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Unveiling a role for Mutant p53 in the regulation of Unfolded Protein Response

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Summary

1. Abstract

Hypoxia, low glucose and low pH characteristics of tumor microenvironment are associated with a condition known as ER stress and, consequently, Unfolded Protein Response activation. Cancer cells are capable to survive in various hostile environments. For instance, they have developed many strategies to take advantages from adverse conditions, present in tumor tissue, by using specific activities of oncogene and mutated proteins. One of the most highly mutated gene in human cancer is the P53 gene. By analyzing expression profile of five different cancer cells bearing mutant p53, we, interestingly, observed, upon depletion of mutp53, an enrichment on global Unfolded Protein Response (UPR) genes. This finding suggest that mutant p53 might have an effect in controlling UPR. In particular, we hypothesized that mutp53 is reprogramming UPR to sustain cancer cells survival. To test this hypothesis, we, firstly, analyzed whether cancer cells bearing mutant p53 were more resistant to treatment with ER stress inducers: Thapsigargin (Tg) and Tunicamycin (Tm). Then, by analyzing the three main mediators of UPR (IRE, PERK and ATF6) and their downstream effectors, we observed that mutant p53 is dampening pro-apoptotic pathways of UPR, CHOP and JNK, and it is, also, promoting ATF6 transcriptional activity. Since ATF6 is crucial for protein quality control and chaperones production, we hypothesized that mutp53 is using ATF6 transcriptional activity to resolve the stress. Interestingly, we observed that ATF6, as well mutant p53, is crucial for cancer cells survival, but, surprisingly, not only upon ER stress induction. We further confirm these observations by using specific inhibitors of ATF6, confirming its pro-survival functions. Finally, we used these findings to test whether, concomitantly inhibition of mutant p53 and ATF6, could represent a promising strategy for cancer treatment. Taken together our data proposed a novel oncogenic function of mutant p53 in the promotion of cancer cells survival and in maintenance cancer cells homeostasis.

2. Introduction

2.1 TP53 and cancer

Cancer, nowadays, represents the major cause of death worldwide. Gene mutations are necessary to induce neoplastic transformation and interest both onco-suppressors and oncogenes.

One of the most highly mutated genes in human cancer is TP53. The P53 protein was first discovered in 1979 (DeLeo et al., 1979) and its role in cancer has become increasingly important in recent years.

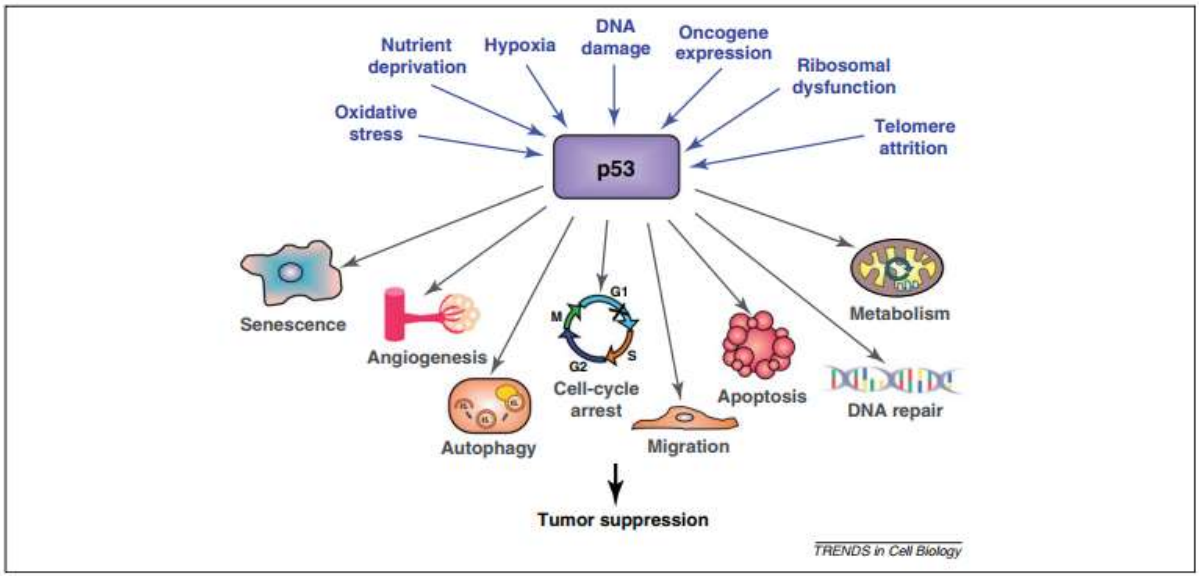

Figure 1: Deconstructing p53 transcriptional networks in tumor suppression. (Attardi, 2012)

P53 is one of the most important players in the response to many types of stress that can lead to loss of genomic integrity and cancer development (Kastan et al. 1991; Vogelstein et al. 2000). P53 responds at these stresses inducing irreversible senescence and consequently apoptosis (Figure1) (Yonish-Rouach et al. 1991; Serrano et al. 1997).

Nevertheless, apoptosis or permanent senescence could compromise organism survival suggesting that p53 activity needs to be tightly regulate. Accordingly, to this, in cells exist a large number of mechanisms that control p53 activity. The most important is mediated by Mdm2, an ubiquitin ligase, which regulates p53 through a negative feedback loop: p53 activates

transcription of the mdm2 gene, whose protein product then targets p53 for ubiquitin-dependent degradation (Freedman et al., 1999). Thus, any increase in p53 activity is accompanied by increased p53 degradation.

Under genotoxic conditions, p53 protein levels are increased upon phosphorylation on Ser20 by the Chk2 kinase, this induces Mdm2 dissociation from p53. This process is under the control of the ATM protein kinase, which is activated in response to DNA DSBs, and play a key role in p53 activation. A similar mechanism regulates the activation of p53 upon block of DNA replication and it is mediate by ATR, an ATM related kinase (Abraham 2001; Iliakis et al. 2003). Other mechanisms of regulation of p53 are numerous post-translational modifications as acetylation of several lysines that enhance p53 transcriptional activity by stabilizing p53 protein, blocking its degradation (Brooks and Gu 2003; Xu 2003). To summarize, p53 stability and transcriptional activity are finely regulated in cells that are exposed to various stress.

2.2 Mutant p53 and its gain-of-function

Mutations in the TP53 gene occur in many tumor types. In fact, about 70% of TP53 mutations are missense and occur in the region encoding the DNA binding domain (DBD) of the p53 protein; some are considered hotspot mutations that are frequently found both in sporadic and in hereditary tumors. DBD mutations induce changes in p53 conformation or prevent binding to DNA, blocking p53 transcriptional activity or altering its interaction with other proteins.

Firstly, TP53 mutations inactivate the oncosuppressive properties of the wild-type p53 protein as a transcription factor – a phenomenon defined as loss-of-function (LOF). In addition, p53 mutant proteins can exert a dominant negative (DN) effect on the wild-type p53 allele in heterozygous conditions, as matter of fact tetramers that present both wt and mutp53 proteins are not functional. Finally, it is well established that mutant forms of p53 can acquire novel oncogenic activities, defined as gain-of-function (GOF) (Figure 2).

In order to sustain tumor development, mutant p53 plays a role in multiple pathways and processes. For example, mutp53 is implicated in the direct inhibition of the p63/p73-mediated tumor suppression (Li and Prives, 2007). This also induces an increase of PDGFRb, normally inhibited by the p73/NFY complex. In fact, high levels of PDGFRb correlates with poor disease-free survival in cancer patients (Weissmueller et al., 2014). Mutant p53 is also related with an

activation of cell cycle drivers, such as Cyclins or in the vitamin D3 receptor signaling (Stambolsky et al., 2010).

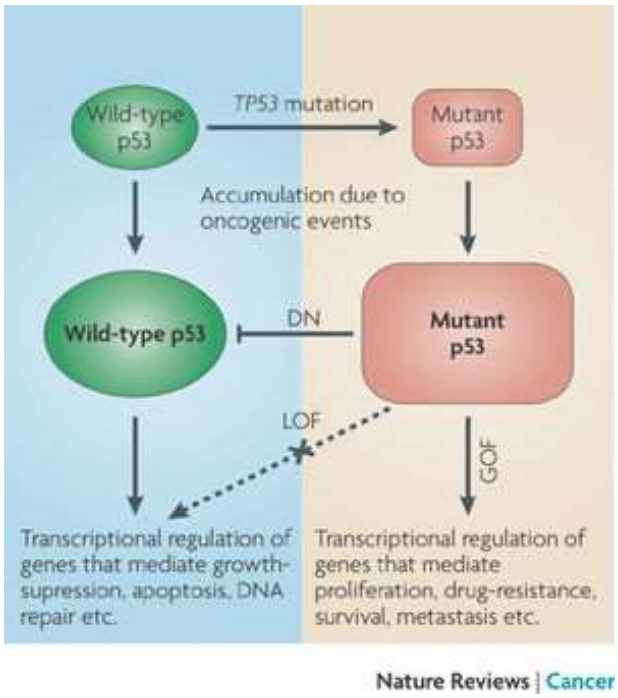

Figure 2: When mutants gain new powers: news from the mutant p53 field. (Brosh and Rotter, 2009)

Despite being incapable of sequence-specific DNA binding, mutant p53 has a strong impact on gene expression by interacting with several other transcription factors, modifying their activity, or promoting selective regulation of specific target genes (Walerych et al., 2015).

For instance, under proteotoxic conditions, mutant p53 uses NRF2 transcriptional activity to induce antioxidant factors and to sustain cancer cells survival. Interestingly, in healthy conditions NRF2 inhibits transcription of some genes that, in tumor cells bearing mutant p53, appear to be crucial for sustaining survival and growth (Walerych et al., 2016).

A similar mechanism involves Heat Shock Factor 1 (HSF1), that is directly bound by mutant p53. This binding facilitates the recruitment of HSF1 to its specific DNA-binding elements and stimulates transcription of heat-shock proteins including Hsp90. Sustaining, in this way, mutant p53 stabilization (Li et al., 2014).

Being very stable, mutant p53 is present both in the nucleus and in the cytoplasm. This cytoplasmic localization gives to mutp53 the possibility to bind to and affect the functions of various proteins that normally do not interact with nuclear wild-type p53. For instance, mutp53 can bind cytosolic AMP-activated protein kinase (AMPK), leading to inhibition of anabolic metabolism and activation of aerobic glycolysis, i.e. the Warburg effect (Zhou et al., 2014). Similarly, by binding the tumor suppressor DAB2IP in the cytoplasm, mutant p53 is able to block JNK phosphorylation and to activate the NF-kB pathway upon TNFa treatment, promoting an aggressive response to inflammation (Di Minin et al., 2014). The inhibition of DAB2iP by mutant p53 can also amplify AKT phosphorylation upon insulin treatment, sustaining tumor proliferation and invasion under conditions of hyperinsulinemia (Valentino et al., 2017).

Mutant p53 can also sustain cancer progression by remodeling the extracellular matrix, inducing secretion of metalloproteases, and various pro-inflammatory and immunomodulatory cytokines.

In addition, by stimulating secretion of lactate, mutp53 induces extracellular acidification regulating the fine crosstalk between cancer and stromal cells (Cordani et al., 2016).

Mutant p53 can also affect steroid biosynthesis, in particular by acting on the mevalonate pathway – this has a profound oncogenic impact, also mediated by activation of YAP/TAZ (Di Agostino et al., 2015) and ID4-regulated angiogenesis (Fontemaggi et al., 2009).

More recently, it was discovered that mutant p53 promotes transcription of the ER localized enzyme ectonucleside triphosphate diphosphohydrolase (ENTPD5). This leads to stimulation of the calnexin/calreticulin-dependent quality control that could help cells to survive in proteotoxic conditions. Importantly, by upregulating ENTPD5 mutant p53 promotes N-glycosylation, folding, and maturation of secreted growth factors and membrane receptors, in turn enhancing cancer cell proliferation and/or survival (Vogiatzi et al., 2016).

2.3 Targeting mutant p53

Given its central role in cancer progression, invasion, and metastasis, targeting mutant p53 function may reveal potential strategies to develop novel anti-cancer therapies (Figure 3).

Considering that mutp53 stability is key to its GOF, one approach is to stimulate its degradation. As for wt p53, MDM2 seems to be a major determinant of mutant p53 levels. Therefore, specifically and selectively activating MDM2-induced degradation may be a strategy to eliminate mutant p53 (Terzian et al., 2008).

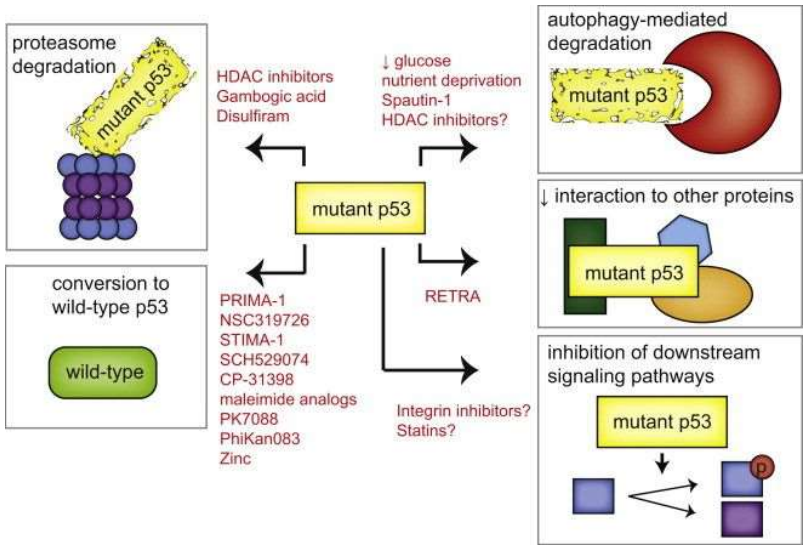

Figure 3: Mutant p53 in cancer: new functions and therapeutic opportunities. (Muller and Vousden, 2014)

Mutant p53 is stabilized by interaction with the HSP90 machinery, which includes HSP90, HSP70 and other co-chaperones (Peng et al., 2001). This complex binds MDM2 and CHiP, blocking their capacity to induce mutant p53 degradation. Disruption of this complex or inhibition of chaperones activity in cultured cells, induces the release of MDM2 and CHiP from inhibition and triggered mutant

p53 degradation (Peng et al., 2001). Based on this knowledge, compounds that inhibit HSP90 were proposed as strategy to impaired mutant p53 stability.

As matter of fact, pharmacological inhibition of HSP90 ATP-dependent chaperone activity by Geldanamycin or 17AAG is able to reduce mutant p53 stability (Whitesell et al., 1998). Several HSP90 inhibitors were show to inhibit tumor growth in pre-clinical models and in patients, and some of them are in phase II clinical trials.

Another class of compounds that reduce mutant p53 protein levels are the histone deacetylase inhibitors (HDACi). Interestingly, these compounds elicit different anticancer responses apart from destabilization of mutant p53. At least three HDACi have been reported to reduce mutant p53 levels in cell lines, FR901228, Trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA), which however were proposed to act through different mechanisms (Wang et al., 2016). SAHA inhibits HDAC6, which normally activates HSP90 by promoting deacetylation of K294. Upon SAHA treatment, MDM2 and CHIP may be released from inhibition by HSP90 complex and induce mutant p53 degradation (Li et al., 2011; Wang et al., 2016). In contrast, the effects of FR901228 and TSA on mutant p53 levels are accompanied by conformational changes in mutant p53 and transcriptional induction of p21 and MDM2, suggesting reactivation of wt-like p53 conformation (Bajbouj et al., 2012; Blagosklonny et al., 2002).

It was shown that phosphorylation of mutant p53 on S15 and S37 contributes to protein stabilization and that the kinase responsible for these modifications is DNA-PK. Targeting DNA-PK by NU7441 reduced mutant p53 phosphorylation on S15 and S37, suggesting a potential use in strategies aiming at inducing mutant p53 degradation (Sunada et al., 2016). Small molecules that directly modify mutant p53 promoting its transition into a wild-type like form, capable of activating the tumor suppressive wild-type p53 transcriptional targets, are also gaining attention. Great interest has been obtained by PRIMA-1 molecule (Teoh et al., 2016), and later by its more potent and less toxic derivative PRIMA- 1MET/APR-246 (Walerych et al., 2016). This compound successfully went through phase I/II clinical trial in hematological malignancies and prostate cancer that included mutant p53 patients (Lambert et al., 2010).

Finally, great interest was raised by evidences that glucose restriction affects mutant p53 stabilization; cancer cell lines bearing mutant p53 showed a marked reduction on endogenous mutp53 levels when grown in conditions of glucose starvation, moreover compounds such as glucocorticoids, that target the mevalonate pathway, are also able to inactivate mutant p53 (William et al., 2013; Parrales et al., 2016). Interestingly, whether on one hand pharmacological

manipulation of glucose metabolism may provide novel tools to induce mutant p53 degradation, through its deacetylation and ubiquitination (Sorrentino et al., 2017), on the other hand it suggests the exciting possibility that dietary regulation may help to restrain tumor progression in cases bearing mutant p53.

