (lower right). (**D**) Endogenous MDM2 expression was analyzed in H1299 cells co-transfected with increasing amounts of p53/47 (0-500 ng) and a fixed amount of p53FL (500 ng). Cell lysates and mRNA levels were analyzed in parallel by western blot (left) and relative RT-qPCR (right), respectively. Values of MDM2 protein expression were normalized against actin. The value 1.0 was set for 500 ng of p53FL-transfected and DMSO-treated cells in western blot quantification. Values of RT-qPCR were normalized against actin and presented as fold change relative to EV-transfected and DMSO-treated cells (mean ± s.d., n = 3 performed in duplicates). For all experiments, 2×10^5 cells were seeded 24 h before transfection in 6-well plates. Thapsigargin (THAP., 100 μM) or DMSO treatments were done for 16 h. MDM2 was detected using 4B2 monoclonal antibody, HA-MDM2 was detected with an anti HA monoclonal antibody and both endogenous and exogenous p53 isoforms were detected using ACMDD serum (rabbit polyclonal antibody raised against peptide MDDLMLSPDDIEQC recognizing the N-terminus of p53/47).[30] BiP expression was used as a positive control for ER stress induction and β-Actin as loading control. Blots represent $n \geq 2$. For all RT-qPCR, primers used to amplify MDM2 are: Forward 5′ ATCTACAGGGACGCCATC 3′ and Reverse 5′ CTGATCCAACCAATCACCTGAA 3′. In B top, Student's t-test compared data to the reference point as indicated. In B bottom and C down left, 2-way ANOVA compared data of the effect of treatment and transfection of p53 on *mdm2* mRNA expression as indicated (for all, $^{**}P < 0.01$; $^{***}P < 0.001$).

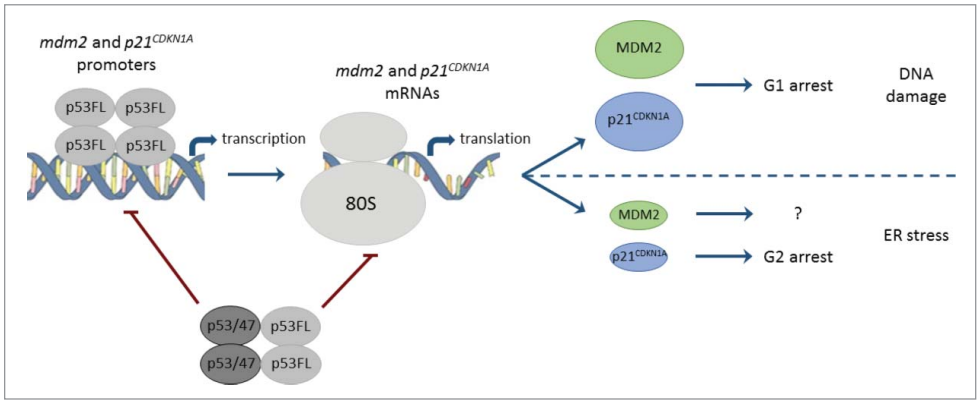

Figure 2. Model depicting how p53 and p53/47 control p21^{CDKN1A} and MDM2 expression differentially during DNA damage and ER stress context. In the DNA damage pathway, p53 full length (p53FL) acts at the transcription level to induce both *p21^{CDKN1A}* and *mdm2* mRNAs. The induction of p21^{CDKN1A} leads to G1 cell cycle arrest. During DNA damage, MDM2 acts as a positive regulator of p53 activity. The suppression of p21^{CDKN1A} and MDM2 during the UPR is attributed to the induction of p53/47, which prevents p53FL-mediated induction of *p21^{CDKN1A}* and *mdm2* mRNA levels. This allows p53 isomer-dependent suppression of *p21^{CDKN1A}* and *mdm2* mRNA translation to become dominant and results in lower p21^{CDKN1A} and MDM2 protein levels. Suppression of p21^{CDKN1A} levels is required to arrest in G2 during the UPR but the physiological role of MDM2 suppression remains to be elucidated.

of proteins in order to facilitate ER repair. However, some proteins required for the ER repair are induced at the level of protein synthesis and it raises the question if translation control during the UPR is a more favorable mechanism whereby protein expression can be controlled. Hence, during the UPR it might be less efficient for p53 to rely on transcription control to regulate gene expression and instead, or additionally, it targets mRNA translation.

Another aspect of these data is the questions of why MDM2 expression is suppressed during the UPR. In the case of p21^{CDKN1A} it was shown that it regulates the stability of 14-3-3σ and unless p21^{CDKN1A} levels are suppressed the cells fail to arrest in G2/M (**Fig. 2**).[17,19] But for MDM2 we still do not know. MDM2 is predominantly known for its role as a regulator of p53 but we have no evidence that MDM2's capacity to regulate p53 stability is different during the UPR as compared to normal conditions. A possible scenario would include MDM2's capacity to interact with ribosomal factors. It is well described that MDM2 interacts with ribosomal factors L5, L11, L23, S7 and the 5S complex.[26,27] The former interactions have been attributed to the control of MDM2 E3 ligase activity toward p53 and the binding to the L5/L11 complex stabilizes p53.[27] However, the role of MDM2 in stimulating p53 synthesis during the DNA damage response opens for the possibility that these interactions might also have the reverse functions and MDM2 might play a role in regulating ribosomal biogenesis. It is possible that such regulation might be acting during the UPR within the global control of protein synthesis and furthermore, it has been described that the MDM2-binding protein Arf which interacts within the same domain as ribosomal factors, inhibits the processing of rRNA.[28] Furthermore, p53 suppresses RNApol III activity via TFIIIB, suggesting that ribosomal biogenesis is interlinked with the p53 pathway and a target for p53 tumor suppressor activity.[29]

This article illustrates that p53 can have opposite functions toward the same gene depending on cellular conditions. A similar observation has been done on MDM2 that targets p53 for degradation during normal conditions but stimulates p53 synthesis following DNA damage.[12] In this case the switch is also mediated by an mRNA, implicating a broader role of mRNA as regulatory switches in the p53 pathway.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Antibodies against p53, MDM2 and HA-Tag were kindly provided by Dr. Borek Vojtesek.

Funding

This work was supported by la Ligue Contre le Cancer, the Inserm and the project MEYS-NPS I-L01413. I. L. is funded by AXA Research Fund.

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p53 binds the *mdmx* mRNA and controls its translation.

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Key words: MDMX, p53, mRNA binding, mRNA translation

NOTE: This article was submitted to Oncogene journal in April 2016 and is currently under review.

ABSTRACT

MDMX and MDM2 are two non-redundant essential regulators of p53 tumour suppressor activity. While MDM2 controls p53 expression levels, MDMX is predominantly a negative regulator of p53 trans-activity. The feedback loops between MDM2 and p53 are well studied and involve both negative and positive regulation on transcriptional, translational and post-translational levels but little is known on the regulatory pathways between p53 and MDMX. Here we show that overexpression of p53 suppresses *mdmx* mRNA translation *in vitro* and in cell-based assays. The core domain of p53 binds the 5' untranslated region (UTR) of the *mdmx* mRNA in a zinc-dependent fashion that together with a trans-suppression domain located in p53 N-terminus controls MDMX synthesis. This interaction can be visualized in the nuclear and cytoplasmic compartment. Fusion of the *mdmx* 5' UTR to the ovalbumin open reading frame leads to suppression of ovalbumin synthesis. Interestingly, the transcription inactive p53 mutant R273H has a different RNA-binding profile compared to the wild type p53 and differentiates the synthesis of MDMX isoforms. This study describes p53 as a trans-suppressor of the *mdmx* mRNA and adds a further level to the intricate feedback system that exist between p53 and its key regulatory factors and emphasizes the important role of mRNA translation control in regulating protein expression in the p53 pathway.

INTRODUCTION

The control of p53 activity under diverse conditions and in different tissues is critical for development and for coordinating the correct response to cellular stresses and damages. Mice lacking either of the two homologues *mdmx* (also called *mdm4*) or *mdm2* die early during development in a non-redundant fashion that is rescued in either case by deletion of p53 (de Oca Luna et al, 1995; Jones et al, 1995a; Parant et al, 2001b). MDM2 and MDMX show strong similarities but also important differences in their activities towards p53. Both proteins bind the N-terminus of the p53 protein but via different interphases and while the Nutlin and similar compounds efficiently compete for p53 binding to MDM2, they have little effect on the p53-MDMX interaction (Wade et al, 2006). MDM2 harbors a C-terminal RING domain and is a well-known E3 ubiquitin ligase towards p53 but despite a similar RING domain, MDMX has no reported ubiquitin ligase activity towards p53. It has however, been implicated in assisting MDM2-mediated ubiquitination of p53 (Gu et al, 2002). During the DNA damage response, the RING domains of both proteins bind the *p53* mRNA and help induce p53 expression following activation by the ATM kinase (Gajjar et al, 2012; Malbert-Colas et al, 2014; Naski et al, 2009). p53 induces MDM2 expression and the feedback loop between p53 and MDM2 plays an important role in activating p53 during the DNA damage response. Interestingly, the negative feedback loop in which MDM2 instead targets p53 for degradation is not required to subdue p53 activity during development (Pant et al, 2013). Furthermore, both MDM2 and MDMX express isoforms that lack the N-terminal p53 binding domains but these retain the capacity to form heterooligomers via their respective RING domains and, thus, to regulate each other's activity Perry et al, 2000; Saucedo et al, 1999; Tournillon et al, 2015).

As compared to the interplay between MDM2 and p53, little is known on the regulatory feedback between p53 and MDMX even though it has been suggested that p53 can activate *mdmx* gene transcription (Phillips et al, 2010). In fact, there are few, if any, suggestions on how the negative activity of MDMX towards p53 is suppressed during p53 activation. Overexpression of MDM2 due to gene amplification is observed in about 17% of cancers and notably in sarcomas (Oliner et al, 1992). The role of MDMX in cancer development is less studied but more recent

reports have shown an overexpression of MDMX in approximately 70% of melanomas implying a post transcriptional regulatory mechanism (Gembarska et al, 2012).

Following cellular stresses or damages, p53 tetramers bind to consensus promoter sites and via the trans-activation capacity of its N-terminal domain, it controls downstream gene expression patterns to best adapt the cellular response to damages. Several point mutations within p53 that prevents its DNA binding capacity harbor gain of function (GOF) activity and several genes have been shown to be differentially regulated by wild type or mutant p53 (Oren & Rotter, 2010). The underlying molecular mechanism for GOF remains elusive and several possible explanations have been proposed, including the interactions with p53 family members p63/p73. However, several reports have shown that in addition to its DNA binding capacity, p53 also harbors an RNA binding capacity (Riley & Maher, 2007) that has been implicated in suppression of its own synthesis and that of FGF2 and cdk4 (Galy et al, 2001a; Galy et al, 2001b; Miller et al, 2000; Mosner et al, 1995). More recently, it was also shown that p53-mediated control of *p21*^{CDKN1A} mRNA translation plays an important role in triggering a G2/M cell cycle arrest following stress to the endoplasmic reticulum (Mlynarczyk & Fahraeus, 2014). Similarly, p53 also has the capacity to suppress MDM2 synthesis sunder similar conditions (López et al, 2015). The information regarding p53 mRNA binding activity is vastly overshadowed by its DNA biding capacity and little is known about its physiological role, its putative relationship with GOF activity or the underlying molecular mechanisms.

More recent works have highlighted the fact that some classic DNA-binding proteins also harbor RNA-binding activity and, *vice versa,* that RNA-binding proteins also use DNA ligands, thereby blurring the previous rather black-and-white view of proteins either affecting DNA- or RNA-relating activities (Cassiday & Maher, 2002; Hudson & Ortlund, 2014; Suswam et al, 2005). In view of this, we set out to more in detail investigate p53's RNA binding activity and its role in selective mRNA translation control.

RESULTS

p53 suppresses MDMX synthesis

There are few commercial antibodies against MDMX and the expression pattern of MDMX in cell lines and tissues is less known as compared to MDM2. We generated a series of monoclonal antibodies against recombinant MDMX protein that recognize the full length MDMX and the alternatively initiated MDMX^{p60}, which lacks the first 127 codons including the p53 binding site (Tournillon et al, 2015). When we overexpressed p53 in Saos-2 cells (p53 null) we observed reduced MDMX expression in the presence of the proteasome inhibitor MG132 (**Figure 1a**). Under the same conditions, we observed a slight but non-significant reduction in *mdmx* mRNA levels (**Supplementary Figure 1a**). This reduction in MDMX levels by p53 was not expected as p53 has been reported to have the capacity to induce *mdmx* RNA levels by binding the *mdmx* promoter (Phillips et al, 2010). As we have previously observed that MDMX supports MDM2-dependent induction of p53's rate of synthesis following activation by the ATM kinase (Malbert-Colas et al, 2014), we were interested to see if the suppression of MDMX by p53 reflects a negative feedback between p53 and MDMX. To test this we transfected cDNA constructs expressing MDMX^{wt} or an MDMX that lacks the C-terminal RING domain (MDMXΔRING) and include the 5' untranslated region (UTR) in human H1299 cells in which endogenous MDMX was not detected. Both constructs contain the 5' UTR. The MDMXΔRING does not bind MDM2 and minimizes the potential role of MDM2 in p53-mediated regulation of MDMX expression. When we co-expressed p53 together with MDMX^{WT} or MDMXΔRING and subjected cells to a ^{35}S-methione metabolic pulse label in the presence of proteasome inhibitors, we observed an inhibition of MDMX^{WT} and MDMXΔRING synthesis (**Figure 1b**). We observed similar suppression of MDMX synthesis using the MDMX*(G57A)* which does not bind the p53 protein (Danovi et al, 2004), supporting the notion that the MDMX-p53 protein-protein interaction is not involved in p53-mediated regulation of MDMX expression (**Supplementary Figure 1b**). We next tested if *mdmx* RNA levels in H1299 cells are modified by p53 using RT-qPCR and we observed an average of 35 % reduction in RNA levels in presence of p53 (**Figure 1c**). However, the lower levels of *mdmx* mRNA that could be unspecific or due to the induction of the miR34a (Mandke et al, 2012), does not explain the near complete

suppression in MDMX synthesis at the same conditions (**compare Figure 1b second and third lane and Figure 1c**). We next tested if p53 has the capacity to directly suppress MDMX synthesis using *in vitro* translation assays. When increasing amounts of recombinant p53 was pre-incubated with the *mdmx* mRNA in the presence of tRNA competitor prior to the addition of the rabbit reticulocyte lysates, we observed a dose-dependent suppression of MDMX synthesis (**Figure 1d** and **Supplementary Figure 2**). Interestingly, p53 did not suppress *mdmx* translation when recombinant p53 was added directly to the *in vitro* translation mix but required a pre-incubation step with the *mdmx* RNA prior to the *in vitro* translation reaction. The synthesis of GFP was not affected under similar conditions (**Figure 1e, upper and lower panels**). These results indicate that p53 does not require the induction of downstream gene products to control *mdmx* mRNA translation.