In summary, pharmacological targeting of mutant p53 functions may have powerful implications for therapy; to this aim, a highly efficient strategy would be to simultaneously target both mutant p53 protein and its downstream activated pathways.

2.4 The Endoplasmic Reticulum and secretory pathway

The Endoplasmic Reticulum (ER) is a network of membrane-bound cavities permeating the entire cytoplasm from the cell membrane to the nucleus (Palade and Porter, 1954). The ER not only is the cradle of most lipids and of membrane and secreted proteins, but also is the major Ca2+ storage in the cell and a hub for signal integration (Meldolesi and Pozzan, 1998). Distinct sub-regions of the ER have specialized tasks. In particular, contact sites with other organelles, such as mitochondria, are fundamental for signaling and lipid transfer. In order to maintain physiological and developmental needs, cells are able to adapt the size and functional capacity of the ER (Shibata et al., 2006).

Secretory proteins in ER compartment acquire fundamental modifications such as N-glycosylation, necessary for their maturation. One of the most important roles of the ER is helping protein folding, thanks to the presence of a large number of chaperones and a favorable environment. Newly synthesized proteins progressively adopt conformations that are energetically more favorable, until the correctly folded 'native' conformation is reached (Ellgaard and Helenius, 2003). Only when proteins are fully folded, all chaperones release and allow their exit to travel further along the secretory pathway. As such, stringent quality control is exerted on the secretory proteome.

Once proteins are correctly folded, they have to exit from the ER lumen. Folding and assembly are fully completed when proteins arrive in the Golgi. In downstream compartments of the secretory pathway, N-linked glycans often are further modified in several ways (De Matteis and Luini, 2008). If immature client proteins exit the ER, they are retrieved through retrograde transport. The ER chaperones and foldases in small numbers do travel to the Golgi, where they can pick up escapes clients. The chaperones carry at their C-terminus a lysine aspartate glutamate

leucine (KDEL) or related tetrapeptide, which is recognized by KDEL receptors in the Golgi. The ternary client– chaperone–KDEL receptor complex is then sorted to COPI-coated vesicles that travel back to the ER for further client folding attempts.

Immature proteins that don't leave the ER lumen are retro-translocated to the cytosol. Here, they are de-glycosylated, ubiquitinated, and, finally, degraded by the proteasome, thanks to a process referred to as ER-associated degradation (ERAD). Rarely, misfolded clients may escape ERAD and accumulate in the ER, sometimes even forming aggregates.

2.5 The Unfolded Protein Response

Different conditions such as hypoxia, low or high glucose, and low PH, can induce a condition known as ER stress, which leads to activation of a highly conserved transcriptional program known as Unfolded Protein Response (UPR). (Cox et al., 1993; Mori, 2000). The UPR can promote both pro-survival or pro-apoptotic outcomes, and in mammals is triggered by three ER-resident receptors: IRE1, PERK, and ATF6 (Figure4).

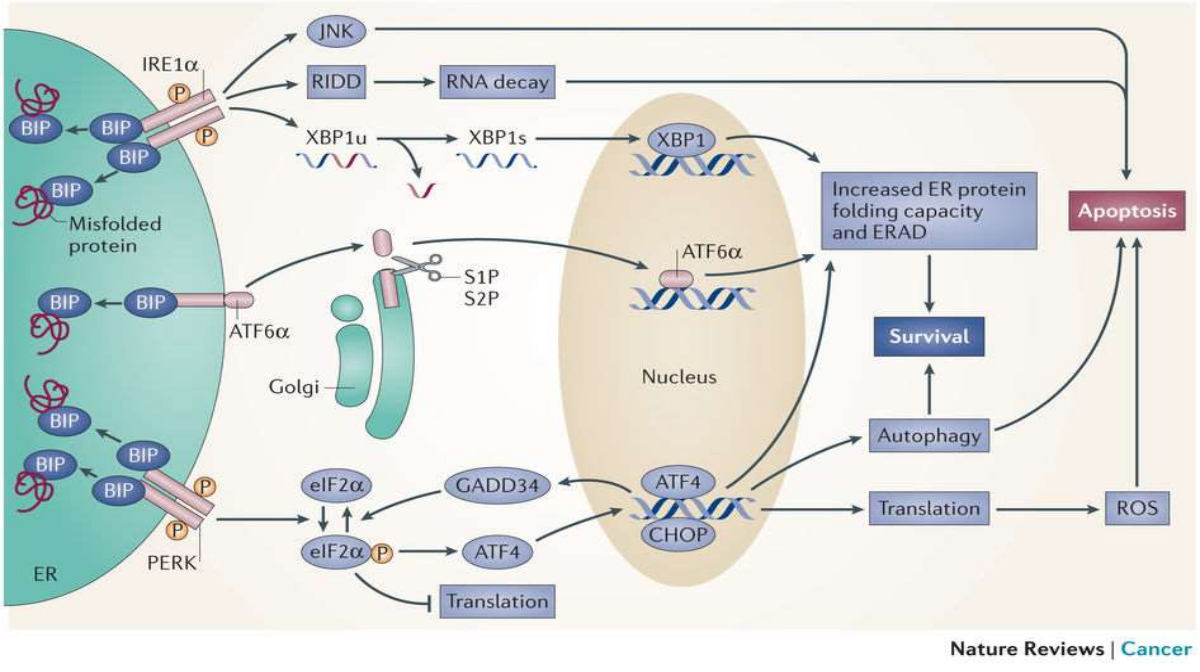

Figure 4: The impact of the endoplasmic reticulum protein-folding environment on cancer development. (Wang and Kaufman, 2014)

Genes induced by the UPR include a full repertoire of ER chaperones and foldases, but also enzymes for membrane lipid synthesis that together sustain expansion of the ER and its folding capacity, whose sustain adaptive responses. In addition to these factors, the UPR can also entail

cell death by apoptosis, where several pro-apoptotic members of the BCL-2 family of proteins are essential for the elimination of irreversibly damaged cells (Carpio et al., 2015).

It is clear that the transition between adaptive UPR programs and the elimination of irreversibly damaged cells by apoptosis depend in part on the duration of ER stress stimulation; when the stress level exceeds the capability of the cells to restore homeostasis, and all adaptive responses have failed, the UPR induces apoptosis.

Maladaptive ER stress responses can lie at the basis of a growing list of pathologies. For example, excessive insulin synthesis and a corresponding 'overzealous' UPR in pancreatic β-cells lead to their demise and, hence, type 2 diabetes; neurodegenerative diseases are also correlated in an over activation of UPR and neurons cell death; finally, UPR is involved in cancer progression and chemoresistance (Wang and Kaufman, 2012)

2.5.1 The IRE1a pathway

IRE1 is the only identified ER stress sensor in yeast and is essential for the UPR in animals and plants (Wang et al., 1998). As an ER transmembrane protein, it monitors ER homeostasis through an ER luminal stress-sensing domain and triggers the UPR through a cytoplasmic kinase domain and an RNase domain (Tirasophon et al., 2000). There are at least two related homologs of yeast IRE1, referred to as IRE1α, constitutively expressed in all cells and tissues, and IRE1β, which expression is restricted to gut epithelial cells, that were identified in both murine and human genomes (Tirasophon et al., 1998; Wang et al., 1998). IRE1 appears as monomer that displays an individual catalytic center (Chen and Brandizzi, 2013). These monomers dimerize, upon induction of ER stress, facilitating trans-auto-phosphorylation and triggering an allosteric change, thus leading to an RNase-active complex (Korennykh et al., 2009), which promotes the

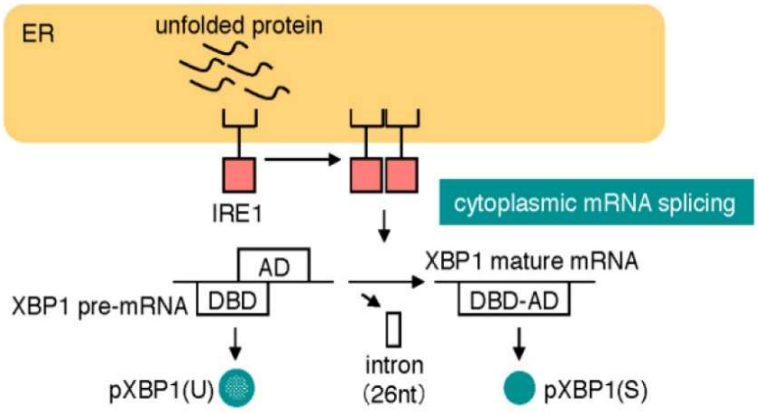

Figure 5: The essential biology of the endoplasmic reticulum stress response for structural and computational biologists. (Wakabayashi and Yoshida, 2013)

unconventional splicing of Xbp1 mRNA, and the degradation of multiple other mRNAs through the so called Regulated IRE1-Dependent Decay (RIDD) (Figure 5).

Through its kinase domain, and consequent recruitment of the TRAF2/ASK1 complex, IRE1 can induce the phosphorylation and activation of JNK, which is sustained by the binding of DAB2IP (DAB2 Interacting Protein), and its consequent pro-apoptotic signaling (Nishitoh et al., 2002; Urano et al., 2000, Luo et al., 2008). Through its RNAse domain, IRE1 promotes both pro-survival and pro-apoptotic pathways. Studies in mammals indicate that XBP1s has a pro-survival output, which is often overactive in cancer, whereas RIDD has a pro-apoptotic output that dominates in other diseases, such as diabetes (Han et al., 2009). Under basal conditions, XBP1 mRNA is not spliced under low ER stress, XBP1 mRNA splicing increases progressively and decreases upon chronic stress (Han et al., 2009). By contrast, RIDD displays constitutive activity under basal conditions, called basal RIDD (Dejeans et al., 2012; Pluquet et al., 2013), increasing proportionally with stress intensity or duration (Maurel et al., 2014). This suggests that RIDD maintains ER homeostasis under low/chronic ER stress, and contributes to apoptosis under irremediable ER stress (Han et al., 2009).

XBP1s is typically associated with pro-survival outcomes, it regulates a subset of UPR-induced genes that participate in folding, quality control, and ERAD (Arensdorf et al., 2013). Moreover, it is proposed that XBP1s regulates genes involved in many aspects of ER function and physiology, and induction of XBP1s results in an increase of ER and Golgi content (Shaffer et al., 2004). Loss of XBP1 in Paneth cells induces a massive disorganization of ER network and subsequent failure of cell development (Huh et al., 2010). XBP1s also promotes Triple Negative Breast Cancer (TNBC) by controlling the HIF1a pathway. In breast cancer cell lines, depletion of XBP1 inhibits tumor growth and tumor relapse. Genome-wide mapping revealed that XBP1 drives TNBC tumorigenicity by assembling a transcriptional complex with HIF1a, that regulates the expression of HIF1a target genes (Chen et al., 2014).

The PERK pathway

The ER luminal portion of PERK contains a stress-sensing domain that is both structurally and functionally related to that of IRE1. The cytoplasmic portion of PERK also has a protein kinase domain that is activated when PERK oligomerizes in stressed cells (Figure 6). Once PERK is activated, it phosphorylates the α-subunit of the eukaryotic translation initiation factor-2 (eIF2α), resulting in globally attenuated translation, thus reducing the protein load at the ER (Harding et al., 1999). Short-lived proteins such as CyclinD1 decay rapidly under these conditions, leading to cell cycle arrest (Brewer and Diehl, 2000). Although cap-dependent translation is inhibited, translation of certain transcripts is increased. In particular, transcription factor as ATF4 is selectively induced in response to eIF2α phosphorylation.

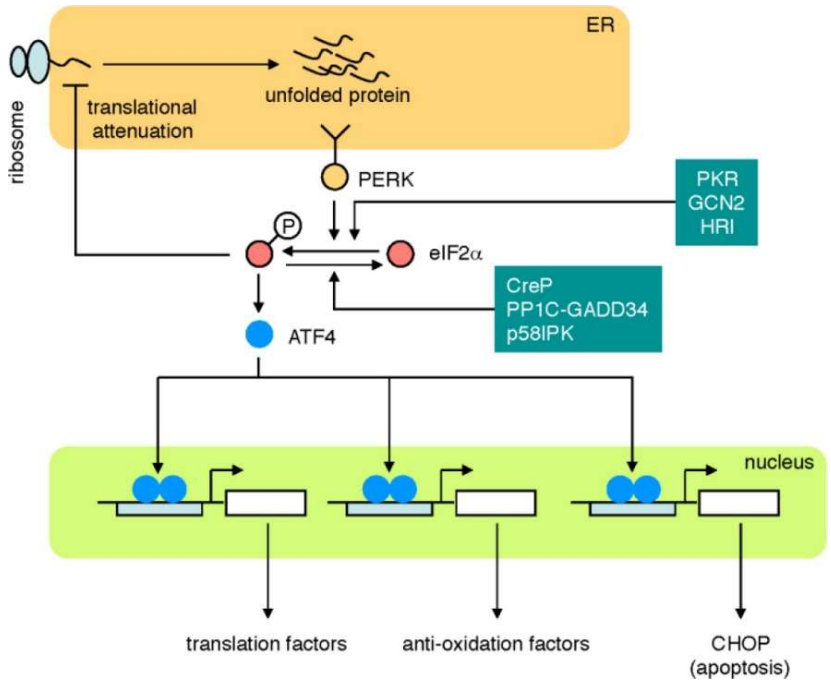

Figure 6: The essential biology of the endoplasmic reticulum stress response for structural and computational biologists. (Wakabayashi and Yoshida, 2013)

ATF4 upregulates the expression of various pro-survival genes including many ER chaperones and antioxidative factors.

Interestingly, ATF4 target genes also include well-known pro-apoptotic factors such as CHOP/ GADD153 and Noxa. In particular CHOP is a transcription factor that inhibits cell proliferation and induces apoptosis. CHOP can act as both a transcriptional repressor and activator. For example, it suppresses the expression of the multi-domain anti-apoptotic protein Bcl-2, but induces expression of GADD34, a subunit of a phosphatase complex that dephosphorylates eIF2α. It has been proposed that eIF2α de-phosphorylation mediated by CHOP-induced GADD34 defines a negative feedback loop that may enhance ER stress-induced apoptosis (Oyadomari and Mori, 2004).

CHOP has been shown to be involved in ER stress-induced apoptosis both in vitro and in vivo. CHOP-deficient MEFs (mouse embryonic fibroblasts) are partially protected against ER stress-induced apoptosis, and kidneys of CHOP−/− mice showed less apoptosis after treatment with the ER stressor tunicamycin, an inhibitor of glycosylation (Zinszner et al., 1998).

CHOP upregulates expression of the BH3-only pro-apoptotic protein Bim, and the pro-apoptotic death receptor DR5, thus sensitizing cells to apoptosis during ER stress (Yamaguchi and Wang, 2004). Another CHOP target gene that may be involved in cell death is ERO1α, which normally contributes to the oxidative environment in the ER, which is crucial for disulphide bond formation (Marciniak et al., 2004).

Thus, transcriptional changes induced by PERK activation can impact the choice between cell survival and apoptosis. It has been proposed that the duration of PERK signaling relative to that of XBP1 activation may influence this critical decision-making process: in response to prolonged ER stress, XBP1 splicing is attenuated, while PERK activity is maintained. This may shift the cell towards a more destructive fate given the above mentioned connections between PERK signaling and the activation of pro-apoptotic proteins (Liu and Ye, 2011).

Notably, in addition to eIF2a, PERK activation induces the phosphorylation of the transcription factor NRF2 (Cullinan and Diehl, 2004). Normally, NRF2 is kept in check by interaction with the protein KEAP1. Upon phosphorylation by PERK, NRF2 is liberated from KEAP1 and translocates to the nucleus where it activates genes encoding antioxidant proteins (Cullinan and Diehl, 2004). This activation of NRF2 by PERK is an important adaptive mechanism, as NRF2-deficient cells displays enhanced apoptosis during ER stress (Cullinan and Diehl, 2004).

PERK's activity is strictly linked with cancer progression; for example, various studies have shown that Nrf2 may protect cancer cells from chemotherapeutic agents, and facilitate cancer progression (Jaramillo and Zhang, 2013). Nrf2 is aberrantly accumulated in many types of cancer, and its expression is associated with a poor prognosis in patients. In addition, Nrf2 expression is induced during the course of drug resistance. Collectively, these studies suggest that Nrf2 contributes to both intrinsic and acquired chemoresistance (Jaramillo and Zhang, 2013). Recently, Walerych et al provided evidence that confirmed the pro-tumoral activity of NRF2; they found that p53 missense mutants can cooperate with Nrf2 (NFE2L2) to activate proteasome gene transcription, resulting in resistance to the proteasome inhibitor carfilzomib (Walerych et al., 2016).

In line with the important role of PERK/NRF2 complex to regulate anti-oxidative responses, loss of PERK in cancer cell lines significantly impaired cellular ROS buffering mechanisms, resulting in oxidative DNA damage and subsequent engagement of the DDR checkpoint (Bobrovnikova-Marjon et al., 2010).