p53 binds the 5' of the mdmx mRNA

The fact that p53 requires pre-incubation with the *mdmx* mRNA to suppress MDMX synthesis *in vitro* shows that p53 by itself does not affect the translation reaction and we next wanted to test the possibility that p53 requires interaction with the *mdmx* mRNA in order to control mRNA translation. This has previously been proposed for p53-mediated translation suppression of *FGF2* and of *p53* mRNAs (Galy et al, 2001a; Galy et al, 2001b; Mosner et al, 1995). We carried out *in vitro* RNA-protein coimmunoprecipitation (Co-IP) assays where we immunoprecipitated p53 derived from insect cell (i.c.) or from bacteria lysates (bact.) and tested for the presence of the full length *mdmx* mRNA in pull downs using RT-qPCR. This revealed that p53 proteins from both sources bind the *mdmx* mRNA with similar affinity (**Figure 2a**). As the recombinant bacteria-derived p53 is not post-translational modified, it suggests that p53 does not require specific post-translational modifications to bind the *mdmx* mRNA. Regulation of mRNA translation is usually mediated via the untranslated regions (UTRs) and in particular the 5'UTRs. We next carried out a deletion series of the *mdmx* mRNA to see if we could identify the p53 interacting sequence. The p53 protein has been shown to bind its own 5'UTR (*5'UTR p53*) and this was used as a positive control. As a negative control we used the structured region of the 5'UTR of the *c-myc* mRNA fused to the ovalbumin coding sequence (*cMyc-Ova*). Using the RNA-protein Co-IP we could observe that p53

bound the 5'UTR of the *mdmx* mRNA and not the coding region of the *mdmx* mRNA alone (**Figure 2b**). We also carried out the proximity ligation assay (PLA) to verify the interaction between the p53 protein and the *mdmx* mRNA. We used a digoxigenin labeled probe against the 3' coding sequence (+10190 - +10122). We then used primary mAb antibodies against digoxigenin the rabbit CM-1 sera against p53 and we could detect cytoplasmic and nuclear interaction between p53 and the *mdmx* probe using the full length *mdmx* mRNA including the 5' UTR. We observed fewer interactions from an mRNA with the coding sequence alone and no interaction using an mRNA lacking the 5' UTR and the first 381 nt of the coding sequence ($MDMX^{p60}$) (**Figure 2c)**. In line with the notion that p53 binds the *mdmx* mRNA to suppress its rate of translation, we also observed that p53 does not affect the synthesis of an mRNA that carries the coding sequence alone (**Figure 2d**). Finally, we could also show that fusion of the 5' UTR of the *mdmx* mRNA to the chicken ovalbumin (Ova) reporter open reading frame (*mdmx-Ova)* resulted in a p53 dose-dependent suppression of synthesis *in vitro* (**Figure 2e**). It is noteworthy that the fusion of the 5' of the *mdmx* mRNA to ovalbumin reduced its rate of translation by itself, in line with previous observations that this sequence harbors a structured regulatory element (Tournillon et al, 2015).

p53 requires the core RNA/DNA binding and a trans-suppression domains to suppress MDMX synthesis.

The RNA binding activity of p53 has not been as well characterized as compared to its DNA binding capacity. *In vitro* RNA-protein Co-IP using a recombinant protein corresponding to p53 core DNA binding domain (p53-DBD, residues 92 to 292) demonstrated an interaction with the *mdmx* mRNA but with slightly less affinity as compared to the full length p53 (**Figure 3a**). A zinc (Zn) atom has previously been shown to stabilize the binding between p53-DBD and the consensus DNA p53-response element (Pavletich et al, 1993) and we found that the presence of Zn also stabilizes the interaction between p53-DBD and the *mdmx* mRNA. This indicates that the RNA and the DNA binding activities of p53 share common structural properties. To further test this hypothesis we carried out protein-RNA ELISA using an *in vitro* transcribed *mdmx* RNA construct containing the 5'UTR of *mdmx* (-120 to +1) together with the recombinant p53-DBD (**Figure 3b, upper panel**). We then used the (-120 to +1) mRNA to compete for binding with different synthetic 40 nt long

RNA oligonucleotides corresponding to indicated sequences of the *mdmx* 5' UTR and to the (-120 to +1) itself. This showed that all oligonucleotides bound to p53-DBD but not in an identical fashion. (**Figure 3b lower panel and Supplementary Figure 3**). When we next used the (-120 to +1) to compete for three different DNA oligomers corresponding to p53 DNA consensus half-sites (decamer 1, decamer 2, decamer 3) (Kitayner et al, 2006), we observed these were dislodged in a similar, but not identical, fashion (**Figure 3c and Supplementary Figure 4**). Having observed that p53-DBD can bind *mdmx* mRNA, we tested if this domain is sufficient to also trans-suppress $MDMX^{WT}$ synthesis. Expression of the DBD alone had no effect on MDMX expression (**Figure 3d**), suggesting that the DBD domain of p53 is necessary but not sufficient to regulate MDMX expression.

A domain adjacent to the p53 trans-activation domain is required for mRNA translation control.

To identify additional domains of p53 that could be involved in its trans-suppression activity we carried out a deletion series of p53. We first tested the p53/47 isoform (ΔN40p53) which is initiated 40 codons downstream of p53 (Grover et al, 2009; Scrable et al, 2005; Yin et al, 2002). This resulted in a partial reduction of the translation suppression capacity, suggesting that the main trans-activation domain of p53 is not required (**Figure 4a upper panel metabolic labeling and lower panel WB**). When we deleted a further three residues (ΔN43p53) and we no longer observed suppression of *mdmx* translation. However, further single point mutations in codons 41, 42 and 43 failed to identify essential residues (**Figure 4b**). This region of p53 contains a helical structure that has been implicated in the interaction with other cellular factors like replication protein A (RPA) (Kaustov et al, 2006; Vise et al, 2005). It is plausible that this region provides the communication with the translation machinery to suppress MDMX synthesis. In this scenario, one would expect that the binding of p53 to any mRNA that is recognized by its core domain would be sufficient to allow p53 to suppress translation via the same factor which is also what we observed when we fused the 5' UTR of the *mdmx* mRNA to a reporter mRNA (**see figure 2d**).

When we introduced a mutation in p53 that abolishes its DNA binding activity (R273H) we observed a partial suppression of full length MDMX synthesis but, interestingly, we also noticed that this construct at the same time induced the expression of $MDMX^{p60}$, while wild type p53

suppresses both isoforms. This indicates that p53 alters the structure of the *mdmx* mRNA which in turn alter mRNA translation initiation (Tournillon et al, 2015). A deletion construct that lacks the 30 most C-terminus residues of p53 (p53ΔC30) was shown to have a similar effect on MDMX synthesis as the wild type protein, showing that positive charged residues in p53 C-terminus do not play a role which further demonstrates the specificity of the p53 interaction with the *mdmx* mRNA. (**Figure 4c and Supplementary Figure 5)** The notion that the p53(R273H) protein interacts differently with the *mdmx* mRNA was observed using RNA-ELISA assays (**Figure 4d**) This, together with the observation that a p53 that lacks the most N-terminal trans-activation domain still suppresses MDMX synthesis support the notion that p53's capacity to induce gene-expression via DNA binding and promoter control, is unlikely to account for the regulation of MDMX expression and supports its role as a translation factor.

To test if the capacity of p53 to control MDMX synthesis is affected by signaling pathway, we tested its capacity to suppress MDMX expression following DNA damage using doxorubicin treatment (1 μM/4 hours). **Figure 4e** shows that p53 effectively suppresses MDMX expression but with no apparent difference treating cells with doxo. We also carried out a metabolic pulse label to confirm that p53's capacity to suppress synthesis of MDMX is indeed on the level of synthesis (**Figure 4f**).

This work shows how p53 efficiently suppresses the synthesis of MDMX via two different domains and, thus, like other RNA binding proteins, p53 requires different modules to achieve its RNA trans-suppression (Lunde et al, 2007). The p53-DBD is sufficient to bind mRNA while an N-terminal trans-suppression domain interferes with mRNA translation. As p53 requires the RNA binding core domain together with a trans-suppression domain it is less likely that the overexpression of p53 *per se* could result in translation suppression simply by unspecific mRNA binding. In support of this we, and others, have found that even though shorter oligonucleotides can bind p53 unspecific, longer mRNA bind p53 with high specificity. This implies that p53 does not recognize RNA sequences *per se,* but more likely structured motifs in line with results from p53 binding the *FGF-2* mRNA (Galy et al, 2001b). However, it has been suggested based on yeast three hybrid assays using 60 nt RNA fragments that this capacity could be unspecific (Riley et al, 2006). It is possible that smaller RNA fragments and the combination with cross-linking can give

unspecific interactions, either via the core RNA binding pocket or to the positive charged C-terminus. The domains that mediate control of transcription or mRNA suppression are not identical. The domain required to suppress MDMX synthesis lies within a previously reported helical structure (aa 37-43) downstream of its conserved BOX-domain (aa. 17-26) that harbors the trans activity that controls expression of genes such as *p21*^{CDKN1A}, *Bax* and *mdm2*. Interestingly, the initiation of the alternatively translated p53/47 (ΔN40p53) at codon 40, might explain why this isoform has a partial effect on MDMX expression.

In further support of the notion that p53 does not affect MDMX synthesis via regulation of transcription we observed that: **i**) p53 suppresses the expression of endogenous MDMX in Saos-2 cells with a minimal effect on *mdmx* mRNA levels; **ii**) the effect of p53 on MDMX synthesis correlates with its binding to the *mdmx* 5' UTR *in vitro* and *in cellulo*; **iii**) recombinant p53 inhibits the synthesis of MDMX and of an 5' UTR *mdmx* reporter mRNA *in vitro*, and finally; **iv**) the transcription inactive p53 mutant R273H retains RNA binding activity and, surprisingly, affects the expression of MDMX isoforms differently.

Gain-of-function mutations (GOFs) in p53 is a well-known phenomenon but the underlying mechanisms are still unclear (Oren & Rotter, 2010). It is early days to speculate if p53's mRNA translation capacity is one possible mechanism to help explain GOF activity but it is interesting that the R273H mutant which has loss of DNA binding activity and the capacity to target p53 downstream promoters has altered activity towards the *mdmx* mRNA. It has been speculated that GOFs are not necessarily "gain" of function in the strict sense but could also reflect loss of one specific p53 activity while others are retained. The capacity of the R273H mutant to alter the expression of MDMX isoforms fits that bill and it will be interesting to see how this capacity might relate to the regulation of other mRNAs.

Little is still known about p53's RNA binding capacity. As we observe the p53-*mdmx* mRNA interaction in the cytoplasm and the fact that p53 has been reported to bind the *mdmx* promoter, this puts p53 in the group of protein with dual RNA/DNA binding capacity that bind the DNA and the RNA of the same gene, supplying a powerful dual mechanisms to control gene expression (Hudson & Ortlund, 2014). This notion is supported by previous observations that p53 controls

transcription and translation of *p21* and *mdm2* mRNAs (López et al, 2015; Mlynarczyk & Fahraeus, 2014).

The results from using bacterial recombinant p53 protein shows that p53 can control *mdmx* mRNA translation without post translational modification. Similarly, p53 can induce gene activation without phosphorylations, even though phosphorylation at p53 N-terminal residues, in particular amino acids 15, 18 and 20 have been associated with an active p53. Hence, despite the fact that a non-modified p53 has this capacity *in vitro* does not rule out the possibility that under physiological conditions certain modifications of p53 might help to activate its trans-suppressor activity.

These results add a level of feedback regulation between p53 and one of its main regulatory factors, MDMX. It has previously been shown that MDMX plays a role in controlling synthesis of p53 during the DNA damage response (Gajjar et al, 2012; Malbert-Colas et al, 2014). In this context, it is interesting that increased levels of p53 could lead to reduced MDMX levels which would allow p53 to become transcriptionally active. This would imply that just like the negative and positive feed-back loops that exist between p53 and MDM2, there is also a feed-back between p53 and MDMX. The levels of MDMX in adult tissues are low but during mouse embryonic development MDMX plays a key role in controlling p53 activity. It was not until recently that the potential role of MDMX in tumour development became clear with overexpression observed in a majority of melanoma tumour samples that are known to harbor wild type p53 (Gembarska et al, 2012). This increase in expression is not reflected by RNA levels and it will be interesting to see if p53-mediated suppression of MDMX is dysfunctional in melanoma cells.

ACKNOWLEDGMENTS

This work was supported by la Ligue Contre le Cancer, the Inserm and the project MEYS-NPS I-L01413. A-S. T. is supported by PACRI. I.L is supported by AXA Research Fund and Fondation pour la Recherche Médicale (FDT20150532276). We thank the IUH platform for technical assistance. The authors claim no conflict of interest.

Supplementary Information accompanies the paper on the *Oncogene* website

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FIGURE LEGENDS

Figure 1. p53 suppresses MDMX synthesis *in vitro* and *in vivo*

a/ Increasing amounts of p53 in the p53 null cell line Saos-2 is accompanied by a decrease in the expression of full length MDMX and the $MDMX^{p60}$ isoforms. MDMX isoforms were detected with the generated monoclonal antibodies. See also supplementary figure 1.

b/ Metabolic pulse label shows the inhibition of $MDMX^{WT}$ and of MDMXΔRING synthesis following expression of p53 in H1299 cells (upper panel). Lower western blots show corresponding steady state levels. Actin serves as loading control. ^{35}S-methionine labeling was performed by culturing cells in methionine-free medium including 10% dialyzed FCS for 1 h and easytag Express Protein Labeling Mix (PerkinElmer, Boston, Massachusetts, USA) was added for 25 to 30 min. Medium were complemented with 25μM of MG132. See also Supplementary Figure 1.

c/ RT-qPCR showing *mdmx* mRNA levels upon co-expression with p53. After RNA extraction from cells performed with RNeasy® Mini kit (Qiagen) (according to the manufacturer's protocol) and reverse transcription using the Moloney Murine Leukaemia Virus M-MLV (Invitrogen) reverse transcriptase and oligo dT (Invitrogen), the quantitative PCR were performed on a StepOne RealTime PCR system (Applied Bioystem) using the PerfeCTa SYBR Green mix (Quanta BioSciences) according to the manufacturer's procedure and using the following primers CAGCAGGTGCGCAAGGTGAA and CTGTGCGAGAGCGAGAGTCTG.

d/ Autoradiograph shows p53's effect on MDMX synthesis *in vitro*. Recombinant p53protein was incubated with *mdmx* mRNA prior to the *in vitro* translation assays in binding buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 0.02 µg/ml yeast tRNA, 0.2 mg/ml BSA for 15 minutes at 37°C. *In vitro* translation assays were performed using Easytag Express Protein Methionine Mix (PerkinElmer, Boston, USA) and Reticulocyte Lysate system (Promega) according to the manufacturer's protocol and capped *in vitro* synthesized *mdmx* mRNA obtained with mMESSAGE mMACHINE T7 kit (Ambion) according with manufacture's protocol.

e/ Autoradiograph of *in vitro* translation like in (d) but comparing the effects of p53 on MDMX synthesis with, or without, pre-incubation with recombinant p53 protein together with the in vitro

transcribed *mdmx* mRNA. The autoradiograph below shows the effect of p53 on GFP synthesis with pre-incubation. See also Supplementary Figure 2.

Figure 2. MDMX 5'UTR is required for p53 to bind *mdmx* mRNA and to suppress its translation

a/ Recombinant p53 from insect cell (i.c.) or bacteria (bact.) binds *mdmx* RNA in *in vitro* RNA-protein co-immunoprecipitation (Co-IP). All binding reactions were carried out for 15 minutes at 37°C in binding buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 0.02 µg/ml yeast tRNA, 0.2 mg/ml BSA. 120 ng of recombinant p53 protein, purified from insect cells (p53 i.c.) or bacteria (p53 bact.) as indicated, and a fixed amount (0.01 pmol) of *mdmx* RNA were used. After incubation, RNA-protein complexes were pulled-down over night at +4°C using anti-p53 (CM1) antibody and protein G beads (Amersham). The unbound fraction was recovered for later analysis and the bound RNA was released from the beads using proteinase K (Sigma). All RNA fractions were then extracted and purified using TRIzol protocol (Invitrogen). RT-qPCR was performed using primers for *mdmx* as described in Figure 1c. The relative binding of mRNAs to proteins was expressed as the ratio between bound and total (bound+free) RNA.

b/ p53 protein binding to *mdmx* RNAs constructs with different size of 5'UTR in *in vitro* RNA-protein Co-IP. *mdmx* constructs correspond to the coding region without 5'UTR (+1) and coding region with 120 or 166 nucleotides of the 5'UTR (-120 and -166, respectively). An RNA construct corresponding to the structured cMyc IRES followed by the coding sequence of the ovalbumin (*cMyc-Ova*) is used as a negative control and the p53 coding region and its 5'UTR (*5'UTR p53*) as a positive control. Protocol is performed as in Figure 2a. *P test <0,01 and ** <0.001.

c) The proximity ligation assay (PLA) using digoxinin labeled probe (+10190 - +10122) against the *mdmx* mRNA and primary mAb antibody against digoxigenin and a rabbit polyclonal against p53 (CM-1) shows the interaction between the p53 protein and the full length *mdmx* mRNA including the 5' UTR (left) predominately in the cytoplasm but also in the nuclear compartment. Less interactions are observed using the coding sequence (CDS) alone (middle) and no interactions

were observed using an mRNA lacking the 5' UTR plus the first 381 nt of the CDS ($MDMX^{p60}$). Using secondary or primary antibodies alone gave no PLA signal (data not shown). White arrows indicate PLA reactions.

d/ Inhibition of *mdmx* translation by p53 protein in *in vitro* translation assays requires the *mdmx* 5'UTR. *In vitro* translation assays were performed as in Figure 1d. See also Supplementary Figures 2 and 3.

e/ *In vitro* translation assay using recombinant p53 and indicated reporter construct in which the -120 to +1 sequence of the *mdmx* mRNA was fused to the ovalbumin (Ova) coding sequence. In vitro translation assays were performed as in Figure 1d.

Figure 3. p53 core DNA-binding domain binds *mdmx* mRNA but does not suppress its translation

a/ The interaction between p53 or the p53 core DNA binding domain (p53-DBD) and *mdmx* RNA in *in vitro* RNA-protein Co-IP. p53-DBD was either produced and purified in presence of zinc (p53-DBD(+)Zn) or without zinc complementation (p53-DBD(-)Zn). RNA-protein Co-IP was performed as described in Figure 2a. See also Supplementary Figure 2.

b/ p53-DBD (aa 92-292) binds *mdmx* 5'UTR (-120 to +1) in *in vitro* RNA-protein ELISA (upper panel). 96 well plates were coated with streptavidin overnight at 4°C and washed 6x 200µl with 0.1 % PBS-Tween and blocked with 100µl of 3 % BSA, 0.1µg/ml of streptavidin overnight. A mix of *in vitro* transcribed biotinylated *mdmx* 5'UTR mRNA (0.1 pmol) (corresponding to nucleotides -120 to +1) and recombinant p53-DBD were incubated in binding buffer (50 mM Tris pH 7.5, 150 mM NaCl, 0.02 µg/ml yeast tRNA, 0.2 mg/ml BSA) for 30 min at 37°C and then added to the plates (50µl/ well) and incubated 1h at RT. The plates were washed 6x 200µl/well with 0.1 % PBS-Tween and the 6His mAB/HRP conjugate (Clontech) was added (50 µl/well) and incubated for 1h at RT and following washes, ECL was added and luminescence was measured. Lower panel shows RNA-protein ELISA competition assays using plates coated with 40-nucleotides synthetic RNAs

fragments corresponding to the indicated region of *mdmx* 5'UTR bound to p53-DBD and subjected to competition with the indicated amount of *mdmx* 5'UTR (-120 to +1). See also Supplementary Figure 3.

c/ RNA-protein ELISA assays using DNA decamer competitors. Using the same protocol as in Figure 3b, plates were coated with fix amount (0.5pmol) of *mdmx* 5'UTR (-120 to +1) and increasing amount of indicated DNA decamer corresponding to p53 consensus half sites. Decamers 1, 2 and 3 correspond to GGGCATGCCC, AGGCATGCCT and GGACATGTCC respectively. See also Supplementary Figure 4.

d/ Metabolic pulse label followed by MDMX IP and corresponding western blot of samples co-expressing $MDMX^{WT}$ and p53 or p53-DBD. The metabolic pulse label was done as described in Figure 1b. The DO-12 mAb (epitope aa. 256-270) was used for p53 immunoblotting and the generated monoclonal antibodies for MDMX.

Figure 4. The p53 trans-suppression domain is located in the p53 N-terminal domain

a/ Pulse labeling in the presence of indicated p53 constructs followed by MDMXΔRING immunoprecipitation (upper panel) and corresponding western blots (below). Pulse label was performed as described in Figure 1b. Polyclonal CM-1 antibody was used to detect p53 isoforms and generated monoclonal antibodies to detect MDMX in western blots.

b/ Pulse labeling followed by MDMXΔRING immunoprecipitation and western blots. MDMXΔRING was co-expressed with p53 constructs carrying point mutations within the amino acids 41 to 43 as indicated.

c/ Pulse label of MDMX full length and $MDMX^{p60}$ in the presence of the wild type p53 or the non-DNA binding and transcriptional inactive p53(R273H) mutant. See also supplementary figure 5.

d/ RNA-ELISA using recombinant p53wt or p53(R273H) and *mdmx* RNA.

e/ Western blot showing the suppression of MDMXΔRING synthesis by p53 in cells treated, or not, with 1 μM doxorubicin (Doxo) for 4 hours.

f/ Pulse label shows the effect of p53 on MDMX synthesis under normal conditions or when cells had been exposed to 1 μM doxorubicin (Doxo) for 4 hours.

Figure 1.

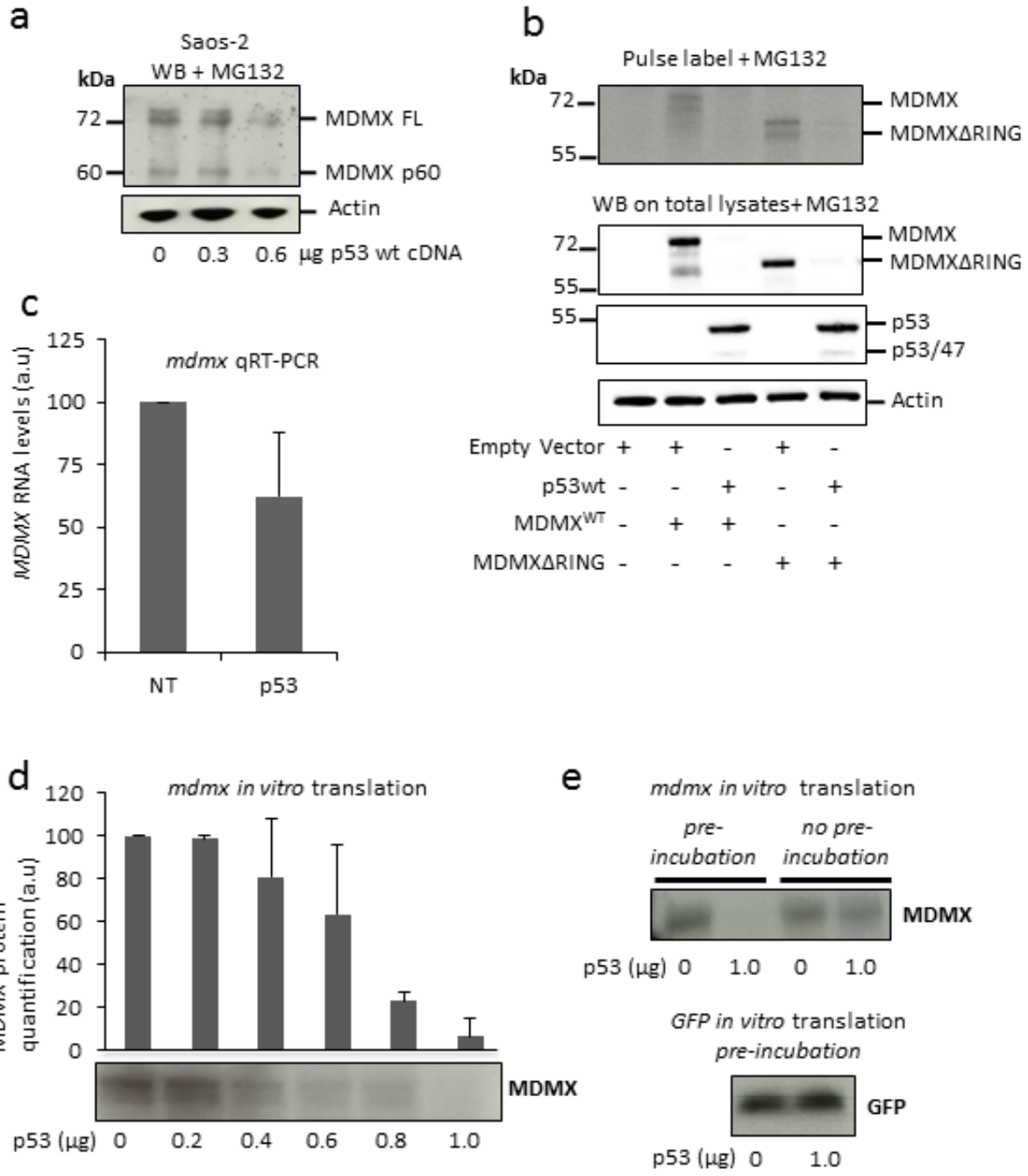

a
Saos-2
WB + MG132
kDa
72
60
MDMX FL
MDMX p60
Actin
0 0.3 0.6 µg p53 wt cDNA
b
Pulse label + MG132
kDa
72
55
MDMX
MDMXΔRING
WB on total lysates+ MG132
72
55
MDMX
MDMXΔRING
55
p53
p53/47
Actin
Empty Vector + + - + -
p53wt - - + - +
MDMX^WT - + + - -
MDMXΔRING - - - + +
c
mdmx qRT-PCR
MDMX RNA levels (a.u)
125
100
75
50
25
0
NT
p53
d
mdmx in vitro translation
MDMX protein quantification (a.u)
120
100
80
60
40
20
0
MDMX
p53 (µg) 0 0.2 0.4 0.6 0.8 1.0
e
mdmx in vitro translation
pre-incubation
no pre-incubation
MDMX
p53 (µg) 0 1.0 0 1.0
GFP in vitro translation
pre-incubation
GFP
p53 (µg) 0 1.0

Figure 2.

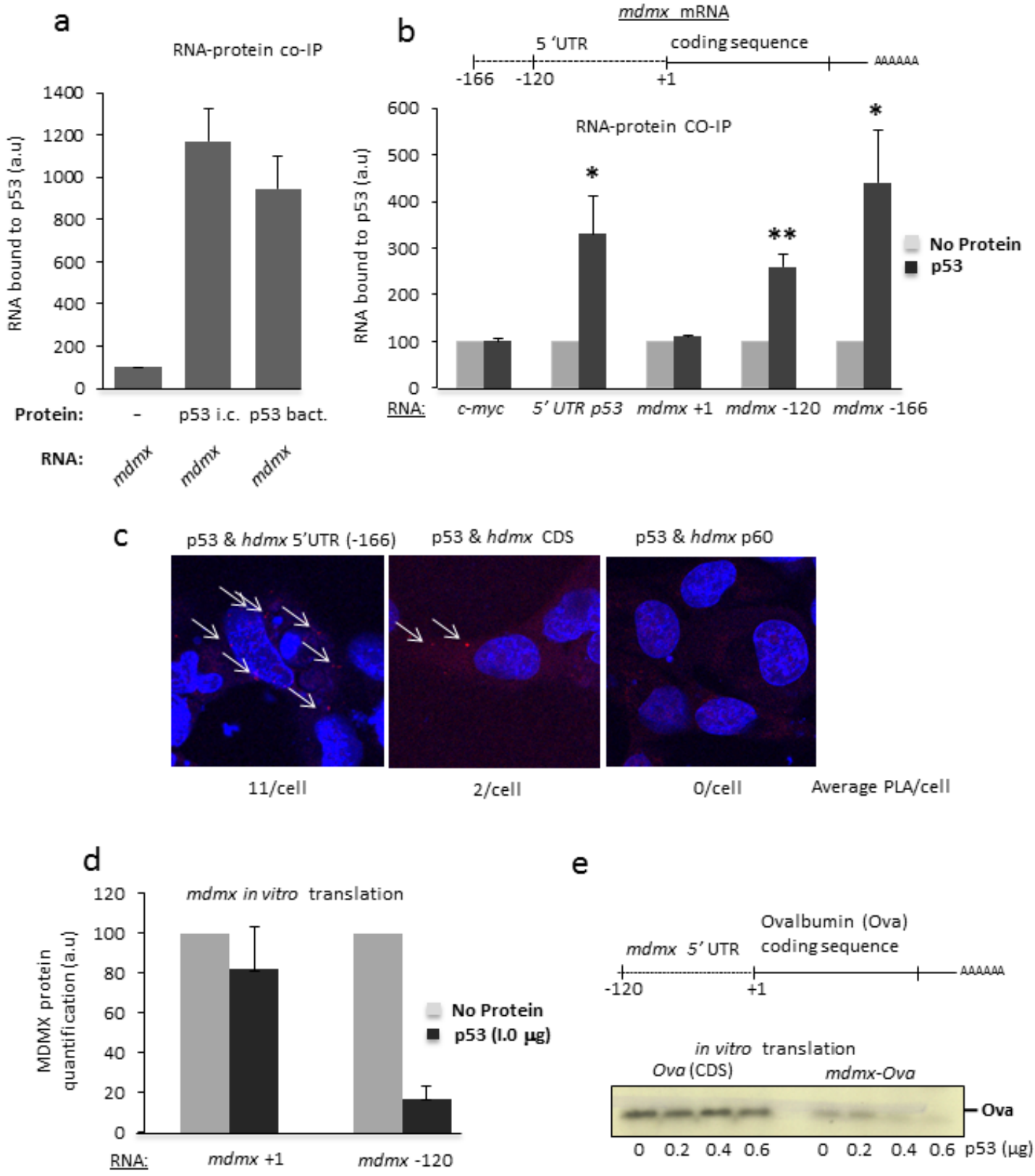

a
RNA-protein co-IP
RNA bound to p53 (a.u)
1400
1200
1000
800
600
400
200
0
Protein: - p53 i.c. p53 bact.
RNA: mdmx mdmx mdmx
b
mdmx mRNA
5 'UTR
coding sequence
AAAAAA
-166 -120 +1
RNA-protein CO-IP
RNA bound to p53 (a.u)
600
500
400
300
200
100
0
*
**
*
No Protein
p53
RNA: c-myc 5' UTR p53 mdmx +1 mdmx -120 mdmx -166
c
p53 & hdmx 5'UTR (-166)
p53 & hdmx CDS
p53 & hdmx p60
11/cell
2/cell
0/cell
Average PLA/cell
d
mdmx in vitro translation
MDMX protein quantification (a.u)
120
100
80
60
40
20
0
No Protein
p53 (I.0 µg)
RNA: mdmx +1 mdmx -120
e
Ovalbumin (Ova)
mdmx 5' UTR
coding sequence
AAAAAA
-120 +1
in vitro translation
Ova (CDS)
mdmx-Ova
Ova
0 0.2 0.4 0.6 0 0.2 0.4 0.6 p53 (µg)

Figure 3.