Interestingly, PERK may also be directly involved in activation of ATF6. In fact, loss of PERK in liver cell lines impaired ER stress responses, and in particular ATF6 transcriptional activity. Authors proposed that ATF4 activity facilitates trafficking of ATF6 from the ER to the Golgi, thus enhancing ATF6 processing and ATF6-dependent transcription of gene involved in regulation of ERAD, chaperones, and vesicular trafficking (Teske et al., 2011).

The ATF6 pathway

The third player involved in the response to ER stress is ATF6. Mammalian ATF6 is an ER transmembrane protein of 90 kDa that under different stress conditions is transported to the Golgi compartment, where it is cleaved by S1P/S2P proteases releasing its cytoplasmic doman, in a similar way to SREBP proteins (Figure 7) (Haze et al., 1999). The ER to Golgi transport occurs with the help of PDIA5 (protein disulfide isomerase A5) and COPII vesicles components sec24/sec23, that are required for full ATF6 activation (Higa et al., 2014; Nadanaka et al., 2006).

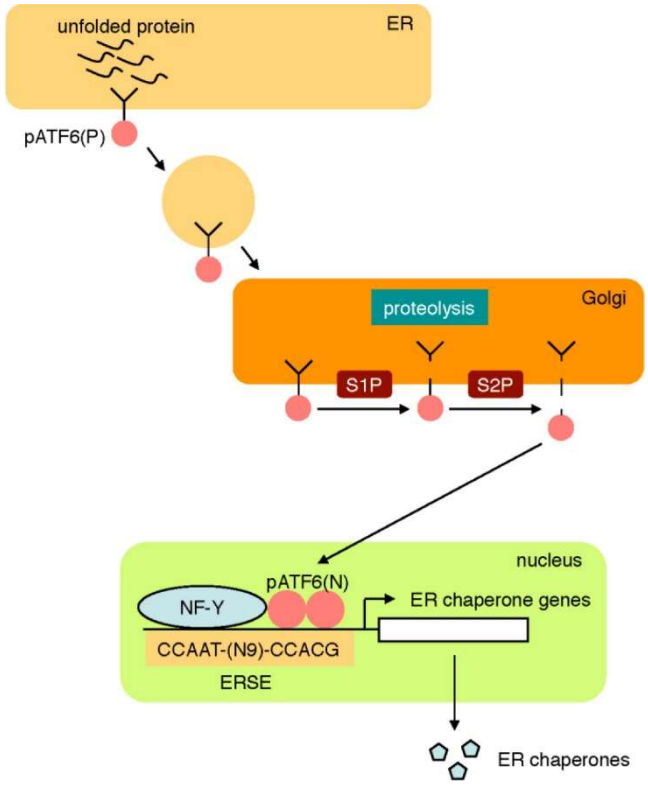

Figure 7: The essential biology of the endoplasmic reticulum stress response for structural and computational biologists. (Wakabayashi and Yoshida, 2013)

The cleaved form of ATF6 (ATF6-f) promotes transcription of UPR target genes through binding of three different consensus sequences: CCAAT-N-CCACG (ERSE-I), ATTGG-N-CCACG (ERSE-II) and TGACGTGG/A (UPRE) (Kokame et al., 2001; Wang et al., 2000). Interestingly, ATF6-f is present in a functional complex with transcription factor NF-Y, and the high-affinity for NF-Y

binding site confers selectivity among different ERSEs (Yoshida et al., 2000). Similarly, interaction with YY1 enhances ATF6-f transcriptional activity (Li et al. 2000).

The main transcriptional targets of ATF6-f are correlated with ER adaptive response, including chaperones and ERAD components, but it is also demonstrated that ATF6-f regulates cholesterol and lipid biosynthesis (Adachi et al., 2008; Maiuolo et al., 2011; MARUYAMA et al., 2013; Yamamoto et al., 2010; Zeng et al., 2004).

As a matter of fact, ATF6 deletion compromises the functionality of the secretory pathway during ER stress and impairs adaptation to chronic ER stress (Wu et al. 2007). Interestingly, it has been reported that ATF6 antagonizes SREBP-2 to regulate the homeostasis of lipids and glucose. In particular, ATF6-f can bind and inhibit nuclear SREBP-2, affecting its lipogenic effect in liver cells (Zeng et al., 2004).

Hereditary mutations in the ATF6 gene are associated to an autosomal recessive disease known as Achromatopsia (Kohl et al., 2015), characterized by cone photoreceptor dysfunction. The authors classified ATF6 mutations in three classes: class 1 ATF6 mutants present impaired ER-to-Golgi trafficking, diminished intramembrane proteolysis, and reduced transcriptional activity; class 2 ATF6 mutants are active even in the absence of ER stress; class 3 mutants present absent or defective β-ZIP domains causing complete loss of transcriptional activity.

Using primary fibroblasts from patients with class 1 or class 3 ATF6 mutations, the authors observed increased cell death in response to ER stress, elegantly proving that ATF6 function correlates with cell survival (Chiang et al., 2016). Thus, exposure to ER-stress during retinal development may contribute to cone dysfunction in children with ATF6 mutations (Chiang et al., 2016). ATF6 also controls transcription of XBP1, and silencing of ATF6 reduced XBP1s levels (Yoshida et al., 2001). Therefore, these two pathways are interconnected.

Since ATF6 and XBP1s can bind the same DNA-consensus sequences, it is difficult to dissociate these two pathways, as ATF6 and XBP1s share a number of common target genes. In order to define specific targets of XBP1s and ATF6, Yamamoto et al. created ATF6-KO mice and demonstrated that both proteins cooperate for the transcription of target genes involved in the regulation of protein quality control, but only ATF6 is implicated in transcription of chaperones (Yamamoto et al. 2007).

The pro-survival and pro-adaptive function of ATF6 may also be involved in neurodegenerative diseases. For instance, Parkinson disease is linked to aberrant accumulation of α-synuclein; in a recent work, authors found that accumulation of α-synuclein inhibits ATF6 function. α-synuclein

directly interacts with ATF6 and indirectly prevents its incorporation in COPII vesicles for Golgi transport. Impaired ATF6 activation results in defective ERAD and apoptosis of neuronal cells (Credle et al., 2015).

In addition to pro-survival functions, ATF6 can also have pro-apoptotic functions, as ATF6-f transactivates CHOP (Gotoh et al. 2002). Indeed, it was demonstrated that ATF6 and CHOP are the main mediators of apoptosis in macrophages treated with Nitric Oxide (NO) (Gotoh et al. 2002).

2.6 Endoplasmic Reticulum Stress and Malignancy

ER stress is a hallmark of many different pathological states, including metabolic syndrome, neurodegenerative diseases, and cancer. As shown in Figure 8, conditions that induce ER stress as dysregulated proliferation, oxidative stress, nutrient and lipid deprivation, hypoxia, and acidic extracellular pH are characteristic for neoplasms. Despite these adverse conditions cancer cells are able to adapt and to survive by activating different responses: autophagy, inflammation and UPR. In particular, the endoplasmic reticulum (ER) regulates this adaptive capacity by coordinating a large number of fundamental cellular processes, including transmembrane and secretory protein folding, lipid biosynthesis, drug detoxification, and calcium storage and signaling.

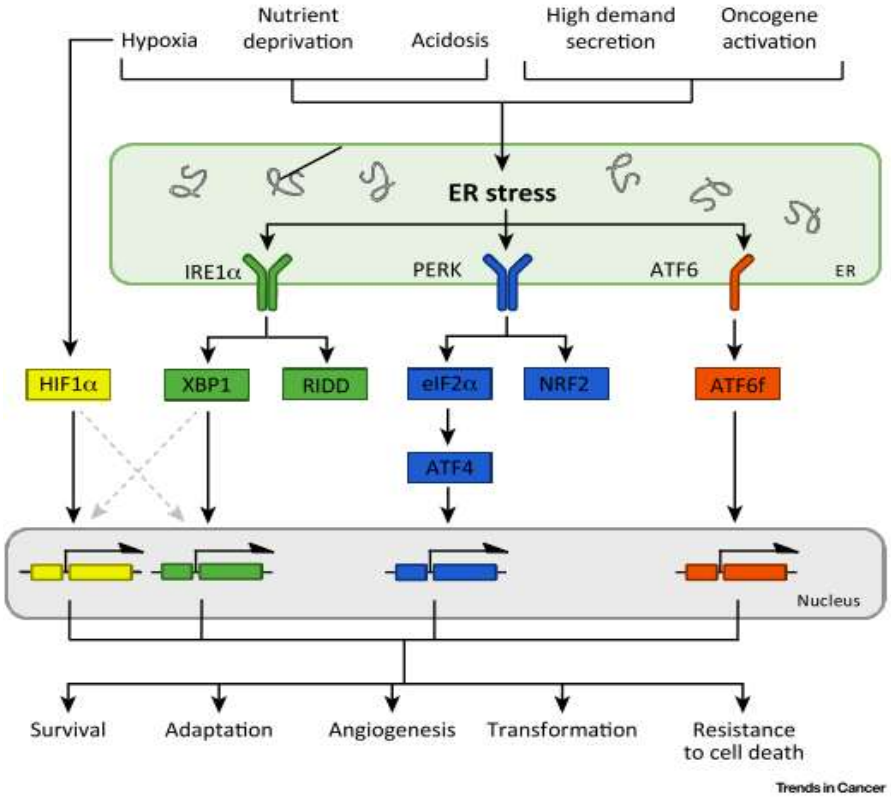

Figure 8: Endoplasmic Reticulum Stress and the Hallmarks of Cancer (Urra et al., 2016)

Understanding the mechanisms of the multiple cellular pathways and outcomes triggered by ER stress will lead to the development of novel therapeutics to prevent or treat disease.

Healthy tissues are able to sense the accumulation of misfolded proteins beyond a tolerable threshold and, depending on the stress burden, to improve ER protein-folding capacity or to induce apoptosis. In contrast, tumor tissues change these responses and take advantages from them to promote cancer progression. Indeed, ER stress responses have been documented to be activated in most major types of human cancer, including breast, pancreatic, lung, skin, prostate, brain, and even liquid malignancies (Wang and Kaufman, 2014). Furthermore, ER stress frequently correlates with advanced-stage disease and chemoresistance.

Cancer cells have developed mechanisms to tolerate persistent ER stress possibly by taking advantage of the UPR response (Urra et al., 2016). UPR activation in transformed cells is regulated by both intrinsic and extrinsic factors.

2.6.1 Cell-intrinsic mechanisms

Cancer initiation and development correlate with an increase in protein synthesis and augmented request of membrane and lipids. As matter of fact, loss of the tumor suppressors p53, PTEN, TSC1, or TSC2 dramatically enhances protein synthesis rates, leading to ER stress (Fu et al., 2008; Kang et al., 2011; Namba et al., 2015). Similarly, enhanced protein synthesis and concomitant ER stress are also observed upon overexpression of oncogenic HRAS (G12E), BRAF (V600E), c-Myc, or Src (Hazari et al., 2016). Interestingly, both UPR signaling and leukemia development induced by conditional PTEN deletion or c-Myc overexpression were reduced or abrogated upon deletion of the key translation rate regulator ribosomal protein RPL24, implicating that protein synthesis rate is a key driver of ER stress and tumorigenicity in vivo (Hazari et al., 2016). It has been proposed that initially transformed cells may undergo ER stress in response to the higher replicative and metabolic demands but can adapt by enhancing steady-state ER protein-folding capacity (Huber et al., 2013).

In addition, de novo genetic mutations giving rise to aberrant proteins, and other cell-intrinsic and cell-extrinsic stresses, likely contribute to the chronic UPR observed in most major cancer types (Wang and Kaufman, 2014).

Cell-extrinsic mechanisms

Many extrinsic mechanisms present in tumor microenvironment are involved in the induction of ER stress. Normal and healthy cells use oxidative phosphorylation or anaerobic glycolysis to generate ATP, in contrast, throught the well know Warburg effect, cancer cells prefer aerobic glycolysis. According to this, cancer cells consume glucose and release large quantities of lactic acid, which lowers the extracellular pH. In addition to acidosis, solid tumors experience limited nutrient availability and hypoxia, all known activators of ER stress.

Low oxygen increases cytosolic ROS production. ROS, are required for both stabilizing HIF1a, the key hypoxia response transcription factor, involved both in ER stress and cancer progression and in generating peroxidized lipids, which could form destructive covalent adducts with various ER chaperones. Deprivation of glucose and glutamine limits metabolic intermediates required for the hexosamine biosynthetic pathway (HBP), crucial for N-linked protein glycosylation and required for successful ER protein folding. Prolin starvation can also induce ER stress, potentially by inducing excessive ROS accumulation (Cubillos-Ruiz et al., 2017).

ER stress and cancer: Friend or Foe?

It was proposed that cancer cells by tacking advtanges from UPR pro-survival activities are able to promote cancer development and homeostasis mantainance (Figure 9). These include sustaining viability under hypoxia and nutrient deprivation, enhancing metastatic spread by supporting EMT, tumor cell dormancy, and stimulating angiogenesis. As ER stress is a common feature of aggressive cancers, understanding how the UPR modulates this disease is critical for identifying promising new clinical strategies.

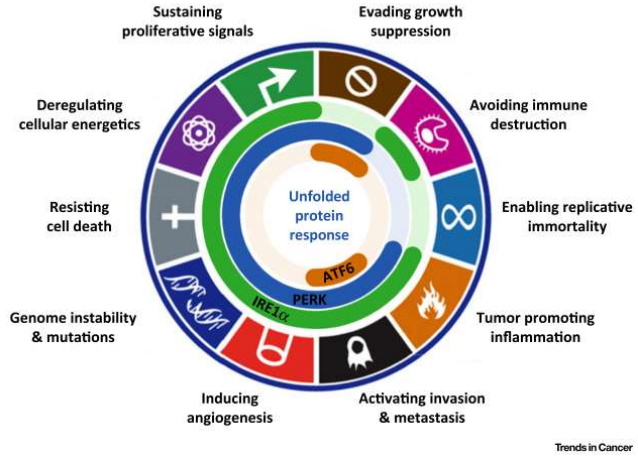

Figure 9: Endoplasmic Reticulum Stress and the Hallmarks of Cancer (Urra et al., 2016)

ER stress dictates cell fate depending on context and signal strength. Prolonged and severe pharmacological ER stress can trigger caspase-mediated cell death through several IRE1a- and PERK-dependent mechanisms. Despite pro-apoptotic mechanisms, mammalian cells

have evolved multiple adaptive mechanisms to ER stress-mediated cell death. For example, MEFs exposed to persistent low-grade pharmacological ER stress resist by sustaining pro-survival Hspa5 (GRP78) mRNA stability and reducing pro-apoptotic Ddit3 (CHOP) mRNA stability (Rutkowski et al., 2006). Furthermore, STAT3 and NF-kB, which can be activated both by IRE1a and PERK, transcriptionally upregulate multiple anti-apoptotic proteins (Tabas and Ron, 2011). Additionally, ATF6-dependent p58(IPK) limits apoptosis during oncogenic transformation by repressing PERK activity (Huber et al., 2013).

In summary, it appears that if cells successfully limit pro-apoptotic UPR outputs, ER stress confers survival advantages during tumor progression. Hypoxia and nutrient deprivation induce XBP1 splicing, which sustains cell growth and viability in human breast cancer cell lines in vitro and in vivo (Chen et al., 2014). PERK-mediated NRF2 stabilization, glutathione synthesis, and HO-1 upregulation collectively reduce cytotoxic ROS levels to facilitate cancer cell growth (Cullinan et al., 2003).

Indeed, invasive cancer cells require PERK-mediated autophagy to resist anoikis, a form of cell death triggered by extracellular matrix (ECM) detachment. (Avivar-Valderas et al., 2011). Indeed, human breast ductal carcinomas also exhibit higher PERK phosphorylation than normal breast tissue, attesting to the physiological relevance of this mechanism (Avivar-Valderas et al., 2011).

The UPR also has a role in sustaining metastasis. In this context, PERK has been reported to have a crucial role in dampening E-cadherin and overexpressing Twist, thereby enhancing migration and tumorsphere formation (Feng et al., 2014). Moreover, ATF4 target genes also correlate with an EMT gene signature in breast, colon, gastric, lung, and mixed origin metastatic cancers. Notably, pre-treating cells with a PERK inhibitor or silencing ATF4 dramatically reduce in vivo lung metastasis (Linares et al., 2017).

Notably, the UPR might be involved in dormancy of metastatic cells. Dormant cells are often quiescent and exhibit reduced metabolic rates, which can insulate them from many anticancer drugs that rely on active proliferation. Disseminated tumor cells in the bone marrow of breast cancer patients exhibit high expression of multiple ER chaperones, including BiP, which protects these cells from hypoxia and glucose deprivation (Yang et al., 2016). Dormancy-associated chemoresistance require both BiP and PERK, as silencing BiP or overexpressing a dominant-negative PERK variant sensitize cancer cells to doxorubicin and etoposide-mediated apoptosis (Yang et al., 2016). In addition, dormant cells showed constitutive ATF6-f nuclear translocation, which was partially dependent on p38 signaling (Teodoro et al., 2012). Despite ATF6 was not

required for tumor cell growth in vitro, silencing ATF6 sensitized cells to rapamycin treatment and reduced tumor nodule size in vivo (Schewe and Aguirre-Ghiso, 2008).