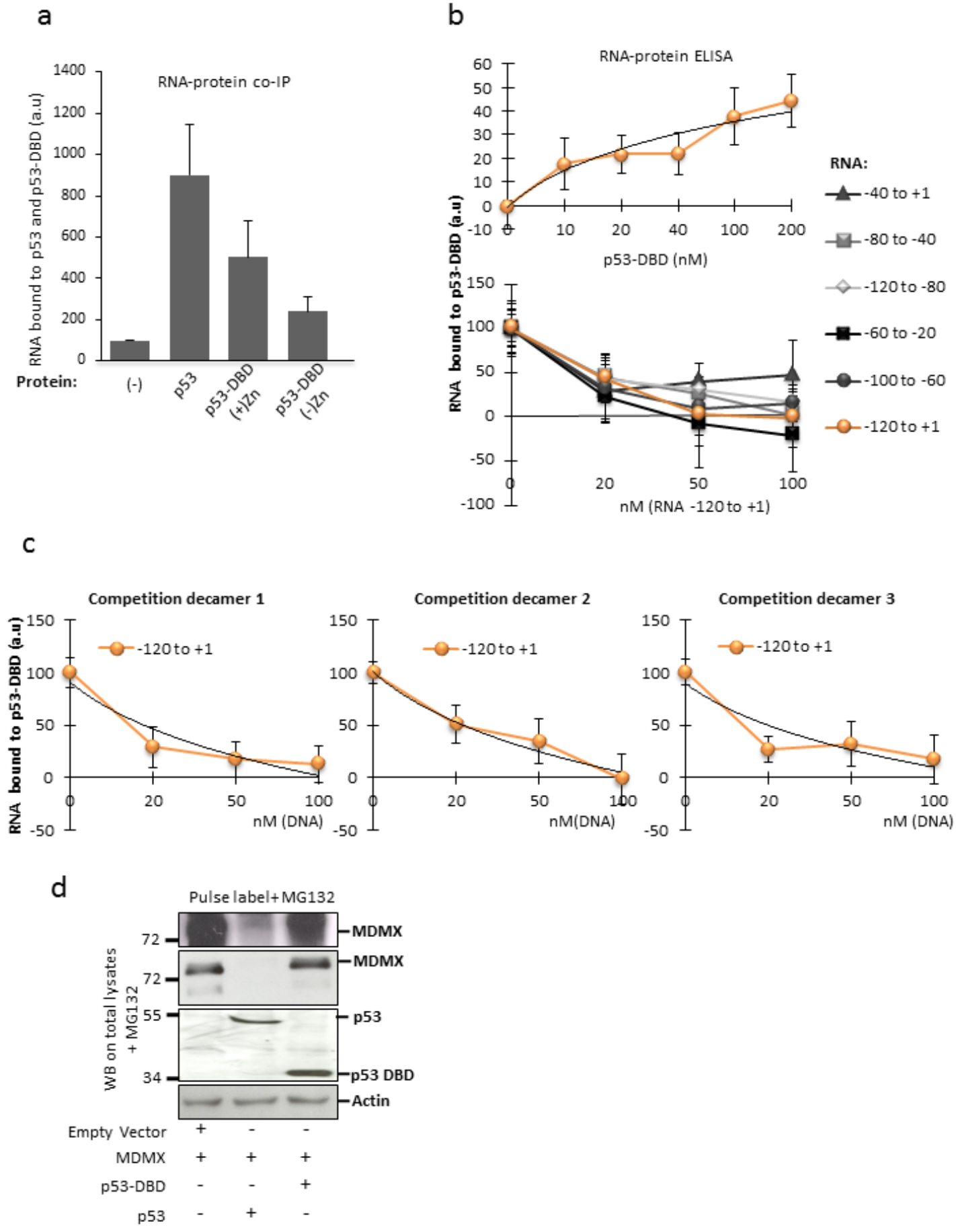
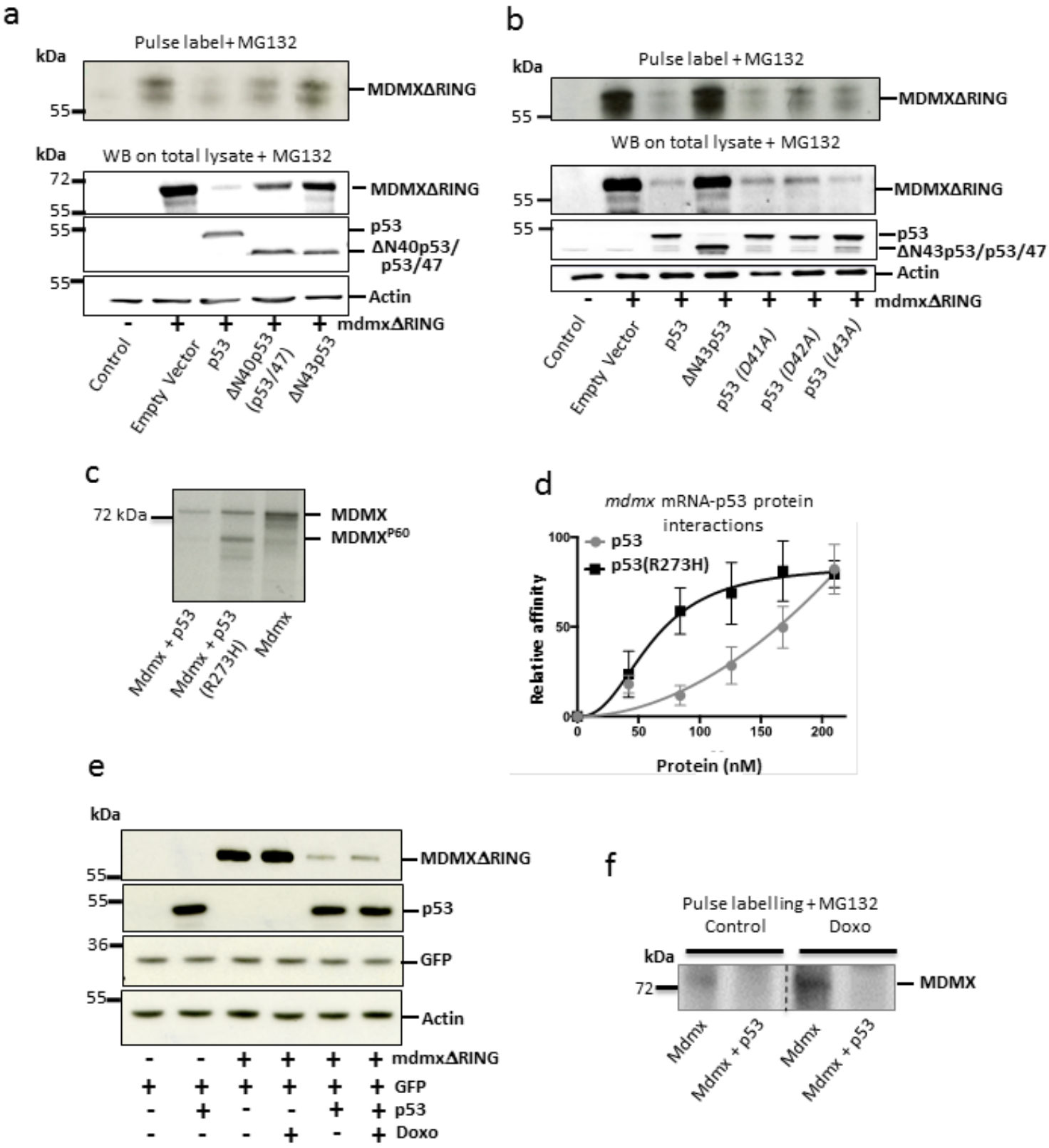
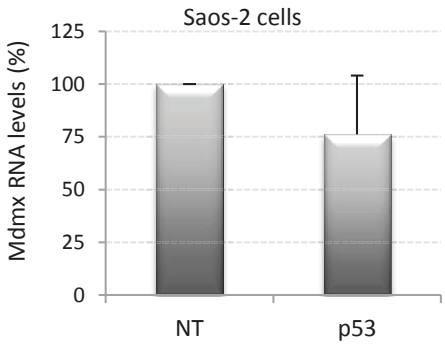

a
RNA-protein co-IP
RNA bound to p53 and p53-DBD (a.u)
1400
1200
1000
800
600
400
200
0
Protein:
(-)
p53
p53-DBD (+)Zn
p53-DBD (-)Zn
b
RNA-protein ELISA
RNA bound to p53-DBD (a.u)
p53-DBD (nM)
RNA:
-40 to +1
-80 to -40
-120 to -80
-60 to -20
-100 to -60
-120 to +1
nM (RNA -120 to +1)
c
Competition decamer 1
Competition decamer 2
Competition decamer 3
RNA bound to p53-DBD (a.u)
-120 to +1
nM (DNA)
nM(DNA)
d
Pulse label+ MG132
WB on total lysates + MG132
72
72
55
34
MDMX
MDMX
p53
p53 DBD
Actin
Empty Vector + - -
MDMX + + +
p53-DBD - - +
p53 - + -

Figure 4.


a
Pulse label+MG132
kDa
55
MDMXΔRING
WB on total lysate + MG132
72
55
p53
ΔN40p53/
p53/47
Actin
mdmxΔRING
Control
Empty Vector
p53
ΔN40p53 (p53/47)
ΔN43p53
b
Pulse label + MG132
WB on total lysate + MG132
p53
ΔN43p53/p53/47
p53 (D41A)
p53 (D42A)
p53 (L43A)
c
72 kDa
MDMX
MDMX^P60
Mdmx + p53
Mdmx + p53 (R273H)
Mdmx
d
mdmx mRNA-p53 protein interactions
p53
p53(R273H)
Relative affinity
100
50
0
0
50
100
150
200
Protein (nM)
e
GFP
Doxo
f
Pulse labelling + MG132
Control
Doxo
Mdmx
Mdmx + p53

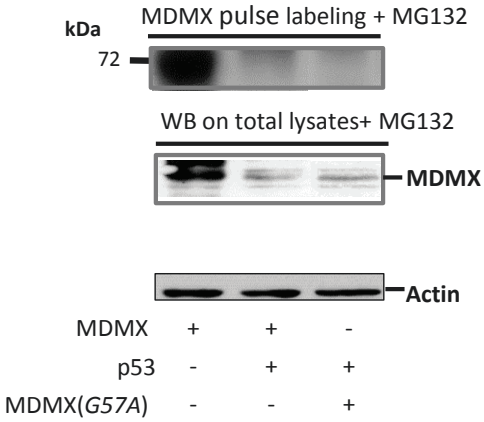
Supplementary Figure 1. (relates to Figure 1)

a



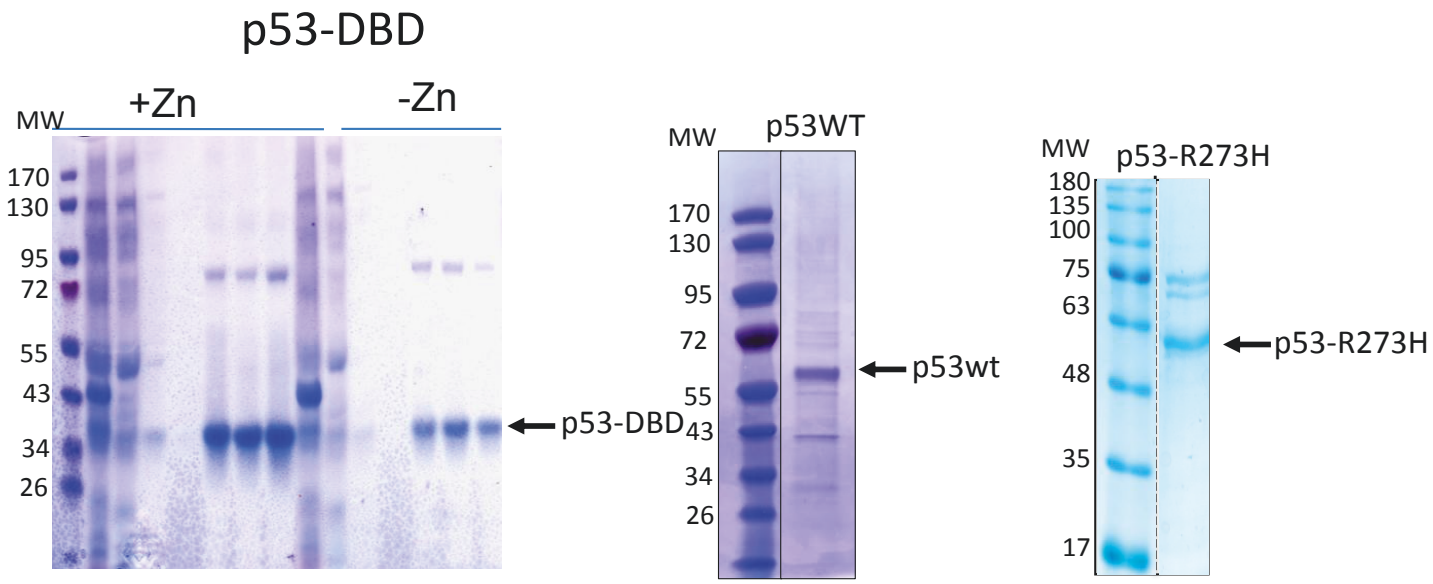
b) The effect of p53 on *mdmx* RNA levels in Saos-2 cells.

b



b) The top autoradiograph shows a metabolic pulse labeling (^{S}35Met) followed by MDMX IP in the presence of p53. Below shows western blots of MDMX (second from top) and MDM2 (third from top) The mutant MDMX(*G57A*) that lacks the capacity to form protein-protein interaction with p53, is also suppressed by p53, suggesting that this inhibition of synthesis does not require p53-MDMX protein-protein interaction.

Supplementary Figure 2. (relates to Figures 1D, 1E, 2A, 2B, 2D, 2E, 3A, 3B, and 3C)



Purification of recombinant protein

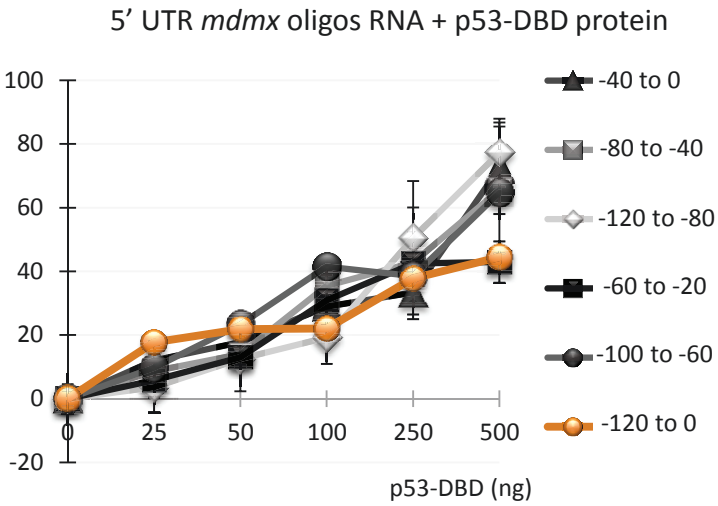
Coomassie stained gels shows purified p53 proteins. Histidine tagged recombinant proteins were produced in BL21 (DE3) E. *Coli* and purified on HiTrap Nickel column (GE healthcare) on ÄKTA purifier system.

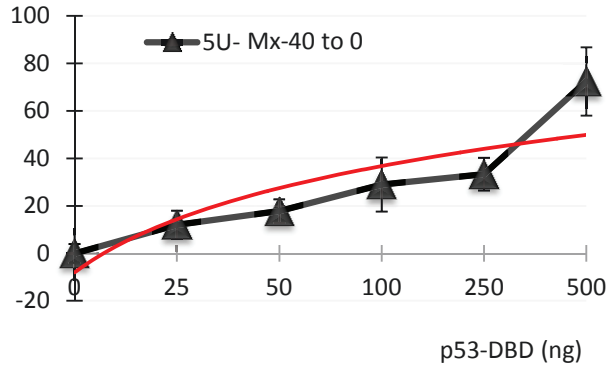
For p53 full length lysis and binding buffer contain 25mM HEPES, 100mM NaCl, 15mM Imidazol, 10% glycerol, 1mM Tris, 10μM $ZnSO_4$ at pH 8 and were complemented with Complete Protease Inhibitor Cocktail EDTA-free Tablets (Roche). Recombinant proteins were eluted from Nickel column with buffer containing 25mM HEPES, 100mM NaCl, 300mM Imidazol, 10μM $ZnSO_4$, 5mM β-Mercaptoethanol, 1mM Tris at pH 8 and dialyzed upon gel filtration step on Superdex 75 in pH8 buffer containing 25mM HEPES, 100mM NaCl, 1mM Tris , 10μM $ZnSO_4$.

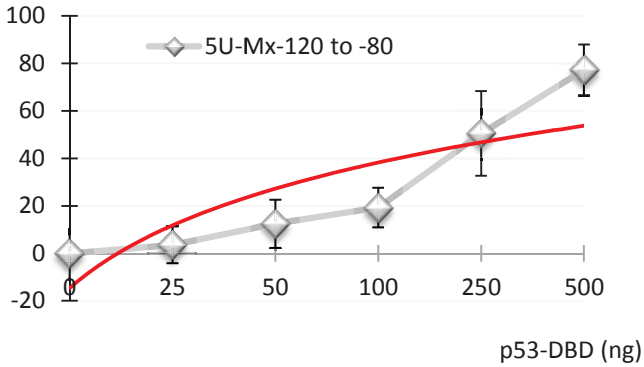
For p53-DBD lysis and binding buffer contain 25mM Citrate, 100mM NaCl, 15mM Imidazol, 10% glycerol, 1mM Tris, 10μM $ZnSO_4$ at pH 6 and were complemented with Complete Protease Inhibitor Cocktail EDTA-free Tablets (Roche). p53-DBD Recombinant proteins were eluted from Nickel column with buffer containing 25mM Citrate, 100mM NaCl, 300mM Imidazol, 10μM $ZnSO_4$, 5mM β-Mercaptoethanol, 1mM Tris at pH 6 and dialyzed upon gel filtration step on Superdex 75 in pH6 buffer containing 25mM Citrate, 100mM NaCl, 1mM Tris , 10μM $ZnSO_4$.

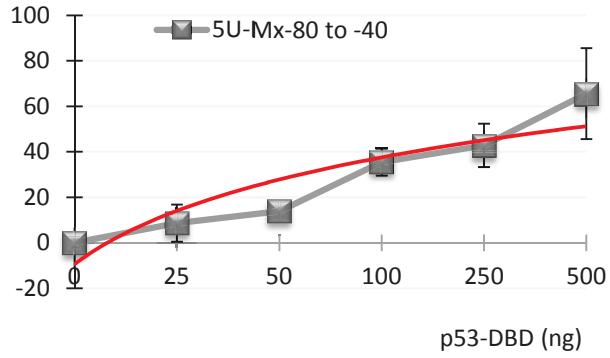
Supplemental Figure 3. (relates to Figure 3B)

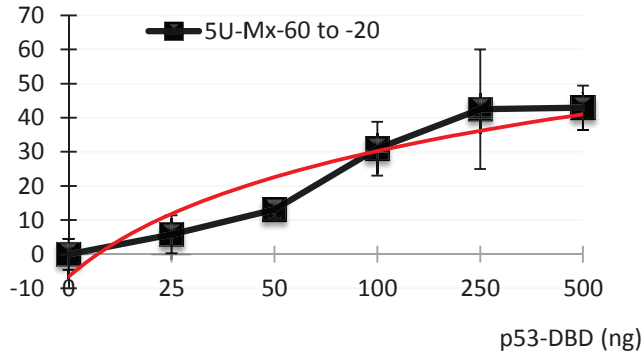
In vitro protein-RNA ELISA : 96 well plates were coated with streptavidin 100µg/ml in 0.1M NaHCO3 blocked with 100µl of 3 % BSA, 0.1µg/ml of streptavidin in PBS. A mix of biotinylated RNA (as indicated on the legend) (0.1 pmol) and p53-DBDwas incubated in binding buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 0.02 µg/ml yeast tRNA, 0.2 mg/ml BSA for 30 min at 37°C and then added to the plates (50µl/well) and incubated 1h at RT. The plates were washed and the 6His mAB/HRP conjugate (Clontech) was added and incubated for 1h at RT. Plates were washed and 50 µl/well of mix of ECL was added and luminescence was measured. 5U is 5' UTR; Mx is MDMX

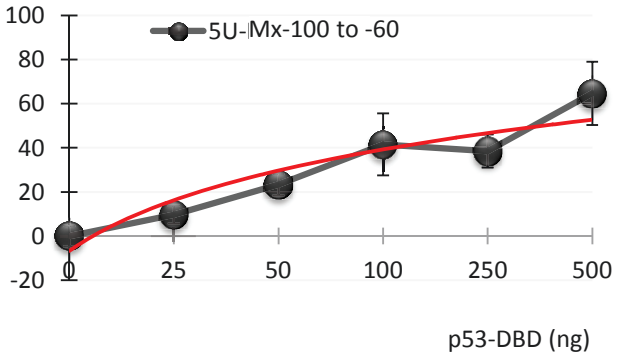


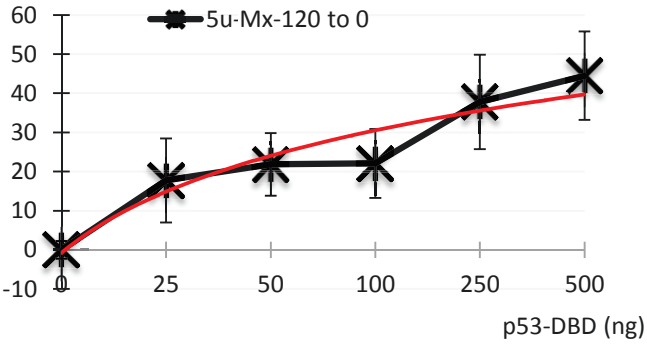




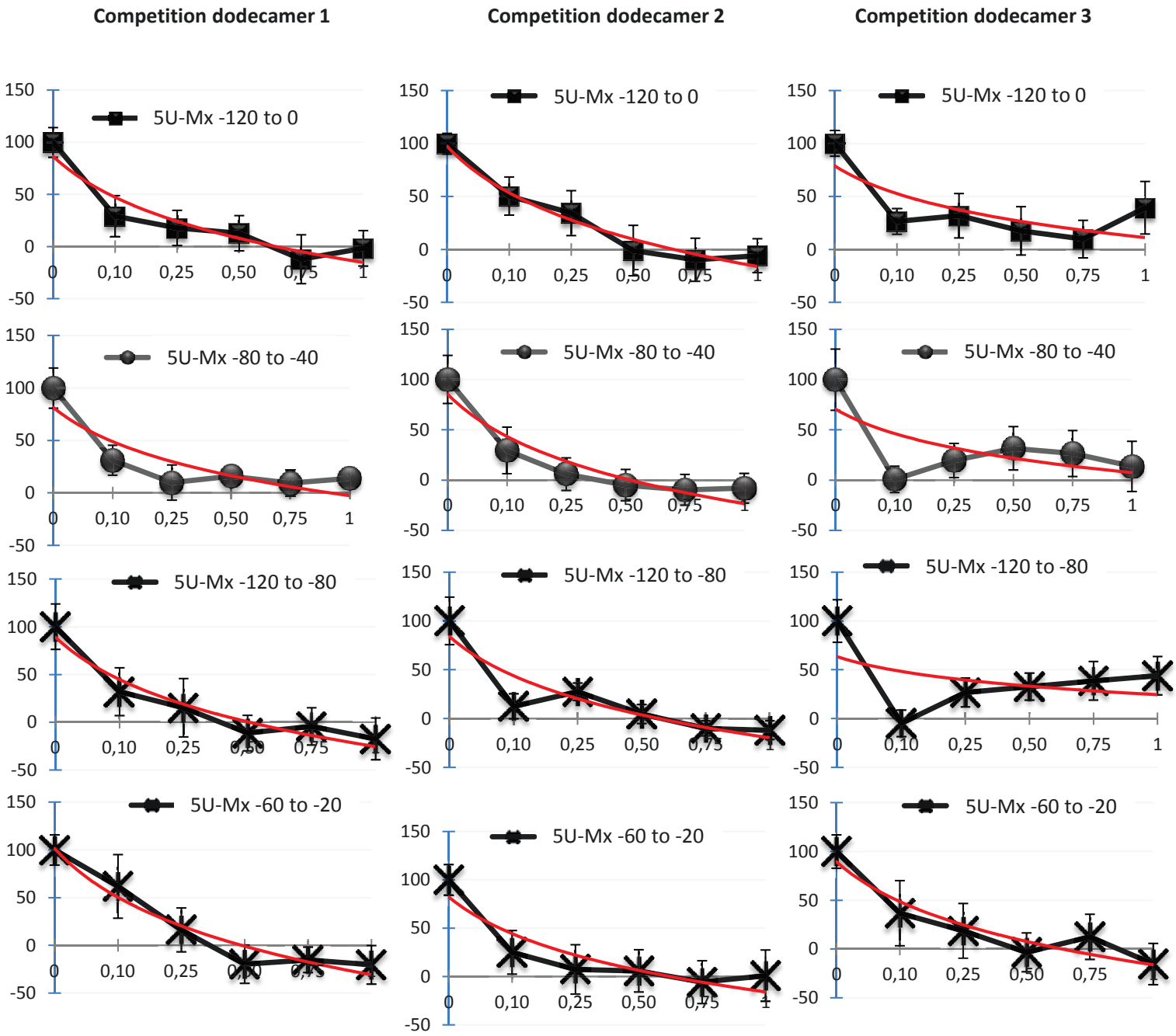








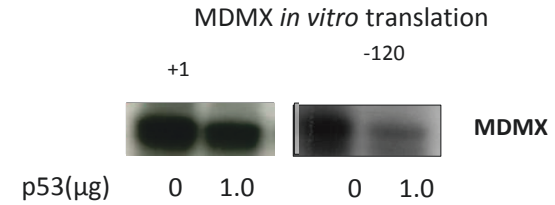
Supplemental Figure 4. (relates to Figure 3C)



In vitro protein-RNA ELISA showing the competition between the DNA decamer (DNA sequence of decamer 1, 2 and 3 are respectively GGGCATGCCC, AGGCATGCCT and GGACATGTCC) and the indicated RNA bound to p53-DBD.

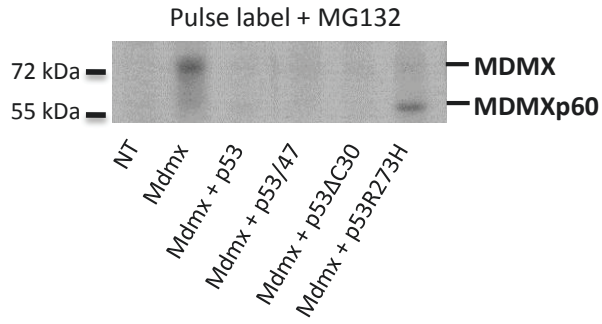
Supplementary Figure 5. (relates to Figures 2 and 4)

a



a) The inhibition of *mdmx* translation *in vitro* by p53 protein correlates with the mdmx 5'UTR.

b



b) The inhibition of *mdmx* translation *in cellulo* by p53 proteins.

Discussion

The work presented here shed some light on two biological aspects. The first one relates to the RNA-binding activity of p53 and its associated capacity to trans-suppress mRNA translation. The second one refers to the physiological responses mediated by p53 during ER stress that depend on control of translation and transcription.

1. Mechanistic concepts

These studies show similarities and differences in the way p53 controls the synthesis of BiP, MDM2 and MDMX. The control of MDM2 and BiP expression relies on their coding sequences while in the case of MDMX, its 5'UTR was shown to be required. Interestingly, down-regulation of MDM2 and BiP synthesis is related to ER stress while suppression of *mdmx* mRNA translation could not be related to neither ER stress not DNA damage. However, a direct interaction between p53 and *bip* or *mdmx* mRNAs was detected while for *mdm2* no binding was reported so far. The latter is similar to the suppression of $p21^{CDKN1A}$ synthesis for which no p53-RNA interaction was yet observed (Mlynarczyk & Fahraeus, 2014).

The difference at the nucleotide sequence presented by *bip*, *mdm2*, *mdmx* and $p21^{CDKN1A}$ is evident. The differences among p53 target mRNA sequences is even higher if the previously reported targets are considered (See Annex 1 "Alignment of target mRNAs"). At this point, even though it is still early days, one can imagine at least two mechanisms of p53 mRNA translation suppression based on a direct interaction between p53 and the mRNA or a non-RNA binding mechanism. The latter could be mediated indirectly by an hitherto unknown target for p53 transcription activity and this fits with the case of $p21^{CDKN1A}$ where a p53 wild-type DNA-binding domain is required to subdue its synthesis (Mlynarczyk & Fahraeus, 2014). However, mutations in the DNA-binding domain of p53 that abolished the effect on $p21^{CDKN1A}$ synthesis do not exclude the possibility of affecting p53-RNA interactions rather than affecting the DNA-binding-dependent trans-activation activity, as discussed below. Although not yet tested and due to the overall similarities in the phenotype, these ideas might also be applied for *mdm2* (see further below).