A genetic polymorphism that increases mRNA expression of ATF6 and its downstream genes has been associated with susceptibility to hepatocellular carcinoma (Wu et al., 2014). More importantly, GRP78/BiP, a direct transcriptional target of ATF6α, has been reported to serve as a malignancy marker for cells. BiP expression not only correlates with cancer cell proliferation and histological grade but also correlates with response to therapies and prognosis (Yao et al., 2015). Indeed, XBP1s also correlates with malignancy, and silencing of XBP1 strongly inhibited mammosphere growth in TNBC cell lines and primary patient samples (Chen et al., 2014). Inducible silencing of XBP1 in established TNBC xenografts significantly reduced primary tumor growth, angiogenesis, secondary metastases, and tumor recurrence after chemotherapy, without enhancing tumor cell death (Chen et al., 2014). Taken together these data suggests that all three UPR branches may promote metastasis by sustaining cell invasion, dormancy, and seeding.

The UPR may also regulate angiogenesis. Solid tumors must produce new vessels to supply oxygen and nutrients for growth and to remove toxic products.. In this particular context, XBP1, ATF4, and ATF6a can transcriptionally upregulate VEGFa under hypoxia or glucose deprivation by directly or indirectly binding the VEGF promoter (Karali et al., 2014). Neovascularization is also mediated by a number of pro-angiogenic cytokines, including fibroblast growth factor 2 (FGF2), interleukin-6 (IL-6), IL-8, and angiogenin, whose expression appears to be sustained by IRE1a activity (Chen et al., 2014).

Finally, the UPR might modulate interaction of cancer cells with their microenvironment. The tumor microenvironment is characterized by the presence and the cooperation of different cellular type: stromal cells, fibroblasts, cancer cells and immune cells (Quail and Joyce, 2013). In the last years, the interaction between the immune system and cancer progression obtained strong interest. Interestingly, upon ER stress cancer cells are able to transmit to immune system cells a signal of proliferation, using a process termed as ‘‘transmissible ER stress’’, which affects the antigen-presenting capacity of bone-marrow-derived dendritic cells (DCs) while provoking overexpression of immunosuppressive molecules (Mahadevan et al., 2012). These studies suggested that ER-stressed cancer cells secrete factors that modulate innate immune cell functions, but whether this process contributed to tolerance and/or immunosuppression in cancer hosts is still unknown (Rodvold et al., 2017). Importantly, subsequent studies

demonstrated that administration of the ER stressor thapsigargin to tumor-bearing mice accelerates cancer progression and stimulates the accumulation and immunosuppressive capability of myeloid-derived suppressor cells (MDSCs), a process that could be alleviated upon treatment of cancer hosts with chemical chaperones that reduce ER stress (Lee et al., 2014).

Chronic inflammation is also associated with UPR, and can contribute to all stages of cancer development and progression. As shown in several pathological conditions, ER stress and UPR activation are involved in the signal transduction and transcriptional regulation of inflammatory mediators (Lerner et al., 2012; Oslowski et al., 2012).

2.7 Targeting UPR in cancer

Giving the pre-existing ER stress induced by intrinsic and extrinsic factors in cancer cells, compounds that increase ER stress levels should induce apoptosis.

One strategy is to increase the amount of unfolded proteins in the ER lumen by inhibiting proteasome-dependent degradation.

To this aim Bortezomib, the first proteasome inhibitor for cancer therapy approved by the US Food and Drug Administration (FDA), obtained success as an ER stress inducer and in treating multiple myeloma. More importantly, the sensitivity to proteasome inhibitors correlates with low XBP1 (or XBP1s) or ATF6α levels suggesting their involvement in tumor progression.

Some new emerging drugs are designed to target specific UPR pathways to inhibit UPR activation, thereby augmenting ER stress in cancer cells (Figure 10).

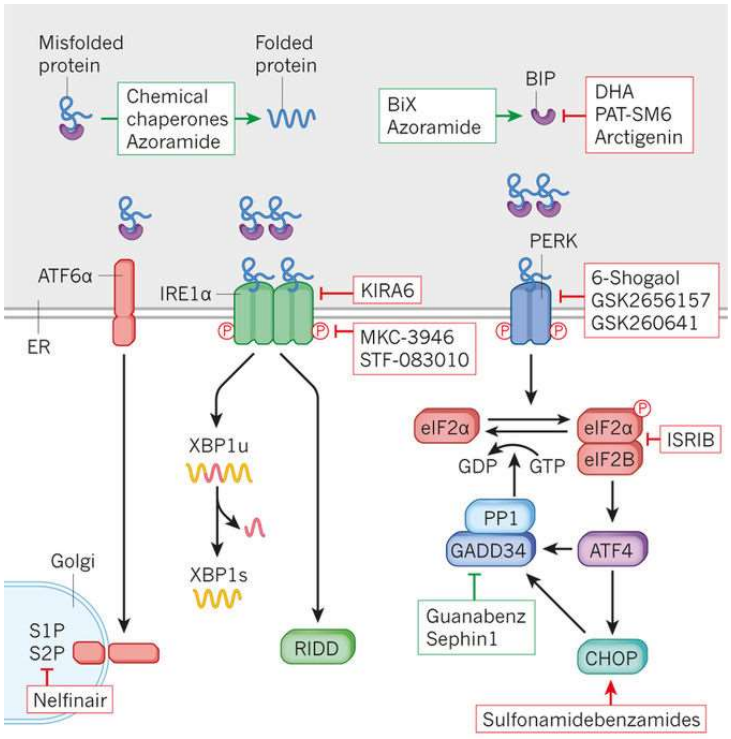

Figure 10: Protein misfolding in The Endoplasmic Reticulum as a conduit to human disease. (Wang and Kaufman, 2016)

2.7.1 IRE1a endo-ribonuclease activity inhibitors

Sustained IRE1a-XBP1 signaling promotes cancer-cell-intrinsic growth, metastasis, and chemoresistance, but its surprising role as a key modulator of myeloid cell function in tumors emerges as an attractive target for cancer immunotherapy. Whereas direct pharmacological inhibition of nuclear XBP1 is difficult due to major technical limitations, targeting its upstream activator, IRE1a, represents a viable strategy.

Indeed, the dual enzyme IRE1a is amenable to small-molecule targeting, and two classes of direct inhibitors have been identified. The first group of compounds directly targets the IRE1a endoribonuclease domain, and some examples of this class include toyocamycin (Ri et al., 2012), STF-083010 (Papandreou et al., 2011), 4m8C (Cross et al., 2012), MKC-3946 (Mimura et al., 2012), and B-I09 (Tang et al., 2014). Notably, these direct IRE1a endonuclease inhibitors are capable of blocking Xbp1 splicing without affecting IRE1a phosphorylation or the PERK and ATF6a arms of the UPR. STF-083010, MKC-3946, and toyocamycin demonstrated therapeutic efficacy in multiple myeloma xenograft models, and B-I09 has been shown to control the aggressiveness of chronic lymphocytic leukemia cells in vivo (Tang et al., 2014). The second group of inhibitors targets the IRE1a kinase domain in order to allosterically disrupt its endoribonuclease function. A recent compound in this category is KIRA6 (Ghosh et al., 2014), which reduced plasma glucose levels and improved glucose tolerance in Ins2+/Akita mice that exhibit chronic ER stress in pancreatic b cells (Ghosh et al., 2014). Moreover, intravitreal KIRA6 injection in the P23H transgenic rat model of retinitis pigmentosa preserves photoreceptor viability and function (Ghosh et al., 2014). Nevertheless, it has not been determined whether treatment with IRE1a inhibitors fully recapitulates the biological effects of IRE1a genetic ablation. Developing novel IRE1a inhibitors with potent in vivo efficacy in the tumor microenvironment could therefore be useful to directly restrain cancer cell survival, metastasis, and chemoresistance, while eliciting protective anti-tumor immune responses via myeloid cell reprogramming.

PERK inhibitors

GSK2606414 was the first reported PERK inhibitor (Axten et al., 2012) and was found to be neuroprotective in mouse models of prion disease (Moreno et al., 2013). Another ATP-

competitive inhibitor of PERK enzymatic activity, GSK2656157, was shown to impede ER-stress-induced PERK autophosphorylation, eIF2a phosphorylation, and subsequent overexpression of ATF4 and CHOP in multiple cell lines (Atkins et al., 2013). Oral administration of GSK2656157 to mice impaired PERK auto-phosphorylation in the pancreas and compromised xenograft tumor growth in immunodeficient hosts (Atkins et al., 2013). However, further studies indicate that inhibition of PERK activity by GSK2656157 does not always correlate with reduced eIF2a phosphorylation and that this inhibitor fails to recreate the biological effects of PERK genetic inactivation (Krishnamoorthy et al., 2014). The integrated stress response inhibitor (ISRIB) is a symmetric bis glycolamide that renders cells resistant to eIF2a phosphorylation, thereby blocking the activation of ATF4 and the accumulation of CHOP during conditions of ER stress (Sidrauski et al., 2015). Whether GSK2656157 or ISRIB could modulate the function or survival of MDSCs in the tumor microenvironment by impeding PERK/eIF2a-mediated CHOP activation is yet to be tested. Given the importance of PERK and IRE1a-XBP1 signaling in organ homeostasis of highly secretory tissues, careful optimization of these inhibitory compounds for in vivo use is essential to minimize potential side effects and toxicity in treated hosts.

ATF6 inhibitors

Nelfinavir is the first identified ATF6 inhibitor. It was created as inhibitor of viral proteases but Nelfinavir and its analogs are also able to inhibit the proteases S1P and S2P, that cleave ATF6 and SREBP1 into the Golgi compartment (Guan et al., 2011; Koltai, 2015). Giving the non-specific action of Nelfinavir, another class of ATF6 inhibitors has been recently discovered by Walter and its group. Ceapins, a new class of pyrazoleamides, have been recently demonstrated to specifically inhibit the ATF6a branch of the UPR by blocking ATF6a processing and nuclear translocation in cells undergoing ER stress (Gallagher and Walter, 2016). Ceapins are rather toxic, therefore further optimization of Ceapins for in vivo use will hence be critical for determining the anti-tumor activity of these compounds alone or in combination with other agents that ablate PERK and/or IRE1a signaling in the tumor microenvironment.

Another important finding, which shows that targeting the UPR may be a promising approach for cancer therapy, is that GRP78/BiP is expressed on the surface on cancer cells but not normal cells. Overexpression of BIP in cancer cells correlates with chemotherapy resistance (Yao et al., 2015).

In addition, expression of BIP seems to be increased in the tumor vasculature, suggesting that targeting BIP will have an impact on both cancer cells and the tumor microenvironment (Roller et al., 2013).

However, as UPR activation has both pro-survival and anti-survival effects on cells, caution is necessary in the design of therapies that target UPR components and in the interpretation of the results. Specific gene targeting experiments are required to dissect the requirement for different UPR transduction pathways in the tumor and the microenvironment. Indeed, ER stress and UPR activation may also alter the cancer cell response to adjuvant therapies, offering an opportunity for drug combination treatments. One therapeutic rationale is to induce ER stress and UPR activation to activate death pathways in cancer cells. Alternatively, preventing UPR activation could sensitize cancer cells to other therapies, as the UPR promotes adaptation and drug resistance. Anti-angiogenesis therapies are not adequate alone, but they may show synergy in combination with anti- UPR agents.

In summary, combination therapies using drugs targeting the UPR together with drugs targeting specific oncogene, for example mutp53, may become promising anticancer approaches.

3. Aim of the thesis

TP53 is one of the most frequently mutated genes in human cancer. Wild-type p53 is involved in the activation of apoptotic outcomes in a variety of different stresses conditions, but its role in the response to ER stress in not fully understood. Cancer associated mutant p53 proteins acquire powerful oncogenic activities (gain of function), and almost nothing is known about mutant p53 and ER stress. What appears clearly from the literature is that mutant p53 can sustain proteasome activity (Walerych et al., 2016), N-glycosylation of proteins (Vogiatzi et al., 2016), and HSF1 transcriptional activity (Li et al., 2014). Although these functions are indirectly connected to ER stress, whether mutant p53 can directly modulate the Unfolded Protein Response (UPR) pathway is not clear.

ER stress is a common feature of different types of blood and solid cancers. Adaptation to ER stress is achieved by the activation of the UPR. Some targets and mediators of UPR favor resistance of cancer cells to ER stress. Moreover, cancer cells are able to transmit this resistance to the other cells present in tumor microenvironment (immunity cells, stromal cells and fibroblasts) favoring tumor growth and chemoresistance. For all these reasons, ER stress and UPR are gaining increasing attention as new targets for cancer treatment.

Based on these premises, we hypothesize that mutant p53 can help maintain ER stress under the threshold levels that induce apoptosis, thus sustaining cancer cell survival. We also hypothesize that mutp53 does so by modulating the signaling responses that are triggered by ER stress.

Aim of this Thesis is to define the role of mutant p53 in the response to ER stress in model cancer cell lines. It also aims to study the molecular mechanisms by which mutant p53 might influence the UPR signaling network. Finally, this work aims to explore the potential benefits of inhibiting selected components of the UPR as a novel approach to reinforce the efficacy of mutp53-targeting drugs.

4. Results

Walerych and colleagues have recently compared transcriptome and proteome of five triple-negative breast cancer cell lines bearing different p53 mutations, and found that mutant p53 (mutp53) sustains expression of genes encoding proteasome subunits. Increase in these genes correlates with cancer cells invasion and chemoresistance and they proposed that these phenotypes were dependent on a new gain-of-function of mutant p53 (Walerych et al., 2016). Interestingly, in addition to the proteasome, their data suggest that mutant p53 regulates other homeostatic processes and in particular the Unfolded Protein Response (Figure 1). Starting from this observation and from the evidences that UPR's pro-survival effects are improved in cancer cells to increase resistance to proteostasis insult, I hypothesized that mutant p53 is controlling Unfolded Protein Response to sustain survival and to maintain homeostasis.

Mutant p53 protects cancer cells from ER stress-induced apoptosis

To deepen our understanding on the role of mutant p53 in UPR, we started evaluating survival of cancer cell lines exposed to ER stress. To mimic severe ER stress conditions, we treated cells with two widely used drugs: Thapsigargin (Tg) and Tunicamycin (Tm). Tg is a specific inhibitor of the sarcoplasmic/endoplasmic reticulum Ca2+-ATPase (SERCA). Treatment with Tg results in a decrease in ER calcium levels, so that calcium-dependent ER chaperones, such as calnexin, lose their activity, leading to the accumulation of unfolded proteins. Tm is an inhibitor of UDP-N-acetylglucosamine-dolichol phosphate N-acetylglucosamine-1-phosphate transferase (GPT); it blocks the initial step of glycoprotein biosynthesis in the ER, so Tm treatment causes accumulation of unfolded glycoproteins in the ER, leading to ER stress (Kim et al., 2009).
To measure cancer cells viability, we used ATP-lite viability assay, which, by monitoring ATP, can assess the proliferative effects of a wide range of drugs, biological response modifiers, and biological compounds. As model, we used MDA-MB-231, a metastatic triple negative breast cancer cell line with mutant p53 (R280K), and measured survival upon depletion of mutp53 and treatment with Tg (1uM) and Tm (5ug/ml) for 48 hours. As shown in Figure 2A, depletion of mutant p53 rendered MB231 cells more sensitive to Tg and Tm treatment. Higher cleaved-PARP levels in mutp53-depleted cells indicated that this reduced viability is due to apoptosis (Figure 2B).

Similar results were obtained with PANC1, a pancreatic cancer cell line with mutp53 (R175H) and with SUM159, a breast cancer cell line with mutp53 (R158InF), (Fig 2C, D); therefore, this phenotype is not a peculiarity of MB231 cells.

Together, these preliminary observations suggest that mutp53 confers a certain degree of resistance to ER stress.

To test whether this activity may be considered as a proper GOF of mutp53, we stably overexpressed two different p53 mutants, R280K and R175H, in the triple negative breast cancer cell line HBL-100, bearing wild-type p53. This model allows us to assess the dominant effects of mutant p53. We performed ATP-lite assays after treating cells with Tg and Tm for 48 hours; HBL-100 cells overexpressing mutant p53 showed higher resistance to treatments (Figure 2E), suggesting that mutp53 is sufficient to promote this phenotype.

To further confirm these observations, we compared mouse embryonic fibroblasts (MEFs) derived from p53 -/- and mutp53 R172H/- mice (Girardini et al., 2011). By FACS analysis we observed that upon 24 hours of treatment with Tg and Tm, MEFs KI for mutant p53 showed significantly fewer apoptotic cells with respect to p53 KO MEFs (Figure 2F).

Together, these data support the hypothesis that mutant p53 is protecting cancer cells from ER-stress induced apoptosis.

Mutant p53 depletion induces different UPR activation patterns.

To better understand which pathway is involved in the phenotypes observed, we started inhibiting, in mutant p53 bearing cells, the main mediators of UPR, IRE, PERK and ATF6, by using GSK-2606414 as PERK inhibitor (PERKi), STF-083010 as IRE1 inhibitor (IREi), and Ceapin_A7 as ATF6 inhibitor (ATF6i) (Gallagher et al., 2016; Hetz et al., 2013; Vincenz et al., 2013). Notably, in basal conditions (i.e., without ER stress-inducing drugs) only ATF6 inhibition had a phenotype, clearly reducing cell viability (Figure 3A) - suggesting that ATF6 may have a role in mutant p53 bearing cancer cells.

Efficiency of inhibitors was confirmed by performing three independent experiments in MDA-MB-231. Western blot experiments were performed to measure PERK phosphorylation and ATF6 cleavage, while XBP1s mRNA levels were measured as a readout of IRE1 alpha activation (Figure 3B).

In order to understand whether mutant p53 is acting on IRE1, PERK and ATF6 expression, we analyzed mRNA and protein levels of IRE, PERK and ATF6 upon mutp53 depletion. Notably, MDA-MB-231 depleted for mutp53, showed increase in mRNA and protein levels of IRE1 and PERK, and, interestingly, showed a reduction in cleaved ATF6 protein (Figure 3C).

This suggests that mutant p53, in unstressed conditions, is maintaining low levels of IRE1 and PERK and is sustaining ATF6 cleavage.

Since, IRE1a and PERK are mainly mediators of ER stress-induced apoptosis (Tabas and Ron, 2011), while ATF6 is largely involved in the transcription of chaperones, ERAD components, and protein quality control enzymes, we hypothesized that mutant p53 is inhibiting IRE1 and PERK to block apoptotic signals, and it is sustaining ATF6 activity to maintain ER homeostasis.

Mutant p53 inhibits IRE1a and PERK functions but promotes ATF6 transcriptional activity.

In order to evaluate, whether upregulation of IRE1 and PERK activities impinge on their activation upon silencing of mutant p53, we measured both kinase and RNase activities of IRE1 by quantifying XBP1 mRNA cytoplasmic splicing and JNK phosphorylation. We performed RT-qPCR for XBP1s and western blot experiments for pan and phospho-JNK proteins. As shown in figure 4A, MDA-MB-231 and SUM159 depleted for mutant p53 showed increase in XBP1s expression and increase in JNK phosphorylation, confirming that IRE1 is more active in mutp53-depleted cells.

As readout of PERK activation, we measured CHOP mRNA levels. As expected, MDA-MB-231 depleted for mutant p53 showed increased CHOP mRNA levels (Figure 4B), indicating enhanced PERK activity.

We observed that mutant p53 depletion reduced the cleaved form of ATF6 (Figure 3A). The ATF6f fragment is a DNA-binding transcription factor, and promotes transcription of UPR target genes (Kokame et al., 2001; Wang et al., 2000); therefore, as readout of ATF6f activity we used a luciferase (LUC) reporter construct harboring five repetitions of the ATF6 binding sites (Shen et al., 2002). We transiently transfected this construct in MDA-MB-231 stably silenced for mutant p53 (shp53), and observed a reduction in luciferase signal upon mutp53 depletion (Figure 4C).

Importantly, the results of loss-of-function experiments were confirmed by gain-of-function experiments, as stable expression of two different p53 mutants in p53-null H1299 cancer cells

reduced CHOP and XBP1s mRNA induction, and enhanced ATF6 transcriptional activity (Figure 4D).

Together, these data support the hypothesis that mutant p53 is dampening IRE1 and PERK activation, while sustaining ATF6 processing and transcriptional activity.

Both JNK and CHOP are required for ER stress-induced apoptosis in mutp53-depleted cancer cells.

As already mentioned, CHOP and JNK are, respectively, downstream pro-apoptotic effectors of PERK and IRE1a. We therefore assessed their role in ER stress-induced apoptosis in the presence or absence of mutant p53. To this aim, we performed ATP-lite assays upon siRNA depletion of JNK and/or CHOP. Interestingly, knockdown of either JNK or CHOP was sufficient to reduce cell death, both in untreated conditions than upon Tg or Tm treatment (Figure 5A); this indicates that JNK and CHOP are both required for ER stress-induced apoptosis in mutant p53 depleted cancer cells.

Since there are many evidences that suggest a cooperation of JNK and CHOP in activation of common target genes, one of which is DR5 (Death Receptor 5), a cell surface receptor for tumor necrosis factor (TNF)-related apoptosis (Oh et al., 2012; Tiwary et al., 2010). We thus hypothesized that mutant p53, by coordinately dampening CHOP and JNK activation, may be blocking DR5 induction upon ER stress. To test this, we measured DR5 mRNA levels in cancer cell depleted for mutant p53. As shown in figure 5B, mutant p53 knockdown significantly increased Tg-induced DR5 mRNA transcription, and such increase was blocked by depletion of either JNK or CHOP.

We next asked whether mutant p53 depletion enhances ER stress-induced apoptosis via CHOP and JNK-mediated DR5 induction. To test this, we repeated ATP-lite experiments in MDA-MB-231 upon double knockdown of mutant p53 and/or DR5. As shown in figure 5 C, knockdown of DR5 significantly increased viability of mutp53-depleted cells, confirming that DR5 is a major effector of ER stress-induced apoptosis in MB231 cells, at least under these conditions.

Together these data indicate that mutp53 depletion, possibly by increasing IRE1 and PERK signaling, sensitizes cancer cells to ER stress-induced apoptosis, and that such apoptosis is dependent on the cooperative activity of both JNK and CHOP converging on activation of a set of pro-apoptotic targets, among which is death receptor DR5.

Mutant p53 sustains ATF6 transcriptional activity by promoting its cleavage.

It has been demonstrated that ATF6 activity ameliorates ER function and protects cells from chronic stress, since it regulates chaperones and protein quality control genes (Adachi et al., 2008; Wu et al., 2007). In line with a pro-survival and pro-adaptive function, ATF6 was recently proposed as an important marker of pre-cancerous lesions associated with colorectal cancer (Hanaoka et al., 2017). We found that ATF6 transcriptional activity is reduced upon depletion of mutp53 in MB321 cells (Figure 4C), and is enhanced by overexpression of mutant p53 in p53-null cells (Figure 4D).

Regarding ATF6 expression, RT-PCR experiments indicated that ATF6 mRNA levels are not significantly changed upon silencing of mutant p53, but immunoblotting revealed a reduction in ATF6f protein levels (Figure 3C). Therefore, it is possible that mutant p53 may sustain ATF6 transcriptional activity by enhancing its translocation from ER to Golgi and consequently its proteolytic cleavage.

To better address this point, we analyzed the kinetics of ATF6 cleavage induced by ER stress in MDA-MB-231, confirming that ER stress-induced ATF6 processing is significantly reduced upon mutant p53 knockdown (Figure 6A). Correspondingly, mutant p53 knockdown also dampened ATF6-dependent transcription as assessed by Luciferase experiments, both in untreated conditions than upon ER stress induction (Figure 6B).

To test if mutant p53 may dominantly promote ATF6 processing, we analyzed ATF6f levels and transactivation in HBL-100 cells (wt p53) stably overexpressing R280K or R175H p53 mutants.

Western blot experiments confirmed that mutant p53 increased ATF6f protein levels (Figure 6C), as well as transactivation of the ATF6-LUC reporter (Figure 6D).

ATF6 is required for cancer cell survival and invasion.

Starting from the evidence that ATF6 inhibition using Ceapins_A7 reduces cell viability (Figure 2B), and that mutant p53 overexpressing cells present higher basal ATF6 transcriptional activity (Figure 6D), we hypothesized that mutant p53 may sustain cancer cell survival, at least in part, by promoting ATF6-dependent chaperone production and ER homeostasis.

To test this hypothesis, we further analyzed the impact of ATF6 depletion on viability of MDA-MB-231 cells exposed to ER stress. In line with results obtained with pharmacological inhibition (Figure 3B), ATF6 depletion compromised basal viability of these cells, and strongly increased their sensitivity to ER stress (Figure 7A). This data further confirmed that in our model (mutp53 TNBC cells) ATF6 has a significant pro-survival activity.

In apparent contradiction with the above results, ATF6 depletion reduced CHOP mRNA induction by ER stress (Figure 7B), thus potentially counteracting apoptosis. This result is not surprising, as literature indicates CHOP as a transcriptional target of ATF6f (Yoshida et al., 2000). Nonetheless, ATF6 depletion also increased ER stress-induced JNK phosphorylation, similar to what happens upon silencing of mutp53 (Figure 7B), a result in line with reduced cell viability.

One of the most important ATF6 target genes is the ER chaperone GRP78/BiP, a key regulator that maintains ER homeostasis, suppresses stress-induced apoptosis, and controls UPR signaling. Notably, increased expression of GRP78, together with other ATF6 targets, correlates with poor prognosis and cancer cells invasion and migration (Roller et al., 2013; Yao et al., 2015).

We therefore hypothesized that mutant p53 could regulate ER homeostasis, enhance cell invasion, and suppress stress-induced apoptosis by sustaining ATF6-dependent expression of GRP78 and other chaperones. To test this hypothesis, we performed matrigel invasion assays with cells depleted for ATF6. We observed a substantial reduction in the number of invasive cells upon ATF6 silencing, supporting the evidence that ATF6 activity correlates with a more aggressive phenotype (Figure 7C).

We also measured GRP78 mRNA and protein levels upon silencing of ATF6 or mutant p53. As expected, we observed a strong reduction in GRP78 mRNA and protein levels upon ATF6 depletion (Figure 7D). Interestingly, GRP78 expression was reduced also after mutant p53 depletion (Figure 7E), confirming the mutant p53-ATF6 regulatory axis.

Together, these data suggest that ATF6-depletion reduces cell viability and cell invasion by affecting multiple parameters, including an increase in JNK activation and less efficient induction

of pro-survival effectors such as GRP78/BiP, and possibly other chaperones and ERAD components. They also suggest that the increased resilience to ER stress observed in cancer cells with mutant p53 may be due to increased ATF6 transcriptional activity.

Starting from the evidence that ATF6 needs the binding of COPII vesicles components sec23/sec24 to be translocated into the Golgi apparatus (Schindler and Schekman, 2009), we asked whether mutant p53 may be enhancing expression of these proteins, since this could increase the ER-to-Golgi translocation of ATF6 in cancer cells.

Intriguingly, we measured a reduction of sec24 mRNA and protein levels in MDA-MB231 cells upon mutant p53 knockdown (Figure 7F). These preliminary data suggest a possible involvement of mutant p53 in supporting expression of sec24, which in turn could promote ATF6 incorporation in COPII vesicles for Golgi translocation, cleavage, and activation.

Targeting ATF6 and Mutant p53 as a new strategy for cancer therapy.

ER stress can be theoretically exploited to kill tumor cells, given their strong dependency on ER functions to sustain proliferation, protein biosynthesis, and secretion of matrix remodeling enzymes, growth factors, and receptors. This could be achieved via two complementary approaches: one focalized on exacerbation of ER stress, the other on inhibition of UPR-dependent pro-survival pathways. Both mutant p53 and ATF6 have been associated with cell survival and progression/aggressiveness in different tumor types (Adachi et al., 2008); we therefore sought to evaluate the possibility to enhance cancer cells' sensitivity to ER stress by acting, alone or in combination, on mutant p53 and ATF6.

To target ATF6 we used NELFINAVIR (NFV), an anti-viral compound used against HIV infection that inhibits the proteases responsible for ATF6 cleavage in the Golgi compartment (Guan et al., 2011, 2015). Using our mutant p53 TNBC model, we examined cell viability upon treatment with NFV, alone or in combination with ER stressors Tg and Tm. A shown in figure 8A, NFV treatment strongly reduced cell survival, both under basal conditions and upon ER stress activation. To confirm that NFV inhibits ATF6 functions, we transfected an expression vector encoding an eGFP-ATF6 fusion protein, that makes it easier to follow processing and subcellular localization of ATF6 by detecting eGFP. Treatment with NFV efficiently blocked Tg-induced cleavage of ATF6 in MDA-MB-231 cells (Figure 8B). Accordingly, NFV also inhibited ATF6 transcriptional activity (Figure 8C).

To target mutant p53, we used the FDA-approved histone deacetylase inhibitor SuberoylAnilide Hydroxamic Acid (SAHA), a drug that reduces mutant p53 protein levels and shows preferential cytotoxicity in mutant p53 cancer cells (Li et al., 2011). We analyzed viability of MDA-MB-231 cells upon treatment with SAHA, alone or in combination with ER stress. As shown in Figure 9A, SAHA treatment reduced cell viability, both under basal conditions and upon treatment with Tg or Tm, confirming that mutp53 has a protective role under conditions of ER stress. We also asked if SAHA, reducing mutant p53 levels, might be affecting ATF6 processing and transcriptional functions; in line with data obtained with mutant p53 silencing, treatment of MB231 with SAHA significantly reduced ATF6f levels (Figure 9B), and transactivation of the ATF6-LUC reporter (Figure 9C).

Nelfinavir and SAHA act on two different cellular targets; we therefore tested the hypothesis that their combination could provide a valid strategy to increase cancer cell sensitivity to ER stress. To this aim, we treated MB231 cells with SAHA and NFV, alone or in combination, in presence or in absence of ER stress inducers. Viability assays confirmed that combination of the two compounds was more efficient in reducing cancer cell viability than treatment with single drugs - in particular after ER stress (Figure 10A). To reinforce these observations, we used another mutant p53-targeted drug, Cerivastatin (CER), a statin that was recently shown to inhibit mutant p53 stability and functions (Sorrentino et al., 2014). We then tested CER alone or in combination with NFV, using low concentrations to minimize toxicity and highlight potential cooperative effects. Under these conditions, combined treatment of MDA-MB-231 cells with NFV and CER recapitulated the results obtained with SAHA, additively reducing cell viability (Figure 10B). In order to evaluate the possible effects of these treatments on non-transformed cells, we performed the same experiments in MCF-10A breast epithelial cells. In contrast to what observed in MDA-MB231, treatment with CER (0,01uM) and/or NFV (12,5) did not reduce viability of MCF10A (Figure 10C,D), providing an important indication that the additive effects of ATF6 and mutant p53 inhibitors can be specific for cancer cells.

7. Figures

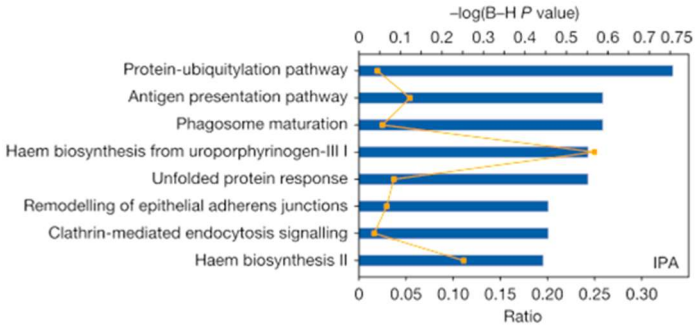

Figure 1. Unfolded Protein Response is one of the biological processes regulated by different missense p53 mutants in a panel of human breast cancer cell lines.

As described in Walerych et al., 2016, transcriptomic profiles of BT-549, HCC-1395, SUM-149PT, MDA-MB-468 and MDA-MB-231 cell lines before or after mutp53 depletion were analyzed to identify genes similarly regulated by different p53 mutants in different TNBC cell lines. Common mutant p53-regulated genes were analyzed for pathway association using IPA (Ingenuity Pathway Analysis – Qiagen). The graph summarizes the most enriched pathways. Bars: adjusted –log (P values) for pathway association. Lines: ratio of the number of genes found in the gene set to the total number of genes associated to each pathway (Walerych et al., 2016).