The second mechanism involves the RNA-binding capacity of p53. The p53 mRNA was the first mRNA target whose translation was reported to be controlled by a direct interaction with the p53 protein (Mosner et al, 1995). This work described that the binding was dependent on the 5'UTR of p53 and that mutations on p53 did not abrogate the interaction. On the other hand, repression of translation in *in vitro* experiments was achieved only with the wild-type protein and not with the MethA mutant, suggesting that RNA binding is not sufficient to control translation and that other domain is required in order to do it. However, the authors did not report which mutation was used in the study. Since the MethA fibrosarcoma tumor cells contains both alleles of p53 mutated (Met234Ile in one allele and p53Cys132Phe/Glu168Gly in the other (Humar et al, 2014)) it is difficult to correlate a p53 domain/function with control of translation of its own mRNA (Mosner et al, 1995). These observations go in the same line as the ones made for *fgf-2* mRNA (Galy et al, 2001a). Control of *fgf-2* mRNA translation was shown to require different regions of the mRNA leader in the 5'UTR and was demonstrated to depend on p53wt. On the other hand, the substitution Val143Ala (a reported cancer mutation that abrogates p53 trans-activation capacity) was not able to inhibit translation of the reporter construct *in vitro* (Galy et al, 2001a). Interestingly, both p53wt and p53Ala143 were able to interact with *fgf-2* mRNA, though less efficiently when the mutant protein was used, as assessed by nitrocellulose filter retention assay. This further supports

the notion that RNA binding is necessary but not sufficient to guide suppression of mRNA translation. In addition, interaction of *fgf-2* mRNA with the p53wt but no the p53Ala143 protein led to a weaker association of the RNA with the 80S particle in comparison to the control reaction without p53. This data suggests that inhibition of translation might be related with a capacity of p53 to modify the formation of translation initiation complexes (Galy et al, 2001a). Moreover, by employing DNA probes complementary to the *fgf-2* mRNA leader and analysis of the RNA/DNA duplex in a shift in probe mobility, it was detected that the accessibility of the DNA probes only occurred in the presence of p53wt and not when the p53Ala143 was used. This points towards a capacity of p53 to modify the secondary structure of the *fgf-2* mRNA leader, a condition that was associated by the authors to the previously described unwinding-annealing activity of p53 (Oberosler et al, 1993). The fact that a pattern on how different mRNAs are regulated by p53 is now starting to emerge will help in future work aimed at addressing the possibility of more than one mechanisms by which p53 controls mRNA translation.

It is worth to mention that FGF-2 expression was also related to apoptosis (see further below). FGF-2 was shown to inhibit the tunicamycin-dependent apoptosis in human hepatoblastoma HEpG2 and breast cancer MCF-7 cells. This activity was related to the capacity of FGF-2 to promote proteasome-mediated degradation of NCK1 (Li et al, 2013b), a known inhibitor of IRE1 signalling towards MAPK and apoptosis (Nguyen et al, 2004). This highlights another way present in cancer cells to avoid death induced by the ER stress (Li et al, 2013b; Manie et al, 2014). Unfortunately, the putative role of p53 binding to and suppression of *fgf-2* mRNA translation during ER stress and its final effect on cell death was not addressed, a link that might be interesting to test in relation to tumor heterogeneity.

It is worth to mention that the mRNA regions involved in the above-mentioned interactions with p53 were predicted to be highly structured and this could be the signal recognized by p53 in a specific manner, rather than sequence (Galy et al, 2001a; Mosner et al, 1995). However, questions about the specificity of these interactions were raised in the past (Riley et al, 2006; Riley & Maher, 2007). The low specificity detected in those studies is related to the recognition of RNA by the C-terminal domain of p53 based on yeast three-hybrid assay that could not be confirmed neither *in vitro* or *in cellulo*. However, another role was attributed to the inespecific p53's C-terminal-RNA binding; that of controlling the oligomerization of p53, and therefore, its capacity to interact with DNA (Yoshida et al, 2004). The sequence-independent p53-RNA interaction was shown to control the recognition of p53's REs by p53 *in vitro*, since a p53 consensus oligonucleotide was able to compete with RNA for interaction with p53 protein while the consensus oligonucleotides of CRE or NF-κB were not. This capacity was shown to be controlled by phosphorylation at Ser392, the same position that was previously suggested to enhance p53wt binding to a consensus DNA sequence *in vitro* (Hupp et al, 1992), an activity dependent on the ability to form tetramers. Thus, as its name points out, the regulatory C-terminal domain of p53 may control the binding of p53 to the different nucleotide chains and this could be related to its capacity to form tetramers. Whether the differential binding of p53 to DNA or RNA depends on its oligomerization status via more promiscuos interactions mediated by the C-terminal domain is not known and further studies must be done to clarify that. In addition, specific binding to RNA might be mediated by the DBD of p53, as shown for *mdmx* mRNA. Moreover, the experiments showing the competition of 5'UTR of *mdmx* mRNA with three different DNA oligomers corresponding to p53 DNA consensus half-sites (decamer 1, decamer 2, decamer 3) (Kitayner et al, 2006), showed that p53DBD is able to bind both DNA and RNA and that the interaction of the 5'UTR *mdmx* with p53 was modified in a similar, but not identical, fashion. Thus, this indicates that the RNA and the DNA binding activities of p53 share common structural properties but are not identical.

The work carried out in this thesis, in particular regarding p53's interaction with the *bip* and the *mdmx* mRNAs support the idea that the interactions are structure-dependent. However, not all structured RNAs bind p53 and this is illustrated by the fact that we use the c-myc IRES as a negative control. This model might actually fit with the results of Riley et al, if we consider the possibility that the mRNAs detected in the yeast three-hybrid system were simply better displayed on the surface of the MS2 coat protein as compared with the follow-up *in vitro* assays using shorter RNA oligos in which interactions were not observed. Previous reported mRNAs bound to p53 were claimed to contain two and/or three-dimensional well folded patterns. Figure 12 shows the predicted folding of these mRNAs along with the targets described in this work. It is clear that although some regions are considered stable in the conditions of the simulations, the structures do not show a consensus feature. However, predicting second and tertiary RNA structures based solely on algorithms is notorious difficult and, thus, making predictions about p53 mRNA targets constitutes a very difficult task and leaves only one door open; empirical approaches.

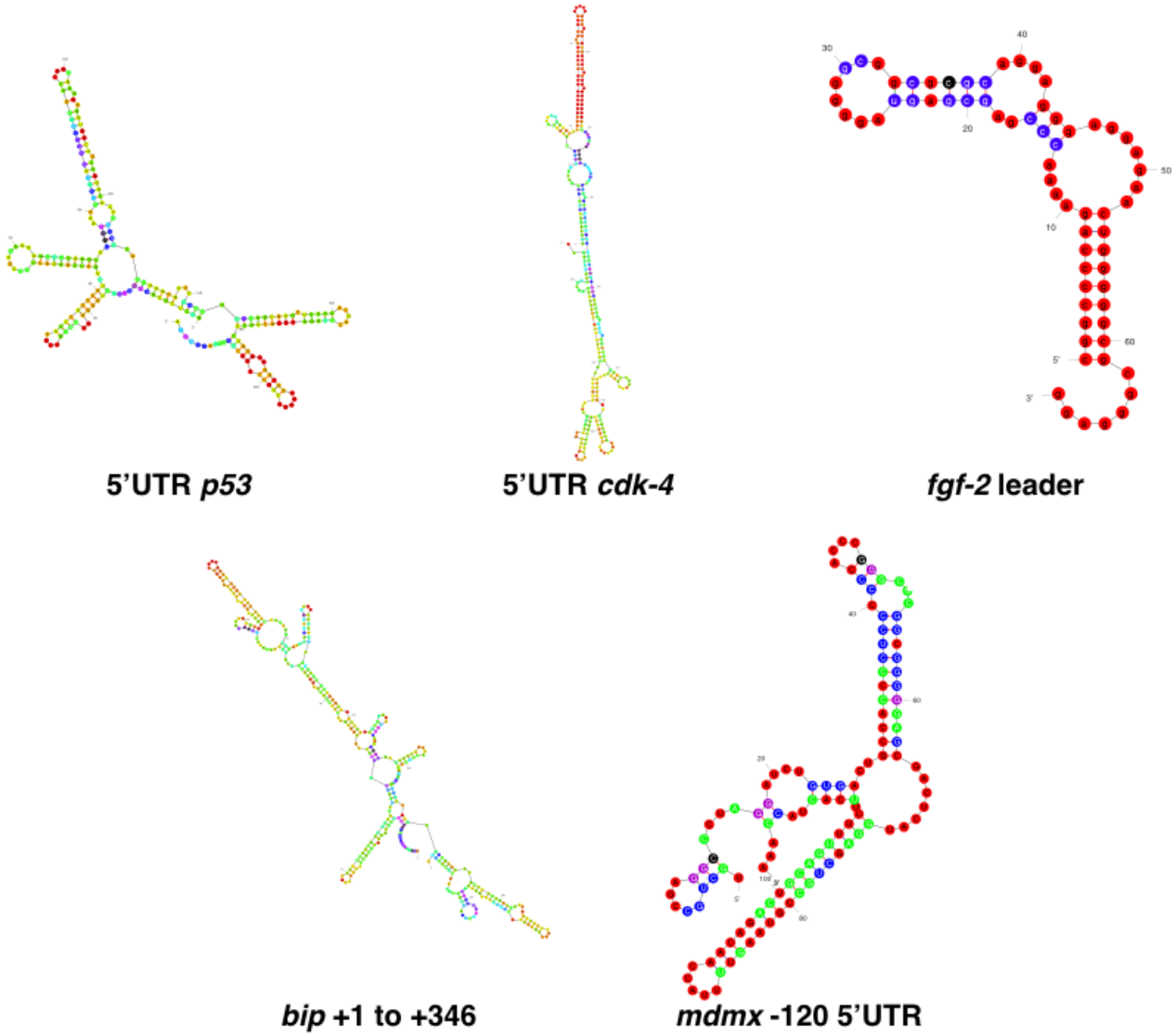


Figure 12. Predicted mRNA folding.
mRNA fold web server was used to predict the secondary structure of the mRNAs bound by p53. The sequences used are the ones reported to mediate the interaction with p53 and are shown in Annex 1. For each mRNA the most stable putative structure according to their Gibbs free energy is shown. Colour code from black to red that corresponds to 0 and

1, respectively, refers to the probability that a base is always single stranded or always paired to a unique partner (Zuker, 2003).

Our results also point out to the statement that binding *per se* does not control the translation of the bound mRNA. As other RNA-binding proteins, and as well as in the case of binding to the DNA and induction of transcription, p53 is a modular protein that requires a core RNA-binding capacity and a regulatory domain. The RNA-binding capacity is located to the DNA-binding and core domain, as suggested by the results with *mdmx* mRNA. Interestingly, the RNA-binding activity presents similitudes to the DNA counterpart as suggested by the requirement of Zn and the competition observed between RNA and DNA, as discussed before. The trans-suppressor domain of p53 that controls expression of BiP and MDMX is located in the TAD II domain.

The TAD II domain starts in the Met40 of the p53FL and represents the first aa of p53ΔN40. This region was important for control of both *bip* and *mdmx* translation. The aa spanning from positions 41 to 47 have been detected to mediate the interaction with different proteins and to adopt a particular secondary structure upon binding, as shown in Figure 13. In particular, Bochkareva et al, described two amphipathic helices in this region that might act together (H1 (residues 41 to 44) and H2 (residues 47 to 55)) and with important differences in binding properties. Indeed, they claim that while helix H2 has the most extensive buried surface and therefore, appears to be the major determinant of the interaction, helix H1 has a smaller interaction surface and smaller changes in NMR resonance frequencies upon binding to RPA70N (the N-terminal domain of the RPA70 monomer of RPA used in the study), suggesting that H1 plays a secondary role in the interaction (Bochkareva et al, 2005). Moreover, Di Lello et al, found that helix H2 mediated the interaction with the p62 (Tfb1) subunit of TFIIH and although they did not attribute any function to helix H1, they showed that p53 phosphorylated at Ser46 (just before the initial position of helix H2 and located in the 7-aa region found to be a requisite to control BiP synthesis), enhanced binding of p53 to both p62 and Tfb1. The interaction was further enhanced by phosphorylation at Thr55 (Di Lello et al, 2006). These results add support to a regulatory role for the region up-stream of helix H2. This behaviour of TAD II is shared with the TAD I that has also been to be flexible in solution but it may adopt transient secondary structures upon association with other factors, as it is exemplified by the p53-MDM2 interaction (Kussie et al, 1996). Thus, the TAD II-dependent down-regulation of BiP synthesis may rely on a protein-protein interaction with a so far unknown factor that is currently the focus of intensive research in our team. Also, post-translational modifications should be kept in mind. This will help us to identify the molecular mechanism leading to translation inhibition, a topic that remains largely unknown.

Translational regulation provides a rapid mechanism to control gene expression and numerous regulatory proteins target the initiation step, often in a way that couples translation to mRNA localization. But the mechanisms of inhibition of the initiation due to the presence of RNA-binding proteins are not well understood. The most clear example is that of ferritin mRNA, whose translation is strongly inhibited by a iron regulatory proteins (IRPs)–RNA interaction occurring at a cap-proximal location (Muckenthaler et al, 1998). The protein–RNA interaction is required to occur at a cap-proximal location, which prevents loading of the 43S complex onto the mRNA but not eIF4F binding to the capped 5′ end. Inhibition is much weaker if the protein-RNA interaction occurs at a distal region, suggesting that in the case of successful loading, the 43S scanning will displace the inhibitory protein. This also seems to depend on the strength of the interaction, since the inhibition of PABP's mRNA translation by binding of PABP protein still occurs even though the binding region is located quite separated from the cap. In this case, that arrives in conditions where there is an excessive amount of free PABP, the scanning process is blocked by PABP rather than

loading of the 43S complex (Jackson et al, 2010). In the case of mRNA control by p53 there are more questions than answers. The only mechanistic evidence comes from the data of *fgf-2*, where a change in the mRNA folding was claimed upon p53 binding (Galy et al, 2001a). However, whether this affects the loading of 43S complex or the initial scanning process remains completely unknown. In addition, since the binding of p53 is not sufficient to suppress synthesis of proteins, it is probably that the effect is mediated by interacting factors/complexes that may impinge on any of the discussed steps.