Figure 2

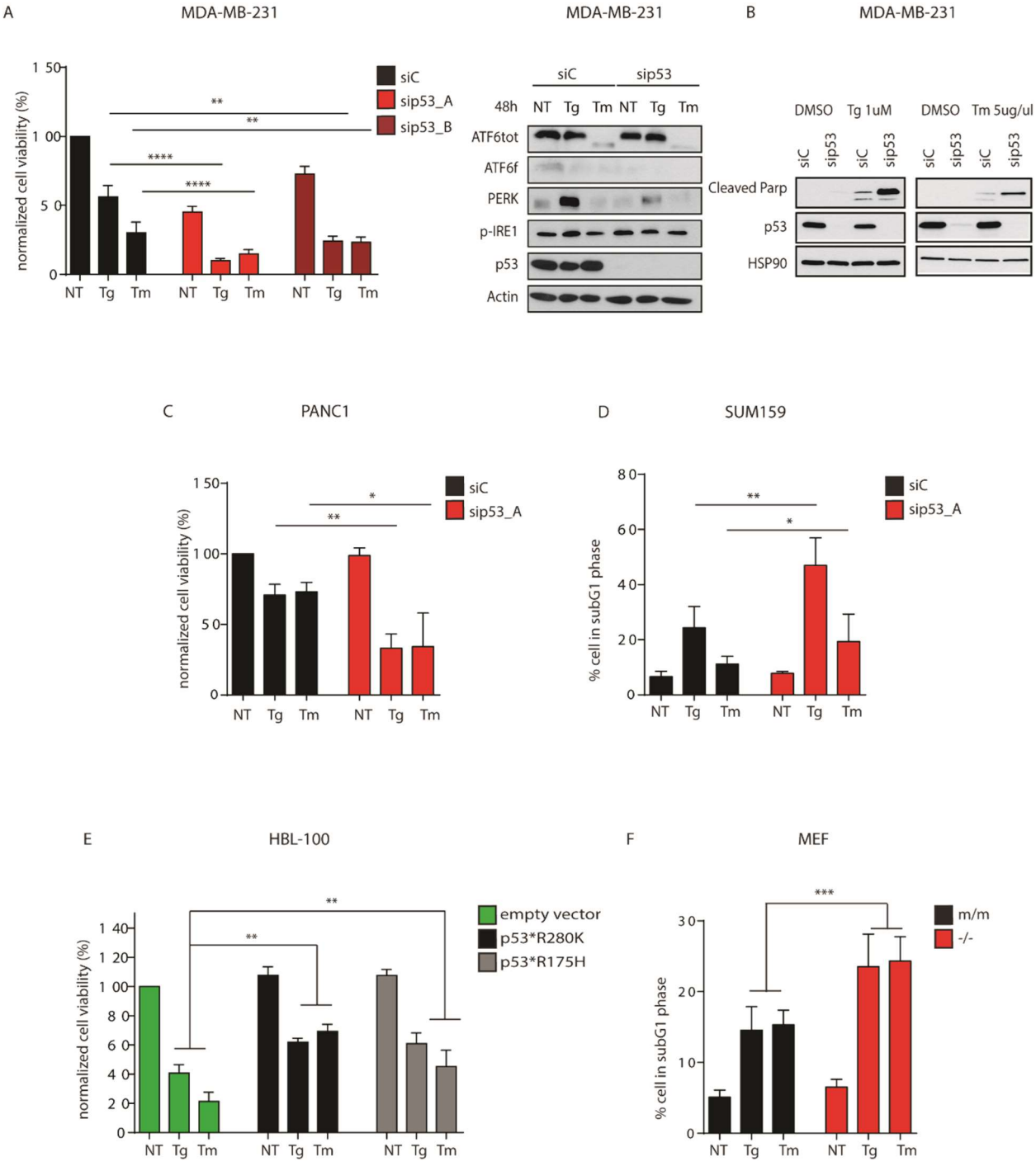
A
MDA-MB-231
normalized cell viability (%)
siC
sip53_A
sip53_B
NT Tg Tm NT Tg Tm NT Tg Tm
MDA-MB-231
siC sip53
48h NT Tg Tm NT Tg Tm
ATF6tot
ATF6f
PERK
p-IRE1
p53
Actin
B
MDA-MB-231
DMSO Tg 1uM DMSO Tm 5ug/ul
siC sip53 siC sip53 siC sip53 siC sip53
Cleaved Parp
p53
HSP90
C
PANC1
normalized cell viability (%)
siC
sip53_A
D
SUM159
% cell in subG1 phase
siC
sip53_A
E
HBL-100
normalized cell viability (%)
empty vector
p53*R280K
p53*R175H
F
MEF
% cell in subG1 phase
m/m
-/-

Figure 2. Mutant p53 sustains cancer cells survival upon ER stress.

A-B) Mutant p53 silencing dampens cancer cell viability and increases apoptosis. A) MDA-MB-231 cells were transfected with two different p53 siRNAs (sip53_A or sip53_B) or control (siC) siRNAs as indicated. 48 hours post transfection; cells were seeded in 96 well. After 24 hours, cells were treated with Thapsigargin (Tg) 1uM or Tunicamycin (Tm) 5ug/ml. After 48 hours, ATP-lite viability assays were performed. Graphs summarize cells viability normalized to untreated siC control (mean ± SD; n=3; ***P<0.001, **P<0.01). ATF6, PERK and p-IRE1 protein levels were analyzed by western blot as markers of Unfolded Protein Response activation. B) MDA-MB-231 were treated as in A, and analyzed by western blot to detect cleaved PARP as a marker of apoptosis. p53 knock-down was confirmed by immunoblotting; HSP90 was used as loading control.

C) PANC1 cells were transfected and treated as in A. Graphs summarize cells viability normalized to untreated siC control (mean ± SD; n=3; **P<0.01; *P<0.1).

D) SUM159 were transfected as in A and treated with Tg or Tm for 24 hours and then processed for FACS analysis. Graphs summarize percentage of cells in sub-G1 phase (mean ± SD; n=3; **P<0.01; *P<0.1).

E) Mutant p53 overexpression protects cells from ER stress. HBL100 cells were stably transduced with retroviruses encoding p53 (R280K) or p53 (R175H). ATPlite assays were performed as in Figure 2A (mean ± SD; n = 3; **p < 0.01).

F) Mouse Embryonic Fibroblasts derived from mutp53 knock-in mice (m/m) or p53 KO mice (-/-), were treated with Tg or Tm for 24 hours and then processed for FACS analysis (mean ± SD; n=3; ***P<0.001).

Figure 3

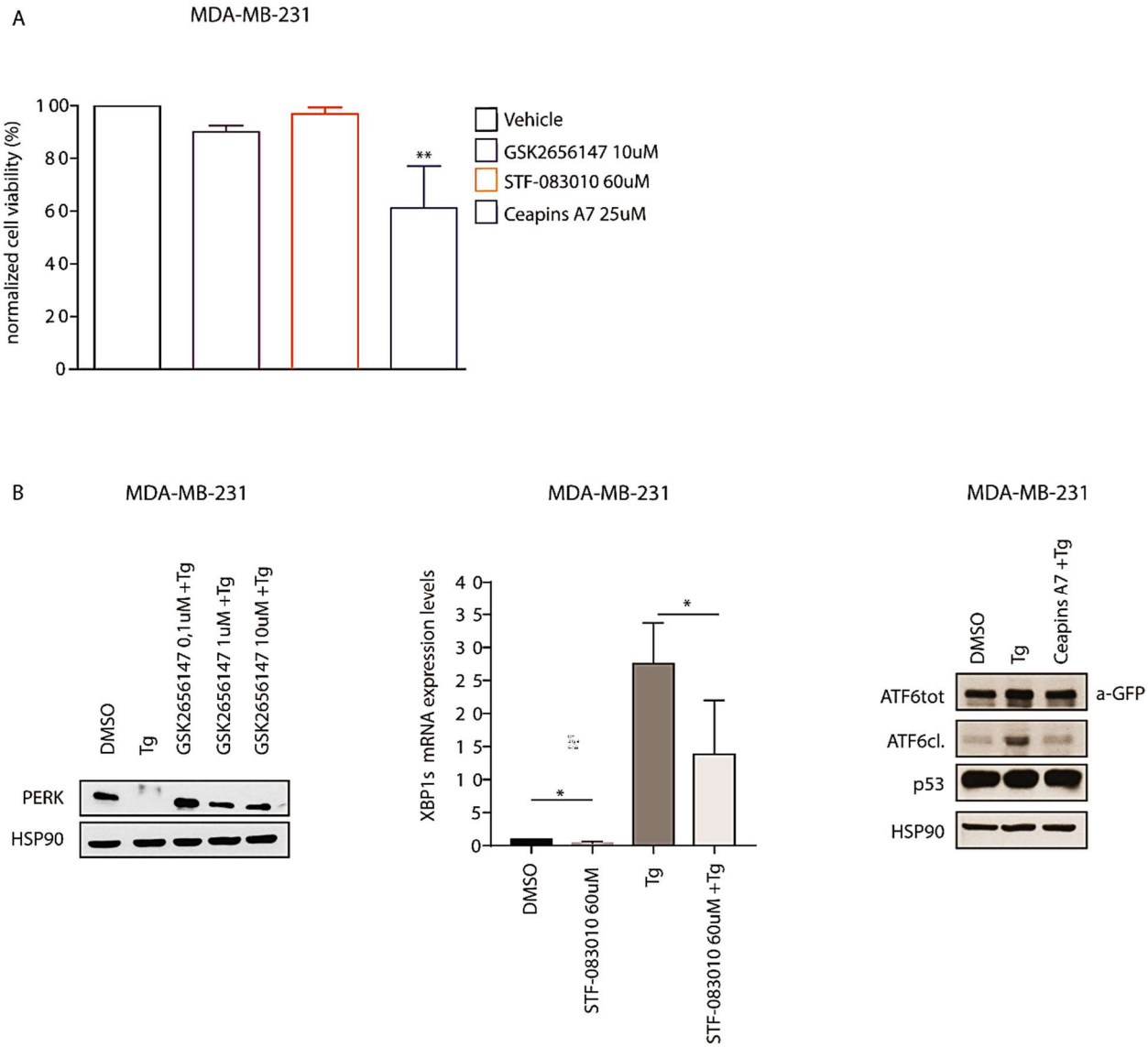
A
MDA-MB-231
normalized cell viability (%)
100
80
60
40
20
0
**
Vehicle
GSK2656147 10uM
STF-083010 60uM
Ceapins A7 25uM
B
MDA-MB-231
DMSO
Tg
GSK2656147 0,1uM +Tg
GSK2656147 1uM +Tg
GSK2656147 10uM +Tg
PERK
HSP90
MDA-MB-231
XBP1s mRNA expression levels
40
35
30
25
20
15
10
5
0
*
*
DMSO
STF-083010 60uM
Tg
STF-083010 60uM +Tg
MDA-MB-231
DMSO
Tg
Ceapins A7 +Tg
ATF6tot
a-GFP
ATF6cl.
p53
HSP90
C
MDA-MB-231
mRNA fold change sip53/siC
5
4
3
2
1
0
***
**
ns
PERK
IRE
ATF6
MDA-MB-231
siC
sip53
IRE1
PERK
p53
HSP90
siC
sip53
ATF6tot
ATF6f
p53
HSP90

Figure 3. Mutant p53 depletion has a different impact on UPR receptors.

A) Only ATF6 inhibition affects cancer cells viability. MB231 cells were treated with specific inhibitors: STF-083010 (IRE1i 60uM), GSK-2606414 (PERKi 10uM) or Ceapins_A7 (ATF6i 25uM). 48 hours post treatment ATP-lite assays were performed as in Figure 2A (mean ± SD; n=3; **P<0.01;).

B) Efficiency of inhibitors was verified in MDA-MB-231. Cells were treated for 48h with GSK-2606414, STF-083010 or Ceapins_A7 at different concentrations, and then treated with Tg for 4h. Phosphorylated PERK and ATF6 cleavage were measured by immunoblotting. Expression of XBP1s was measured by RT-qPCR after 8h of Tg 1uM (mean ± SD; n = 3; *p < 0.1).

c) Mutant p53 depletion increases IRE1 and PERK mRNA and protein levels, but decreases ATF6f. MDA-MB-231 cells were transfected with mutp53 (sip53) or control (siC) siRNAs. 48 hours post transfection; cells were harvested for RNA or protein extraction. RT-qPCR data are normalized to H3 gene, and compared to expression levels of untreated cells transfected with siC. Immunoblots are normalized by Hsp90.

Figure 4

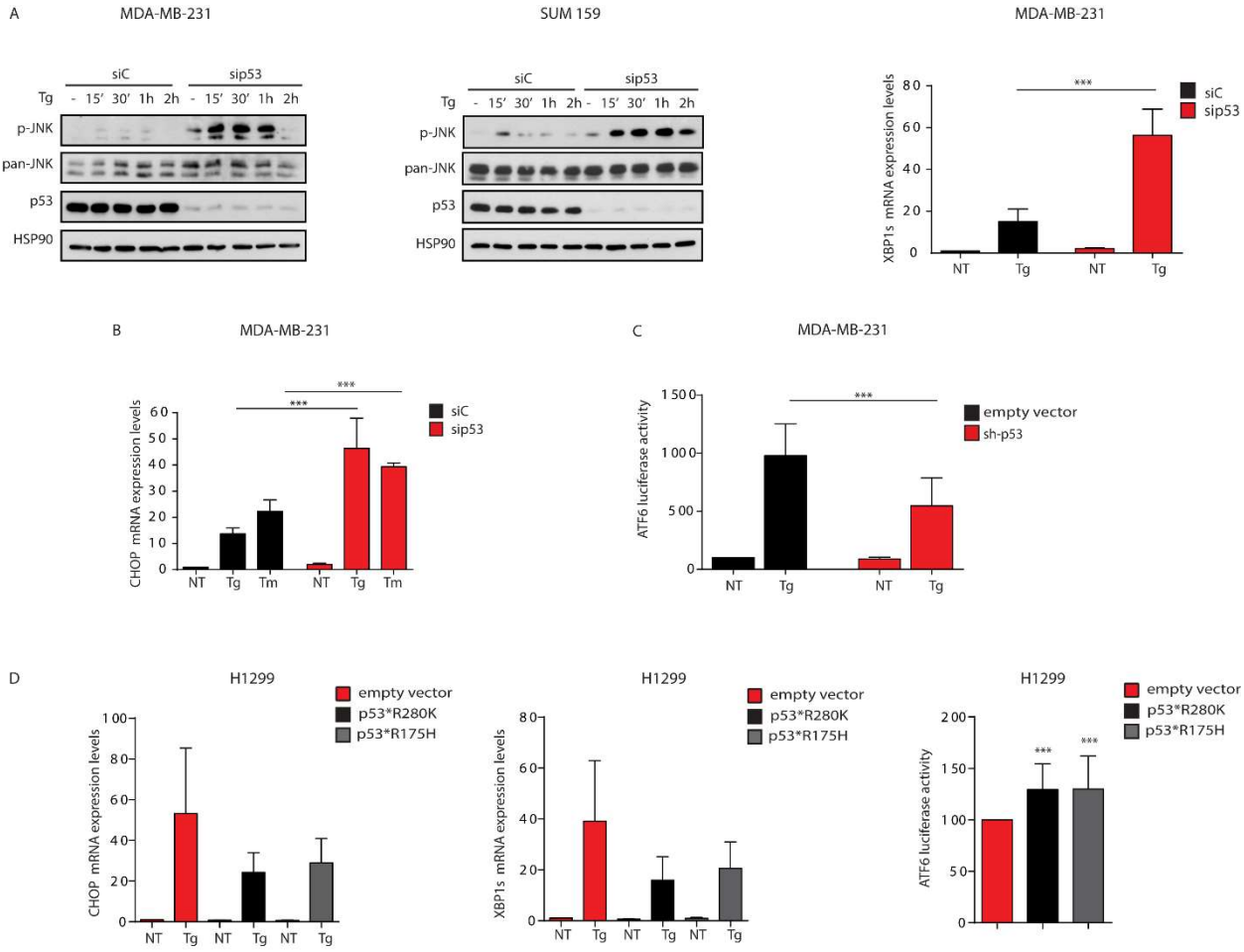
A
MDA-MB-231
siC
sip53
Tg - 15' 30' 1h 2h - 15' 30' 1h 2h
p-JNK
pan-JNK
p53
HSP90
SUM 159
MDA-MB-231
XBP1s mRNA expression levels
siC
sip53
NT Tg NT Tg
B
MDA-MB-231
CHOP mRNA expression levels
NT Tg Tm NT Tg Tm
C
MDA-MB-231
ATF6 luciferase activity
empty vector
sh-p53
D
H1299
CHOP mRNA expression levels
empty vector
p53*R280K
p53*R175H
H1299
XBP1s mRNA expression levels
H1299
ATF6 luciferase activity

Figure 4. Mutant p53 dampens IRE1a and PERK functions and sustains ATF6 transcriptional activity.

A) Mutant p53 depletion increases IRE1a activation. MDA-MB-231 were transfected with p53 (sip53) and control (siC) siRNAs. After 48 hours, cells were treated with Tg (1uM) for the indicated times. Phosphorylated and total JNK were measured by immunoblotting. Expression of XBP1s was measured by RT-qPCR after 8hr of Tg 1uM (mean ± SD; n = 3; ***p < 0.001).

B) Mutant p53 depletion increases PERK activation. Expression of CHOP mRNA was measured by RT-qPCR in MB231 cells treated exactly as in A (mean ± SD; n = 3; ***p < 0.001).

C) Mutant p53 depletion reduces ATF6 transcriptional activity. MDA-MB-23, stably infected with control (empty vector) and p53 (sh-p53) retroviral constructs, were transfected with p5xATF6-GL3 and pCMV-renilla constructs Transcriptional activity of ATF6f was measured by dual-luciferase assay in cells treated as in A (mean ± SD; n = 3; ***p < 0.001).

D) Mutant p53 overexpression decreases CHOP and XBP1s mRNA levels and promotes ATF6 luciferase activity. H1299 were stably infected with retroviruses encoding p53 (R280K) or p53 (R175H). RT-qPCR were performed as described in B and luciferase assays were performed as in C (mean ± SD; n = 3; ***p < 0.001).

Figure 5

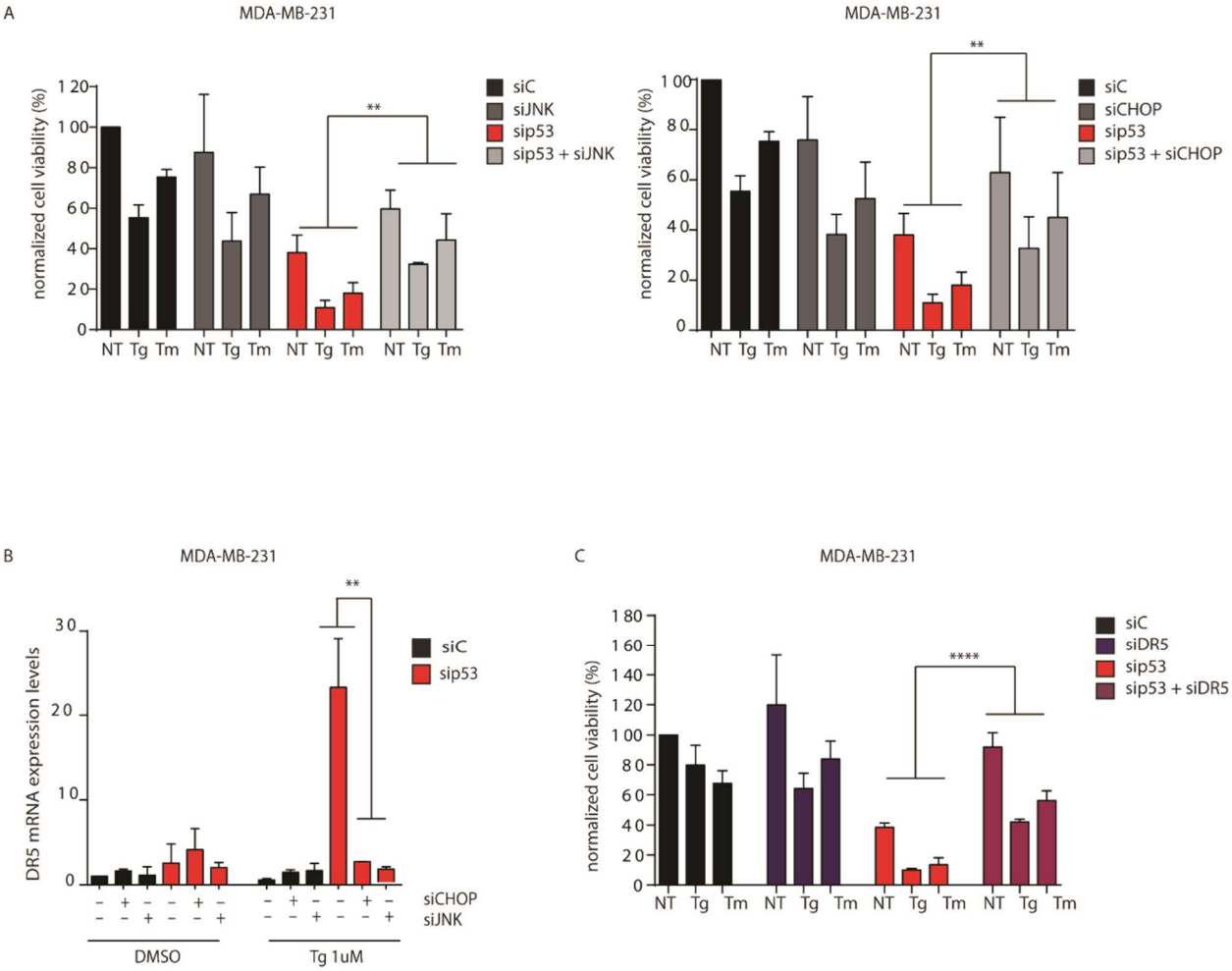
A
MDA-MB-231
normalized cell viability (%)
120
100
80
60
40
20
0
**
siC
siJNK
sip53
sip53 + siJNK
NT Tg Tm NT Tg Tm NT Tg Tm NT Tg Tm
MDA-MB-231
normalized cell viability (%)
100
80
60
40
20
0
**
siC
siCHOP
sip53
sip53 + siCHOP
NT Tg Tm NT Tg Tm NT Tg Tm NT Tg Tm
B
MDA-MB-231
DR5 mRNA expression levels
30
20
10
0
**
siC
sip53
siCHOP
siJNK
DMSO
Tg 1uM
C
MDA-MB-231
normalized cell viability (%)
180
160
140
120
100
80
60
40
20
0
****
siC
siDR5
sip53
sip53 + siDR5
NT Tg Tm NT Tg Tm NT Tg Tm NT Tg Tm

Figure 5. Both JNK and CHOP are required for enhanced ER stress-induced apoptosis after mutant p53 depletion.

A) JNK and CHOP are required for mutant p53 depletion-dependent ER stress induced apoptosis. MDA-MB-231 were transfected with JNK (siJNK), CHOP (siCHOP), p53 (sip53) or control (siC) siRNAs. After 48h, cells were harvested and treated as in Figure 2A. Viability was measured by ATPlite, and normalized to control untreated cells (mean ± SD; n = 3; **p < 0.01).

B) DR5 is a common target of CHOP and JNK upon ER stress. MDA-MB-231 were transfected with JNK (siJNK), CHOP (siCHOP), DR5 (siDR5), p53 (sp53) or control (siC) siRNAs as indicated. After 48hr, cells were treated and processed as in Figure 2A. DR5 expression levels were detected by RT-qPCR. Data are normalized to H3, and compared to untreated cells transfected with siC (mean ± SD; n = 3; **p < 0.01).

C) DR5 depletion reduces ER stress-induced apoptosis in mutp53 knockdown cells. MDA-MB-231 were transfected with DR5 (siDR5), p53 (sip53) or control (siC) siRNAs as indicated. After 48hr, cells were harvested and treated as in Figure 2A. Viability was measured by ATPlite (mean ± SD; n = 3; ****p < 0.0001).

Figure 6

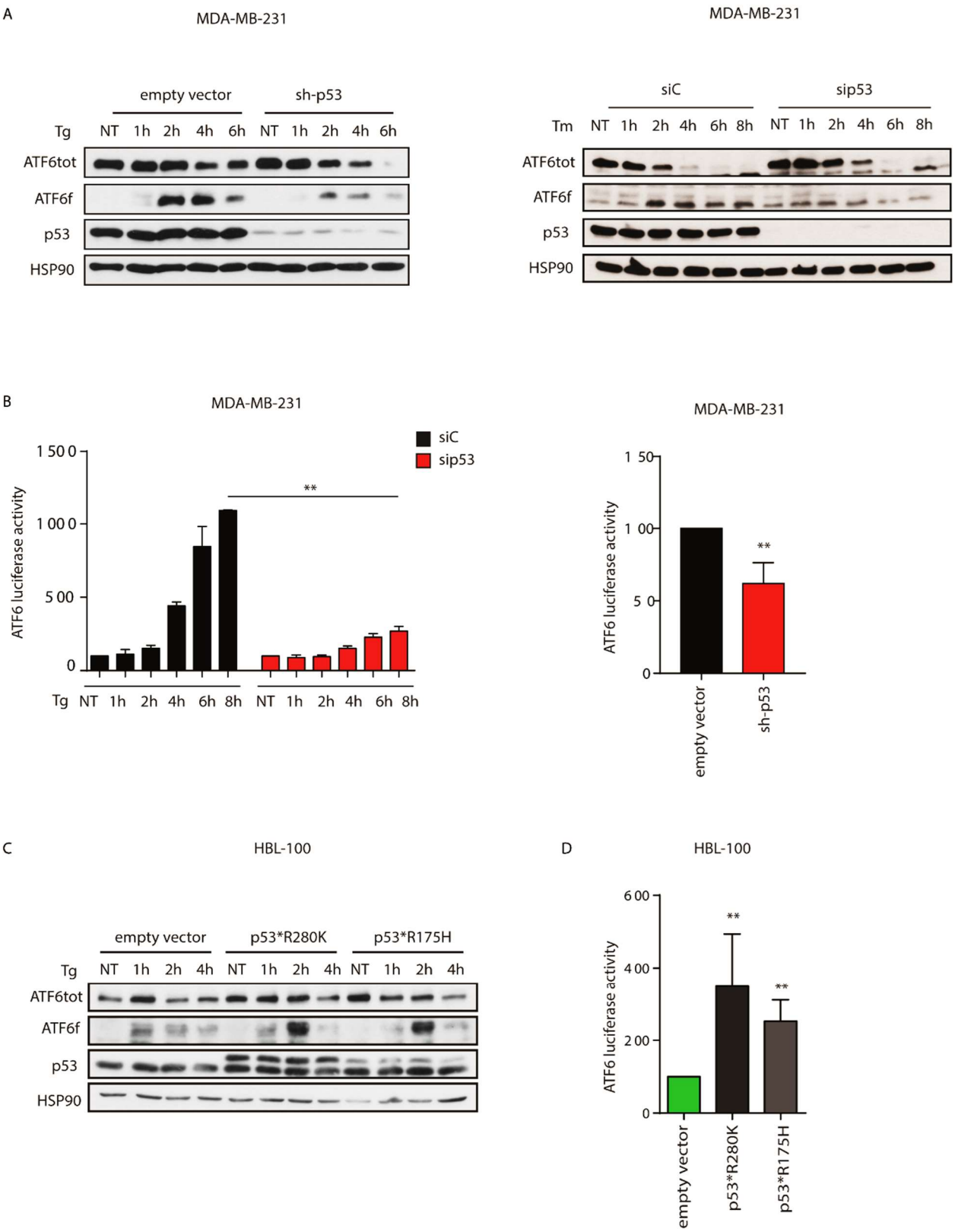
A
MDA-MB-231
empty vector
sh-p53
Tg NT 1h 2h 4h 6h NT 1h 2h 4h 6h
ATF6tot
ATF6f
p53
HSP90
MDA-MB-231
siC
sip53
Tm NT 1h 2h 4h 6h 8h NT 1h 2h 4h 6h 8h
ATF6tot
ATF6f
p53
HSP90
B
MDA-MB-231
siC
sip53
ATF6 luciferase activity
1 500
1 000
500
0
**
Tg NT 1h 2h 4h 6h 8h NT 1h 2h 4h 6h 8h
MDA-MB-231
ATF6 luciferase activity
1 50
1 00
5 0
0
**
empty vector
sh-p53
C
HBL-100
empty vector
p53*R280K
p53*R175H
Tg NT 1h 2h 4h NT 1h 2h 4h NT 1h 2h 4h
ATF6tot
ATF6f
p53
HSP90
D
HBL-100
ATF6 luciferase activity
600
400
200
0
**
**
empty vector
p53*R280K
p53*R175H

Figure 6. Mutant p53 sustains ATF6 cleavage and transcriptional activity.

A) Mutant p53 depletion reduces ATF6 cleavage. MDA-MB-231 cells were silenced for mutp53 using transient siRNA transfection (sip53 vs siC) or stable shRNA expression (sh-p53 vs empty vector). Cells were treated with Tg (1uM) for the indicated times. Western blot experiments were performed to detect total (ATF6 tot) and cleaved (ATF6f) forms of ATF6.

B) Mutant p53 depletion reduces ATF6-dependent transactivation. MDA-MB-231 cells, stably transfected with p53 (sh-p53) or control (empty vector) shRNA constructs, were transfected with the p5XATF6-GL3 reporter to detect ATF6 transcriptional activity. After 48hr, cells were treated with or without Tg (1uM) for the indicated times before processing for dual Luciferase assays (mean ± SD; n = 3; **p < 0.01).

C) Mutant p53 overexpression increases ATF6 cleavage. HBL100 cells were transduced with retroviruses encoding mutp53(R280K) or mutp53(R175H). After 48hr, cells were treated with Tg (1uM) for the indicated times. Western blot experiments were performed to detect endogenous total (ATF6 tot) and cleaved (ATF6 f) forms of ATF6.

D) ATF6 transcriptional activity is sustained in presence of mutant p53 in basal conditions. The indicated cell lines, stably infected with retroviruses encoding p53 (R280K) or p53(R175H), were transiently transfected with p5XATF6-GL3 to detect ATF6 transcriptional activity by dual-Luciferase assays (mean ± SD; n = 3; ***p < 0.001;**p < 0.01).

Figure 7

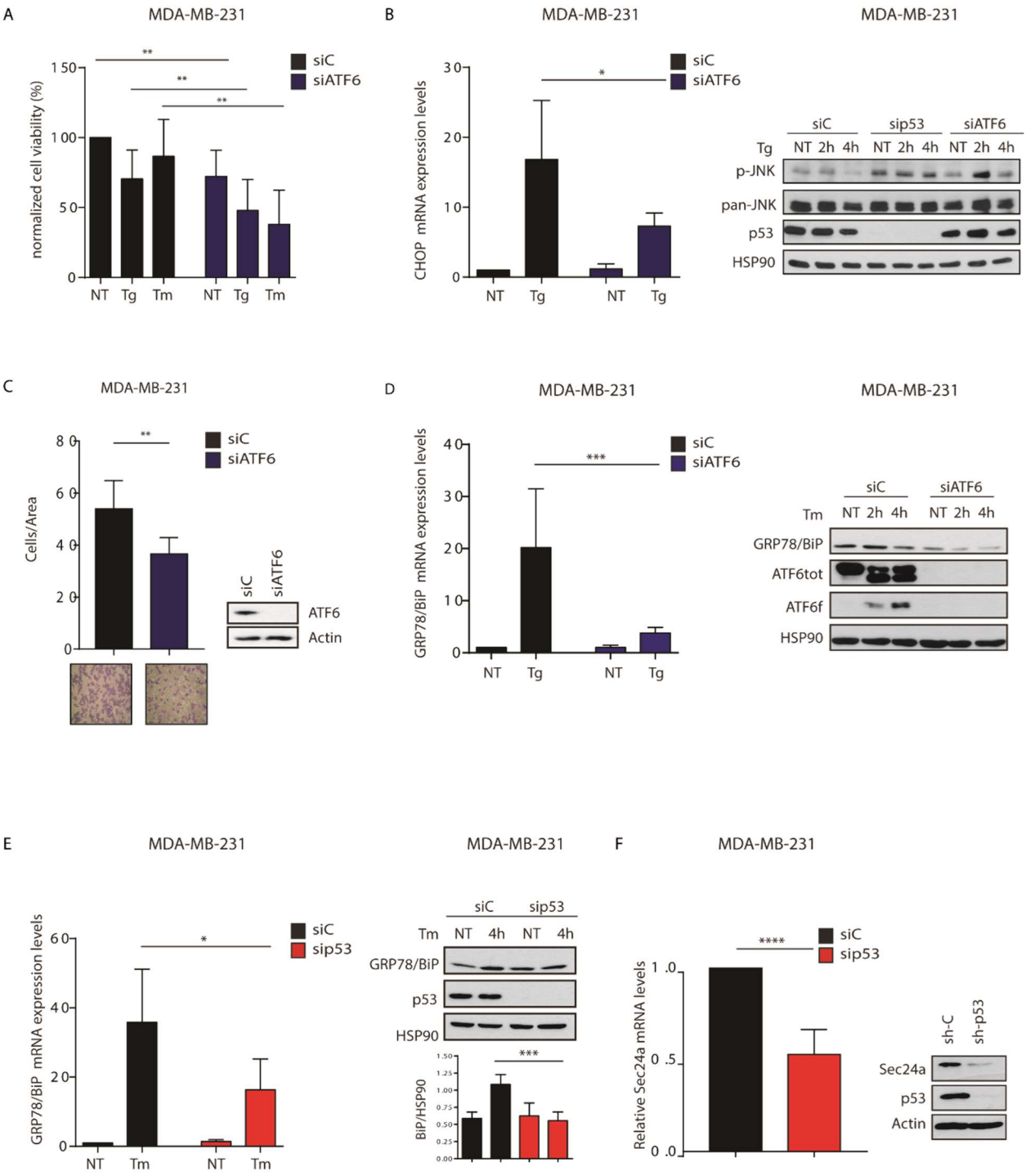
A
MDA-MB-231
normalized cell viability (%)
siC
siATF6
NT Tg Tm NT Tg Tm
B
MDA-MB-231
CHOP mRNA expression levels
siC
siATF6
NT Tg NT Tg
MDA-MB-231
siC sip53 siATF6
Tg NT 2h 4h NT 2h 4h NT 2h 4h
p-JNK
pan-JNK
p53
HSP90
C
MDA-MB-231
Cells/Area
siC
siATF6
ATF6
Actin
D
MDA-MB-231
GRP78/BiP mRNA expression levels
siC
siATF6
NT Tg NT Tg
MDA-MB-231
siC siATF6
Tm NT 2h 4h NT 2h 4h
GRP78/BiP
ATF6tot
ATF6f
HSP90
E
MDA-MB-231
GRP78/BiP mRNA expression levels
siC
sip53
NT Tm NT Tm
MDA-MB-231
siC sip53
Tm NT 4h NT 4h
GRP78/BiP
p53
HSP90
BiP/HSP90
F
MDA-MB-231
Relative Sec24a mRNA levels
siC
sip53
sh-C sh-p53
Sec24a
p53
Actin

Figure 7. Inhibition of ATF6 activity reduces cancer cells viability and invasion.