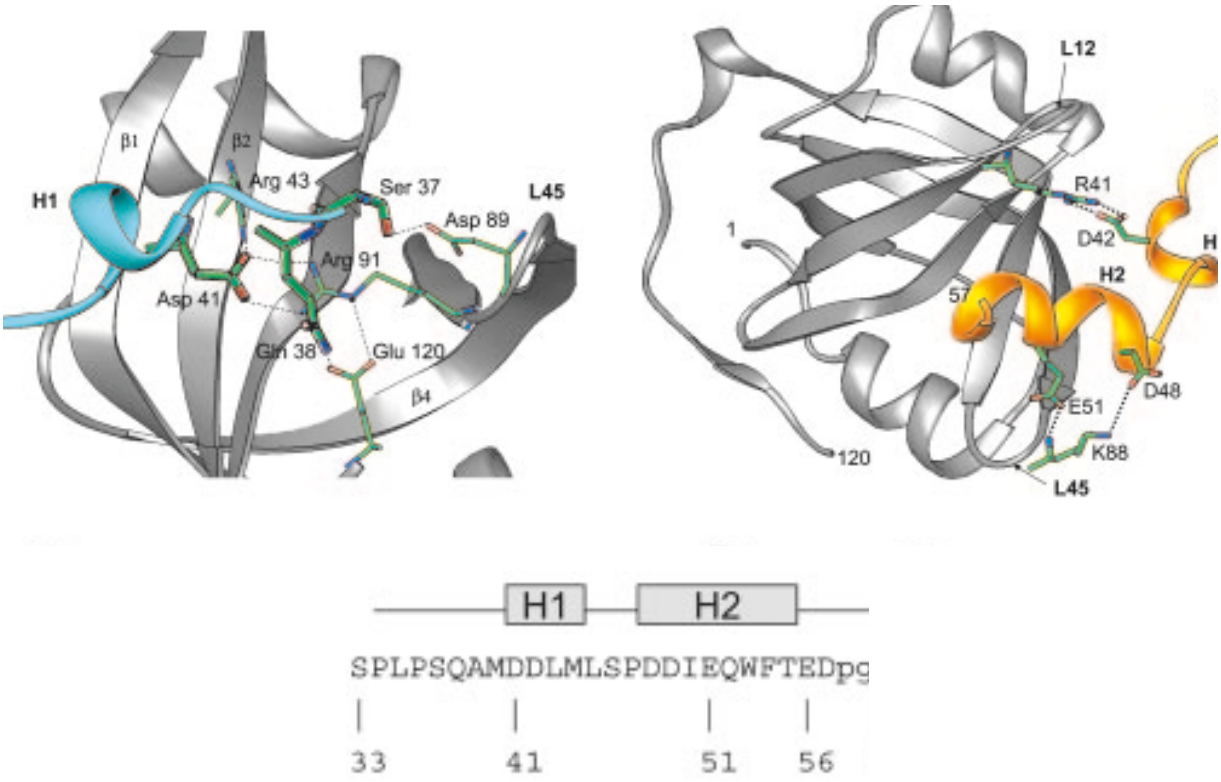


Figure 13. Structural model of the p53 TAD II interacting with RPA.

Structural details of the p53 interaction with the fusion partner RPA70N (upper left) and symmetry-related RPA70N (upper right). Several particularly interesting residues are highlighted. H1 and H2 are shown. Lower panel shows the amino-acidic sequence of part of the TAD II and the regions forming the H1 and H2 helixes. Numbers below refers to the position in the full-length p53 protein. Taken from Bochkareva et al, 2005.

2. MDM2 (and p21^{CDKN1A}) down-regulation under ER stress and its implications

Down-regulation of MDM2 under ER stress conditions was suggested to depend on two activities of p53. First, it relies in the counteracting effect of p53ΔN40 towards induction of *mdm2* transcription by p53FL, in line with previous reports (Courtois et al, 2002; Yin et al, 2002). Secondly, *mdm2* mRNA translation is diminished by both p53FL and p53ΔN40 isoforms. It is interesting to note that repression of translation was also detected in normal conditions using an exogenous construct containing only the coding sequence of MDM2. This argues towards the idea that this activity is constitutive of p53. However, the down-regulation of endogenous MDM2 only became evident when the cells were faced to ER stress. ER stress leads to up-regulation of p53ΔN40 and a consequent inhibition of p53-mediated *mdm2* transcription and, thus, it allows the trans-suppression activity to become important and to be detected. The inhibitory effect of p53ΔN40 was also shown for transcription of *p21^{CDKN1A}* by our group and others (Ghosh et al, 2004; Mlynarczyk & Fahraeus, 2014; Yin et al, 2002). Similar to MDM2, p21^{CDKN1A} expression was also diminished during ER stress by a combined effect of the inhibition of transcription by p53ΔN40 and translation repression by the two isoforms. The opposite effects of p53 towards MDM2 and p21^{CDKN1A} during DNA damage and UPR suggests the possibility that different aspects of p53 response are prominent during different cellular conditions. Under ER stress, the cells trigger a general suppression of protein synthesis via phosphorylation of eIF2α as a pre-requisite to favour repair and avoid apoptosis (Holcik & Sonenberg, 2005; Jackson et al, 2010), and it is possible that control of translation is a more favourable mechanism to control gene expression in response to this type of stress, which goes in line with the results concerning MDM2 and p21^{CDKN1A}.

While down-regulation of p21^{CDKN1A} was exquisitely proved to be required to stabilize 14-3-3σ and trigger G2 arrest (Mlynarczyk & Fahraeus, 2014), the reasons to suppress MDM2 expression during the UPR are at this point merely speculative. The more I think about it the more I convince myself that this might be related to stability of p53. Since synthesis of p53FL is largely suppressed and its degradation is enhanced during the UPR (Baltzis et al, 2007; Pluquet et al, 2005; Qu et al, 2004), MDM2 down-regulation might avoid a complete loss of the p53 full-length protein, keeping a bit to orchestrate responses that rely on p53's trans-activation capacity due to the presence of TAD I. It should also be kept in mind that during DNA damage, p53 is prevented from degradation by MDM2 via ATM activity. Interestingly, this is done by two mechanisms. Firstly, ATM-mediated phosphorylation of p53 prevents it from becoming a target for MDM2 ubiquitynation and proteasomal-dependent degradation. Also, activated ATM phosphorylates both MDM2 and MDMX and renders them positive regulators of p53 synthesis. This, very interestingly, is mediated by direct binding of p53 mRNA first by MDMX that offices as an RNA chaperone that induces a conformational change on the mRNA that mediates the subsequently interaction of MDM2, which will induce p53 protein synthesis (Candeias et al, 2008; Malbert-Colas et al, 2014; Naski et al, 2009). Thus, this also adds more support to the importance of mRNA translation control in the p53 pathway in response to different cellular conditions. We do not know the status of ATM during the UPR, but so far, activation of ATM has not been described. Therefore, down-regulation of MDM2 expression offers an alternative mechanisms to ensure p53 expression. MDM2 has the capacity to promote degradation of p53ΔN40 when in hetero-dimer formation with full-length protein and it is thus possible that down regulation of MDM2 safeguards these complexes. However, p53-independent functions have been described for MDM2, such as apoptosis, cell cycle regulation, DNA replication and DNA repair, the two latter related to loss of genome stability (Bouska & Eischen, 2009). It is thus possible that down-regulation of MDM2 during ER stress might be related to other activity not related to controlling p53 expression.

3. Effect on apoptosis

Activation of PERK and subsequent phosphorylation of eIF2α lead to inhibition of global cap-dependent translation (Holcik & Sonenberg, 2005; Jackson et al, 2010). This appears intuitive to favour repairing processes since it lowers the protein input and, thus, represents a rapid protective response of the UPR (Szegezdi et al, 2006). This is in line with the fact that *Perk*$^{-/-}$ mouse embryonic fibroblasts, when challenged with ER stress-inducing agents, failed to block protein translation and exhibited increased cell death (Szegezdi et al, 2006) and by the hypersensitivity to ER stress observed when a non-phosphorylable form of eIF2α was over-expressed (Harding et al, 2000; Urra et al, 2013). However, the PERK arm of the UPR also leads to induction of CHOP, which is one of the main inducers of apoptosis in response to ER stress reported so far (Hetz et al, 2013; Szegezdi et al, 2006; Urra et al, 2013). CHOP was previously shown to control the expression of members of the BCL-2 family. BCL-2 itself was shown to be repressed by CHOP at the transcription step (McCullough et al, 2001) while BIM and PUMA were reported up-regulated upon CHOP induction during ER stress (Puthalakath et al, 2007; Reimertz et al, 2003). More recently, however, a ChIP-seq and mRNA-seq analysis of ATF4 and CHOP demonstrated that they interact to directly induce genes encoding protein synthesis-related factors including eleven aminoacyl-tRNA synthetases and four initiation factors as well as members of the UPR. This correlated with an increased protein synthesis rate, as assessed by incorporation of ^{35}S in total extracts of *Chop*$^{-/-}$ MEFs where ATF4 and/or CHOP were over-expressed compared to their mock-transfected counterparts. In addition, it also correlated with less cell viability due to increased ROS production and ATP depletion. Interestingly, an enrichment in cell death-related genes that were previously reported to play a role in ER stress-induced apoptosis, including *Bim*, *Bcl2*, *Bax* and *Bad*, were not found (Han et al, 2013). These different results (probably related to differences in experimental settings) along with the fact that the same event (i.e. down-regulation of translation) favours repair and apoptosis (presumably due to differential magnitude and exposure time to the insult) suggest that the cellular response might change according to the tissue type and opens for the role of other factors/mechanisms promoting apoptosis. The observation that CHOP-deficient cells still undergo apoptosis also argues in that direction (Urra et al, 2013; Zinszner et al, 1998), along with our results showing that down-regulation of CHOP by siRNA in thapsigargin-treated cells in the presence of p53 did not completely abrogate apoptosis, as estimated by cleavage of PARP-1. Indeed, IRE1 activation was also linked to apoptosis pathway via induction of P58^{IPK} that binds and inhibits PERK and therefore promotes translation recovery, a pre-requisite to trigger apoptosis. In addition, IRE1 binds TRAF2 and they recruit ASK1 kinase that induces the pro-apoptotic JNK (Szegezdi et al, 2006; Urra et al, 2013). Herein, we presented data supporting the idea that p53 might also contribute to ER stress-induced apoptosis.

The role of p53 in inducing apoptosis during ER stress is not new. Indeed, it was shown that p53 promotes transcription of *Noxa* and *Puma* (although only PUMA protein was shown to follow the increased mRNA pattern) to induce apoptosis in MEFs (Li et al, 2006). However, we could not detect induction of these two pro-apoptotic members in our system, in line with previous reports (Han et al, 2013). Therefore, we focused our attention into other players. We were able to see that a down-regulation of BiP and an induction of BH-only BIK expression and its activation by escaping from BiP, mediated by p53FL and p53ΔN40, correlated with thapsigargin-induced apoptosis.

There is data supporting the inhibitory effect of BiP on apoptosis induction. It was proposed that interaction with caspase-7 reduced apoptosis in cultured cells treated with several DNA-damaging drugs (Reddy et al, 2003) and that the interaction with caspase-7 and -12 at the ER

membrane prevented release of caspase-12 from there and reduced the ER stress-induced apoptosis (Rao et al, 2002). Also, *in cellulo* studies have reported an interaction between BiP and BIK proteins at the ER membrane that was essential to counteract estrogen starvation–induced cell death by competing with BCL-2/BIK interaction (Fu et al, 2007; Zhou et al, 2011). All these interactions supposed a localization of BiP to the ER membrane or the cytoplasm although BiP's primary structure does not contain any putative trans-membrane domain. Nevertheless, the interactions could be stablished with the domains of the interacting proteins that reach the ER lumen or through a complex with other factors, two ideas that need to be investigated in the future. Interestingly, a cytoplasmic variant of BiP was reported, as well as the localization of the canonical protein to the mitochondria and to the cellular membrane upon ER stress (Lee, 2014; Sun et al, 2006). Thus, upon ER stress, BiP expression seems to be modified both quantitatively and qualitatively.

Further support of its anti-apoptotic activity is given by the fact that BiP knock-out mice are not viable and die before implantation due to massive increase in apoptosis in the inner cell mass (ICM) (Luo et al, 2006). Also, knock-out of BiP in various tissues led to caspase activation and tissue atrophy, while in breast, prostate and leukaemia cancer models, heterozygous and/or conditional homozygous knock-out of BiP increased tumour apoptosis and impeded tumour progression, as reviewed in Lee, 2014. These properties of BiP are not unique and are actually shared with other chaperones. Expression and/or activity of members of HSP27, HSP70 and HSP90 families is abnormally high in cancer cells and further increased after many different death stimuli. They are powerful anti-apoptotic proteins, establishing direct associations or indirectly modifying key apoptotic factors and cell signaling pathways (from AKT and JNK, BCL-2 members and death receptors DR4 and DR5 to effectors like APAF-1) and thereby blocking cell death process at different levels. For example, expression of HSP70 has been associated with therapeutic resistance, metastasis, and poor clinical outcome. In malignantly transformed cells, HSP70s protect cells from the proteotoxic stress associated with abnormally rapid proliferation, suppress cellular senescence, and confer resistance to stress-induced apoptosis including protection against cytostatic drugs and radiation therapy. Moreover, preclinical trials have proved that overexpression of the HSPs increases tumor growth, metastatic potential, and resistance to chemotherapy in rodent models. Thus, control of HSP90, HSP70 and/or HSP27 expression is emerging as a novel strategy for cancer therapy, as reviewed elsewhere (Radons, 2016; Wang et al, 2014).

BiP itself was proposed as a clinical target to treat cancer since it has been implicated in tumour resistance. For example, primary cultures of human brain endothelial cells derived from blood vessels of malignant glioma tissues (TuBEC) are substantially more resistant to apoptosis that non-malignant tissues and this was shown to be related to BiP. Indeed, its expression is generally highly elevated in the vasculature derived from human glioma specimens, both *in situ* in tissue and *in vitro* in primary cell cultures. In particular in TuBEC tissues, BiP was reported to be over-expressed without concomitant induction of other major UPR targets. Moreover, the cells were resistant to chemotherapeutic agents such as CPT-11, etoposide, and temozolomide in a BiP-dependent fashion (Virrey et al, 2008). Also, at least part of the estrogen receptor-positive breast tumours that lose drug sensitivity and become endocrine-resistant show an elevated expression of BiP that was shown to directly affect the anti-estrogen therapy (Cook et al, 2013). In addition, the capacity of a population of MCF-7 cell line displaying characteristics of stem cells that resist to ionizing radiation (IR) when transplanted into mice, was attributed to BiP over-expression. These breast cancer-initiating-like cells (CICs) showed less resistance to IR when BiP was knocked-down (Li et al, 2013a). Moreover, a quite promising drug (bortezomib) that inhibits the

proteosomal activity, leads to accumulation of protein inside the ER and triggers UPR-dependent apoptosis is counteracted by high secretion of BiP (Kern et al, 2009). Bortezomib (also known as Velcade® and PS-341) is the first U.S. FDA approved selective inhibitor of the proteasome for the treatment of multiple myeloma and mantle cell lymphoma. Cells in general may not respond similarly to proteasome inhibition and several types of cancer cells have actually been found to be more sensitive to pro-apoptotic effects of proteasome inhibition than normal cells and this provides the essential basis for proteasome inhibitors as anticancer drugs (Mujtaba & Dou, 2011). Although BiP does not bind bortezomib, it seems it impinges on p53, ERK and AKT pathways to inhibit apoptosis and promote cell growth (Kern et al, 2009). All this information highlights the potential of BiP as a therapeutic target to treat cancer progression. In light of this, many BiP inhibitors and cytotoxic agents that target cell surface BiP have been reported and some of them are being tested in clinical trials (Lee, 2014).

Targeting BiP appears very promising because the pleiotropic effects of modifying its activity or expression would be minimized due to the small changes that are needed to modify the cell behaviour. This is supported by the observation that half reduction of BiP expression, as seen by heterozygous knock-down mice models, does not hamper normal tissues but on the other hand, it impedes the growth of the tumour cells that highly depend on BiP expression (Wey et al, 2012). This was further supported by bi-allelic conditional knock-out mouse model of *bip* and *Pten* (a plasma membrane lipid phosphatase that antagonizes the PI3K signalling pathway and major tumour suppressor gene) in the bone marrow. This showed that heterozygous knock-down of BiP in PTEN-null mice is sufficient to restore the hematopoietic stem cell population back to the normal percentage and to suppress leukemic blast cell expansion. The effect was shown to depend on suppression of the PI3K/AKT/mTOR pathway by BiP knock-down in leukemia cell lines (Wey et al, 2012). This goes in line with our results, where the modest difference in terms of BiP expression we have detected in our system is accompanied by significant changes in cellular output.

The role of BIK in controlling apoptosis is, at least to me, more intuitive. As a member of the BH3-only pro-apoptotic family, BIK promotes apoptosis by either inhibiting the anti-apoptotic BCL-2 proteins or by directly activating the effectors BAX and BAK, as presented in the introductory section (Chinnadurai et al, 2008; Germain et al, 2005; Gillissen et al, 2007; Mathai et al, 2005). Importantly, these two mechanism are not exclusively and may act in parallel to enhance BIK activity. Activation of *bik* transcription was previously shown to be both dependent (Germain et al, 2002; Mathai et al, 2002) and independent (Paquet et al, 2004) on p53. However, this is the first time this is clearly related to the activity of the short p53ΔN40 isoform, in line with previous results showing induction of BIK in a wrongly described p53-null environment as it is the HCT116 $p53^{-/-}$ cell line, since they still produce p53ΔN40 (Chinnadurai et al, 2008; Real et al, 2006).

The role of BIK in promoting apoptosis during ER stress is related to its normal location at the ER membrane. Form there and in the context of the “direct” model presented in the introductory section, BIK was able to induce oligomerisation of BAK and BAX and the concomitant ER Ca^{2+} depletion that leads to the mitochondrial apoptotic pathway (Germain et al, 2005; Mathai et al, 2005).

Binding of BIK to BiP was suggested to control BIK activity. Our results show that loss of BIK/BiP complexes is the response most related to the combined effect of p53 and ER stress. While down-regulation of BiP expression by p53 occurs *in cellulo* in resting and stress scenarios as well as in *in vitro* experiments, and BIK expression was also proven to occur by both p53wt and p53ΔN40 in both conditions, the dissociation of BIK from BiP was found to occur only in the presence of p53 in ER-stressed cells. This opens for the interesting idea that BiP expression, in addition to be modified in terms of amount, is also qualitatively controlled by p53 during the UPR.

The quantitative side of this relationship could highlight the possibility that suppression of BiP only plays a determinant role when its level becomes decisive for the cellular response, that is in a highly demanding ER during stress, as described above. In terms of quality, this might be related to the above-discussed re-localization of BiP upon ER stress to different organelles apart from the ER. In terms of quality, it is valid to speculate that p53 might be involved in re-defining the localisation of BiP during prolonged ER stress and in this way permit apoptosis by releasing BIK.

Finally, it is important to point out that retention of at least part of the capacity of the p53FL to induce apoptosis by p53ΔN40, constitutes a very important result since it is the isoform preferentially expressed in conditions of ER stress, as suggested by its enhanced protein synthesis and stability and by the high rate of p53FL degradation (Baltzis et al, 2007; Bourougaa et al, 2010; Candeias et al, 2006; Pluquet et al, 2005; Qu et al, 2004; Yamasaki et al, 2007). This observation adds to the notion that the TAD I (aa 1 to 40) and TAD II (aa 40 to 60) domains of p53 have specific cell biological down-stream targets acting at the transcription level. It is thus tempting to (re-)postulate that the TAD I is associated to control of cell cycle arrest and repair, as shown by p53 derivatives lacking TAD I but retaining TAD II that are unable to trans-activate, for example, *p21*^{CDKN1A} (Ghosh et al, 2004; Mlynarczyk & Fahraeus, 2014; Phang et al, 2015). On the other hand, the TAD II will impinge on apoptosis-related genes as supported by the induction of *Bax* (Yin et al, 2002) and here on *Bik*. The capacity of p53ΔN40 to induce apoptosis upon exposure to a variety of stress signals also includes genes such as *Fas*, *Dr5*, *Api1* and *Pig3* (Phang et al, 2015). In addition, the inability to activate *p21*^{CDKN1A}-like genes was shown to depend on lack of acetylation on K382 that requires the presence of N-terminal domain of p53 (Phang et al, 2015). Moreover and attending to the results presented here, the TAD II will also impinge on apoptosis via controlling of mRNA translation, particularly in this case via BiP.

Annexes

Annex 1. Alignment of mRNA targets

The alignment of the nucleotide sequence of the target mRNAs presented here (*bip*, *mdm2*, *mdmx*) and the previously reported (*p53*, *cdk-4*, *fgf-2* and *p21*^{CDKN1A}). The region used for each mRNAs is the one reported to play a role in the control by p53, weather there is a direct interaction or not. p53, 5'UTR of p53; cdk-4, 5' UTR of cdk-4; fgf-2, mRNA leader of fgf-2; p21, nucleotide region spanning the codons 76 and 165 of p21CDKN1A; mdm2, 1107 N-terminal nucleotides of mdm2; mdmx, 120 nucleotides spanning the region -120 to +1 of the 5' UTR of mdmx; bip, first 346 nucleotides of the bio coding sequence, segment +1 to +346. The BioEdit sequence alignment software was used. See next page.


p53
cdk4
fgf-2
p21
mdm2
mdmx
bip

Annex 2. Abbreviations

14-3-3σ; 14-3-3 sigma (stratifin)
^{35}S; Sulfur ^{35}S isotope
4E-BP; 4E-binding protein
A; adenine
aa; amino-acid
ADP; adenosine diphosphate
AGO: argonaute
Ala; alanine
AMP; adenosine monophosphate
AMPK; AMP-activated protein kinase
APAF1; apoptosis protease-activating factor-1
Arg; arginine
ASK1; apoptosis signal-regulating kinase 1
Asp; aspartate
ATF4; activating transcription factor 4
ATF6; activating transcription factor 6
ATM; ataxia telangiectasia mutated
ATP11C; Phospholipid-transporting ATPase IG
BAD; Bcl-2-associated death promoter
BAX; bcl-2-like protein 4
BCL-2; B-cell lymphoma 2
BCL-W; Bcl-2-like protein 2
BCL-XL; B-cell lymphoma-extra large
BER; base excision repair
BFL1/A1; BCL2-related protein A1
BH (1 to 4); Bcl-2 Homology
BID; BH3 interacting-domain death agonist
BIK; BCL-2 interacting killer
BIM; Bcl-2-like protein 11
BiP; Binding immunoglobulin protein
BMF; Bcl-2-modifying factor
BNIP3; BCL2/adenovirus E1B 19kDa interacting protein 3
BOK; Bcl-2 related ovarian killer
bp; base pair
BSA; Bovine serum albumin
bZIP; basic leucine zipper motif
C; cytosine
Ca^{2+}; Calcium
CBP/p300; CREB-binding protein
CCL2; chemokine (C-C motif) ligand 2
Cdc2; cell division cycle protein 2 (CDK1)
CDK; Cyclin-dependent kinase
CDK-4; Cyclin-dependent kinase 4
cDNA; copy deoxyribonucleic acid
CDS; coding sequence

CHAPS; 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate
ChIP; Chromatin Immunoprecipitation
CHK2; Checkpoint kinase 2
CHOP; C/EBP homologous protein
CLIP; cross-linking immunoprecipitation
CMV; Cytomegalovirus
COP-1; constitutive photomorphogenetic 1
DAPI; 4',6-diamidino-2-phenylindole
DBD; DNA-binding domain
DISC; death-inducing signaling complex
DMS; dimethyl sulfide
DMSO; dimethyl sulfoxide
DNA; deoxyribonucleic acid
DNMT1; DNA (cytosine-5)-methyltransferase 1
DNMT3A; DNA (Cytosine-5-)-Methyltransferase 3 alpha
DPH (1, 3 and 4); diphthamide biosynthesis 1, 3 and 4
DR 4 and 5; death receptors 4 and 5
DRBP; DNA- and RNA-binding protein
DRP1; dynamin-related protein
DSB; double strand base repair
dsDNA; double-stranded DNA
DTT; Dithiothreitol
E2F1; Transcription factor E2F1
EBV; Epstein-Barr virus
EDTA; Ethylenediaminetetraacetic acid
eEF#; eukaryotic elongation factor #
EFsec; Sec-tRNA$^{[Ser]Sec}$-specific elongation factor
EGCG; (-)epigallocatechin gallate
EGF; epidermal growth factor
EGFR; epidermal growth factor receptor
EGTA; ethylene glycol tetraacetic acid
eIF#; eukaryotic initiation factor #
ELISA; enzyme-linked immunosorbent assay
EMSA; electrophoretic mobility shift assay
ENO1; Enolase 1, (Alpha)
ER; endoplasmic reticulum
ERAD; Endoplasmic reticulum-associated protein degradation
eRF#; eukaryotic termination factor #
ERK; Extracellular signal–regulated kinases
ERO1; ER oxidoreductin 1
ERQC; ER protein quality control
ERSE; ER stress response elements
EV; empty-vector
FACS; Fluorescence-activated cell sorting
FADD; Fas-Associated protein with Death Domain
FasL; Fas ligand
FasR; Fas receptor

FDA; Food and Drug Administration
FGF-2; Fibroblast Growth Factor 2 (Basic)
FITC; Fluorescein isothiocyanate
FOXO3A; Forkhead box O3
G; guanidine
GADD#; Growth arrest and DNA damage-inducible protein family #
GAPDH; Glyceraldehyde 3-phosphate dehydrogenase
GCN2; general control non-derepressible-2
GCR; glucocorticoid receptor
GDP; guanosine diphosphate
GFP; green fluorescent protein
Gln; glutamine
Glu; glutamic acid
Gly; glycine
GOF; gain-of-function
GRP#; glucose-regulated protein #
GSK-3; glycogen synthase-3
GST; glutathione S-transferase
GTP; guanosine triphosphate
His; histidine
HIV-1; human immunodeficiency virus 1
HIV-1 NC; human immunodeficiency virus 1 nucleocapsid protein
hnRNP; heterogeneous nuclear ribonucleoproteins
HPV; Human papillomavirus
HRD1; ERAD-associated E3 ubiquitin-protein ligase
HRI; haem-regulated inhibitor kinase
HRK; harakiri protein
HRP; horseradish peroxidase
HSP#; heat shock protein #
HSPA5; heat shock 70 kDa protein 5
ICAM3; Intercellular adhesion molecule 3
ICM; inner cell mass
IF; immunofluorescence
IL-2; interleukin-2
Ile; isoleucine
INFγ; Interferon gamma
IPTG; isopropyl β-D-1-thiogalactopyranoside
IRE1; inositol-requiring enzyme 1
IRES; internal ribosome entry site
IRPs; iron regulatory proteins
ITAF; IRES trans-acting factors
JAK–sTAT; janus kinase/signal transducers and activators of transcription
JNK; c-Jun N-terminal kinase
KDa; Kilo Dalton
Leu; leucine
lncRNA; long non-coding RNA
LOH; loss of heterozygosity

MAPK; mitogen-activated protein kinases
MCL1; induced myeloid leukemia cell differentiation protein
MDM2; murine double minute 2
MDMX; murine double minute X
MEF; mouse embryonic fibroblasts
Met; methionine
Met-tRNA^{Meti}; initiator Methionyl-tRNA-methionine
Mg^{2+}; magnesium
MHC-I; major histocompatibility complex type I
min; minutes
miRNA; microRNA
MNK#; MAP kinase interacting Ser/Thr kinase #
MOMP; mitochondrial outer membrane permeabilization
mRNA; messenger RNA
MS2; the coat protein of the RNA bacteriophage MS2
mTOR; mammalian target of rapamycin
$NaAsO_{2;}$ Sodium arsenite
NAD+; Nicotinamide adenine dinucleotide
NBD; nucleotide-binding domain of BiP
NCK1; NCK Adaptor Protein 1
NEFs; nucleotide exchange factors
NES; nuclear export signal
NF90; NF of activated T cells 90 kDa
NF-Y; nuclear factor-Y
NF-κB; NF-kappa-B transcription factor
NLS; nuclear localization signal
NMR; Nuclear magnetic resonance spectroscopy
nt/s; nucleotide/s
OMM; outer mitochondrial membrane
ORF; open reading frame
OS9; osteosarcoma 9
OVA; chicken ovalbumin
PABP; poly(A)-binding protein
PAI-1; plasminogen activator inhibitor
PANDA; P21-associated ncRNA DNA-damage activated lncRNA
PARP-1; poly (ADP-ribose) polymerase-1
PAZ; domain named after the proteins Piwi Argonaut and Zwille
PBS; phosphate buffered saline
PCR; polymerase chain reaction
PDI; protein disulfide-isomerase
PERK; protein kinase RNA-like ER kinase
PFA; paraformaldehyde
PI; propidium iodide
PI3K; phosphatidylinositol-4,5-bisphosphate 3-kinase
PIG3; p53 inducible gene 3
PIP3; phosphatidylinositol-(3,4,5)-trisphosphate
PIWI; name of the domain is derived from P-element induced wimpy testis in Drosophila

PKR; protein kinase RNA
PLA; proximity ligation assay
PRR; Proline Rich Region
PTB; polypyrimidine tract-binding protein
PTC; peptidyl transferase center
PtdSer; phosphatidylserine
PTEN; Phosphatase and tensin homolog
PUMA; p53 upregulated modulator of apoptosis
qPCR; quantitative polymerase chain reaction
R; purine
RE; responsive element
REDOX; reduction/oxidation reactions or processes
RIDD; IRE1-dependent decay of mRNA
RING; really interesting new gene
RMST; rhabdomyosarcoma 2-associated transcript lncRNA
RNA; Ribonucleic acid
RNApolII; RNA polymerase II
ROS; Reactive Oxygen Species
RPA; replication protein A
rpL#; ribosomal protein of large subunit #
RsmE; ribosomal RNA small subunit methyl-transferase E
RT; retro-transcription
SBD; substrate-binding domain of BiP
SBP2; SECIS binding protein 2
SDS-PAGE; sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sec; selenocysteine
SECIS; selenocysteine insertion sequence
Ser; serine
SH3; SRC Homology 3 Domain
siRNA; small interfering RNA
SOX2; sex determining region Y-box 2
ssDNA; single-stranded DNA
STAT1; Signal transducer and activator of transcription 1
SV40; Simian virus 40
T; thymidine
TAD I II; trans-activation domain I and II
TBP; TATA-binding protein
TET; tetramerization domain
TGF-β; Transforming growth factor beta 1
THAP; thapsigargin
TNFR1; Tumor necrosis factor receptor 1
TNF-α; Tumor necrosis factor alpha
TOM20; translocase of outer membrane 20
TRADD; Tumor necrosis factor receptor type 1-associated death domain protein
TRAF2; TNF receptor-associated factor 2
tRNA; transfer RNA
TSE; tobaco smoke extract

Tsix; from the reverse of Xist
uORF; up-stream open reading frame
UPR; Unfolded Protein Response
UPS; ubiquitin-proteasome mechanism
UTR; untranslated region
UV; ultraviolet light
Val; valine
W; weak bases
WB; western blotting
WT1; Wilms' tumor 1 transcription factor
XBP1; X-box binding protein 1
Xist; X-inactive specific transcript
Xkr8; XK, Kell Blood Group Complex Subunit-Related Family, Member 8
Y; pyrimidine
Zn; Zinc
zVAD-fmk; carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone
α2M; plasma proteinase inhibitor α2-macroglobulin

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