A) ATF6 depletion reduces cell viability upon ER stress. MDA-MB-231 cells were transfected with ATF6 (siATF6) or control (siC) siRNAs. Cells were treated as in Figure 2A, with viability measured by ATP-lite assays (mean ± SD; n = 3; **p < 0.01).

B) ATF6 depletion affects CHOP and JNK activation by ER stress. MDA-MB-231 were transfected as in A and treated with Tg for the indicated times. CHOP mRNA levels were measured by RT-qPCR. Data are normalized to H3, and compared to untreated cells transfected with siC (mean ± SD; n = 3; *p < 0.01). JNK phosphorylation and total protein levels were detected by immunoblotting under the same conditions. P53 knockdown was confirmed by western blot. HSP90 was blotted as loading control.

C) ATF6 depletion reduces cancer cells invasion. MDA-MB-231 were transfected as in A and cell invasion was measured by matrigel transwell assays. Graphs summarize migrated cells per area (mean ± SEM; n=3; ** P<0.01, *** P<0.001). Depletion of endogenous p53 was checked by western blot. Representative images of migrated cells, fixed and stained with Crystal Violet are also shown (bottom).

D) GRP78/BiP is a transcriptional target of ATF6. Cells were transfected with ATF6 (siATF6) or control (siC) siRNAs, and treated with Tg or Tm for the indicated times. GRP78 mRNA induction upon Tg treatment was measured by RT-qPCR as in A (mean ± SD; n = 3; ***p < 0.001). GRP78 protein induction upon Tm treatment was analyzed by immunoblotting. ATF6 was blotted to confirm efficient depletion. HSP90 was blotted as loading control.

E) GRP78/BiP induction upon ER stress is reduced in mutant p53-depleted cells. Cells were transfected with p53 (sip53) or control (siC) siRNAs, and treated with Tm for 4h. GRP78 mRNA induction upon Tg treatment was measured by RT-qPCR (mean ± SD; n = 3; *p < 0.05). GRP78 protein induction upon Tm treatment was analyzed by immunoblotting. P53 was blotted to confirm efficient knockdown. Bottom: the ratio of GRP78 levels compared to HSP90 was quantified by densitometry on autoradiography film (mean ± SD; n = 3; ***p < 0.001).

F) Sec24a expression is reduced upon mutant p53 knockdown. MDA-MB-231 cells were silenced for mutant p53 using transient siRNA transfection (sip53 vs siC) and Sec24a mRNA levels were detected by RT-qPCR (mean ± SD; n = 6; ****p < 0.0001). Western blot experiments were performed to detect sec24a protein in MDA-MB-231 stably depleted of mutant p53 by expression of a specific shRNA.

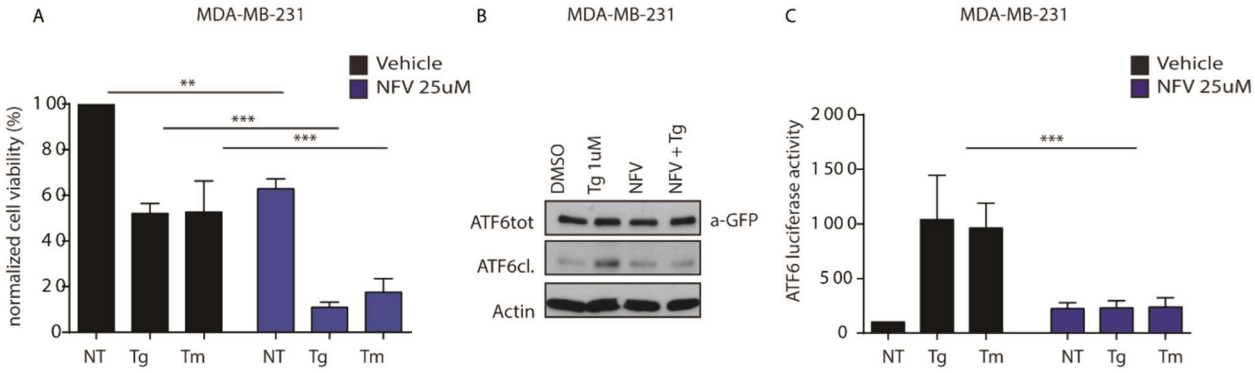

Figure 8. ATF6 inhibition reduces cell viability and increases sensitivity to ER stress.

A) Nelfinavir (NFV) treatment reduces cell survival upon ER stress. MDA-MB-231 cells were treated with Tg (1uM), Tm (5ug/ml) or DMSO (vehicle), with or without NFV (25uM). After 48hr cell viability was measured by ATPlite assays (mean ± SD; n = 3; ***p < 0.001 **p < 0.01).

B) NFV inhibits ATF6 cleavage. MB231 cells were transfected with peGFP-ATF6 for 48h, and treated with DMSO or NFV for additional 24 h. ATF6 cleavage was detected by GFP immunoblotting after 8 hours of Tg treatment.

C) NFV inhibits ATF6 transcriptional activity. MB231 cells were transfected with p5xATF6-GL3 for 48hr, and pre-treated with NFV for additional 24hr. ATF6f transcriptional activity was measured by dual-Luciferase assay after 8 hours of Tg or Tm treatment (mean ± SD; n = 3; ***p < 0.001).

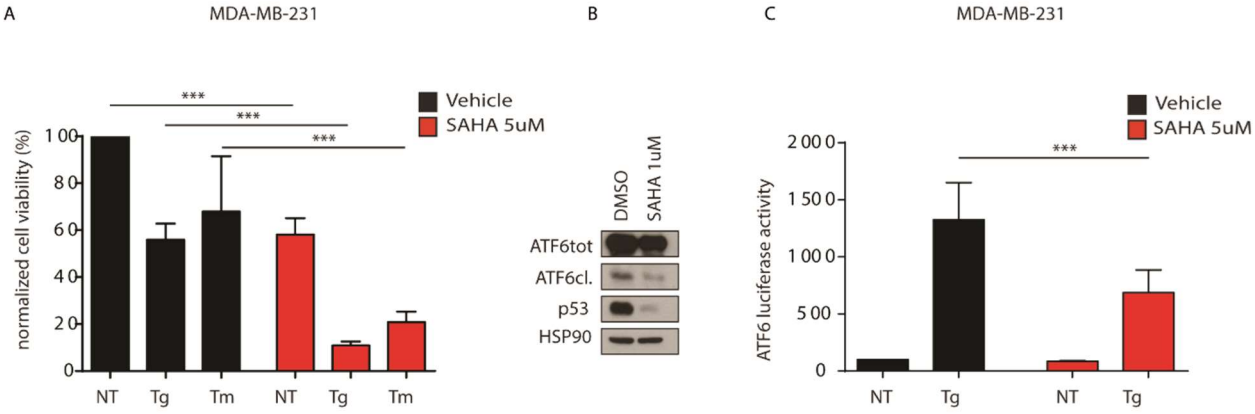

Figure 9. Pharmacological inhibition of mutant p53 increases sensitivity to ER stress.

A) SAHA treatment reduces cell survival upon ER stress. MDA-MB-231 cells were treated with Tg (1uM), Tm (5ug/ml) or DMSO (vehicle), with or without SAHA (5uM). After 48hr cell viability was measured by ATPlite assays (mean ± SD; n = 3; ***p < 0.001).

B) SAHA reduces ATF6 cleavage. Endogenous ATF6 isoforms and mutp53 protein were detected by immunoblotting in the absence of drugs.

C) SAHA inhibits ATF6 transcriptional activity. MB231 cells were transfected with p5xATF6-GL3 for 48hr, and pre-treated with SAHA for additional 24hr. ATF6f transcriptional activity was measured by dual-Luciferase assay after 8 hours of Tg treatment (mean ± SD; n = 3; ***p < 0.001).

Figure 10

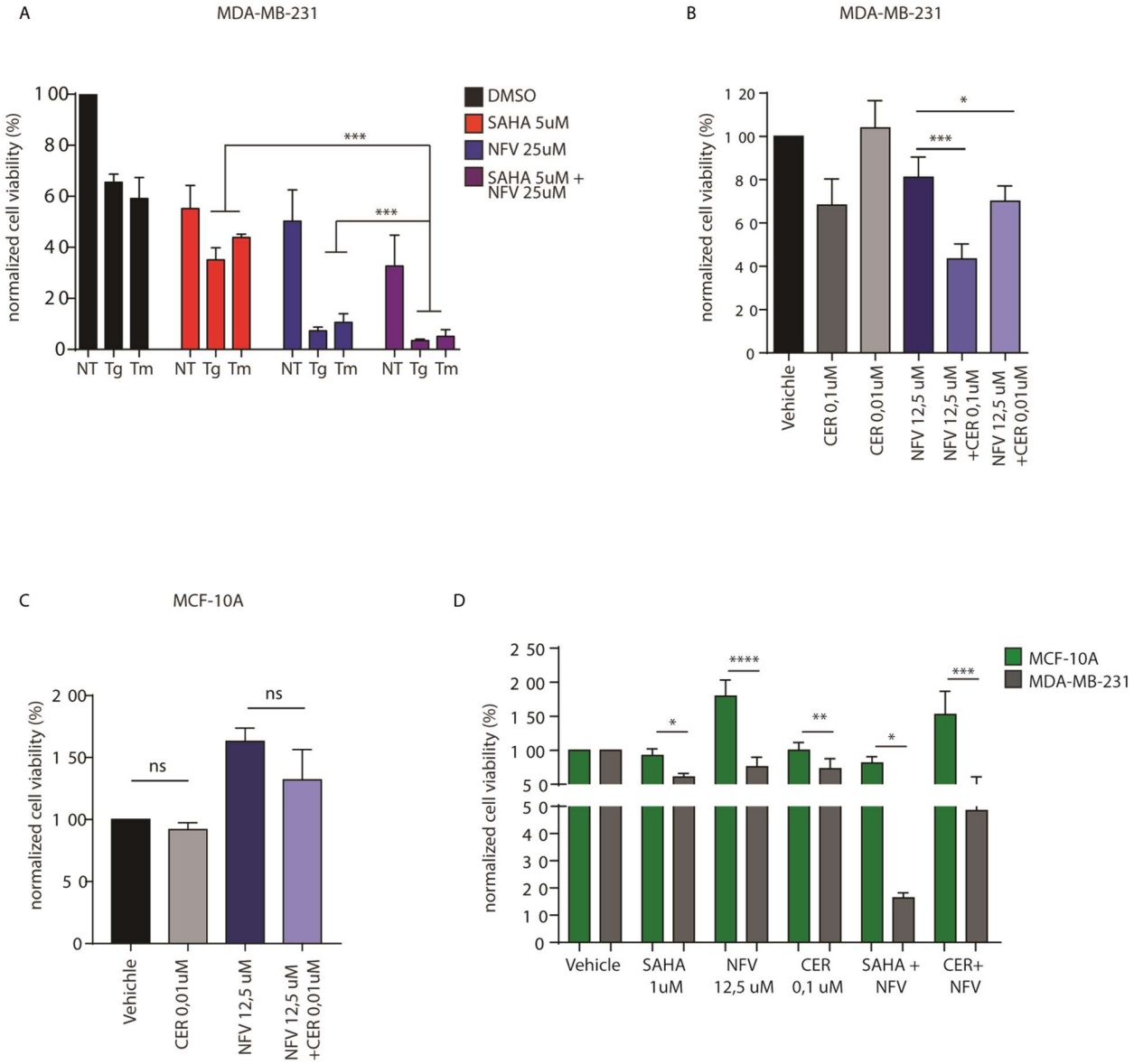
A
MDA-MB-231
normalized cell viability (%)
1 00
80
60
40
20
0
NT Tg Tm NT Tg Tm NT Tg Tm NT Tg Tm
***
***
DMSO
SAHA 5uM
NFV 25uM
SAHA 5uM +
NFV 25uM
B
MDA-MB-231
normalized cell viability (%)
1 20
1 00
8 0
6 0
4 0
2 0
0
*
***
Vehichle
CER 0,1uM
CER 0,01uM
NFV 12,5 uM
NFV 12,5 uM
+CER 0,1uM
NFV 12,5 uM
+CER 0,01uM
C
MCF-10A
normalized cell viability (%)
2 00
1 50
1 00
5 0
0
ns
ns
Vehichle
CER 0,01uM
NFV 12,5 uM
NFV 12,5 uM
+CER 0,01uM
D
normalized cell viability (%)
2 50
2 00
1 50
1 00
5 0
5 0
4 0
3 0
2 0
1 0
0
****
*
**
*
***
MCF-10A
MDA-MB-231
Vehicle
SAHA
1uM
NFV
12,5 uM
CER
0,1 uM
SAHA +
NFV
CER+
NFV

Figure 10. Mutant p53 and ATF6 inhibitors additively sensitize MDA-MB231 cells to ER stress.

A) Additive effect of SAHA and Nelfinavir (NFV) in reducing cancer cells survival upon ER stress. MDA-MB-231 cells were treated for 48 hours with SAHA (5uM), NFV (25uM), Tg (1uM), Tm (5ug/ml) or DMSO (vehicle) as indicated. Cell viability was measured by ATP-lite assays (mean ± SD; n = 3; ***p < 0.001).

B) Additive effect of Cerivastatin (CER) and NFV. MDA-MB-231 cells were treated for 48 hours with CER (0,1uM or 0,01uM), NFV (25uM), or DMSO (vehicle) as indicated. Cells viability was measured by ATP-lite assays (mean ± SD; n = 3; ***p < 0.001).

C) Viability of non-transformed cells is not reduced by treatment with ATF6 and mutp53 inhibitors. MCF10A were treated as in B, and cell viability was measured by ATP-lite assays.

D) The additive effect of Cerivastatin and Nelfinavir is observed in mutp53-bearing MDA-MB-231, but not in wtp53-bearing MCF10A. Cells were treated as in B, and viability was measured by ATP-lite assays.

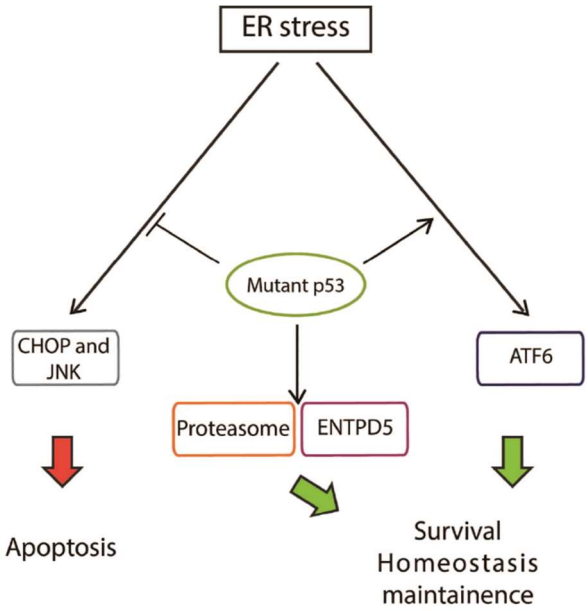

Figure 11. Working model

Mutant p53 protects cells from ER stress-induced apoptosis by acting at multiple levels. It has been shown that mutp53 enhances expression of proteasome subunits, potentially alleviating proteotoxic stress (Walerych et al., 2016). It has also been reported that mutp53 controls expression of ENTPD5, an enzyme important for N-linked glycosylation and quality control of secretory and membrane proteins [2]. In this Thesis, we find that mutp53 acts on multiple branches of the UPR. Specifically, pro-apoptotic effectors such as CHOP and JNK are dampened in presence of mutant p53, thus reducing cell death. The pro-survival effector ATF6 is activated by the presence of mutant p53, favoring ER homeostasis and promoting adaptation to stress.

6. Discussion

In normal and healthy tissue, in response to accumulation of unfolded proteins, cells react by activating a transcriptional program known as Unfolded Protein Response (UPR), which first aims at restoring homeostasis and resolving stress. However, when stress levels exceed the capability of the cells to recover normal conditions, cells undergo apoptosis. In the tumor context, instead, cancer cells exposed to the same stress frequently do not activate apoptosis. On the contrary, they are more prone to resist, expressing pro-survival effectors and inhibiting pro-apoptotic signaling, thus sustaining neoplastic transformation.

The biological role of the UPR in oncogenesis, cancer development, and resistance to chemotherapies is well established (Clarke et al., 2014); however, the specific role of the three main UPR sensors, IRE1, PERK, and ATF6, is not so clear. Depending on the duration and persistence of stress, all these three receptors could activate both pro-survival and pro-apoptotic outcomes. In this context, genetic mutations that support the pro-survival homeostatic functions of the UPR are likely to contribute to cancer development and progression – and may also become candidate targets for therapy.

It has been convincingly demonstrated that mutant p53 (mutp53) can act on different crucial processes associated with cancer cells' homeostasis: for instance, by promoting transcription of proteasome subunits, or by enhancing ENTDP5 expression, mutp53 has effects on the protein degradation and modification machineries, ameliorating the protein landscape of cancer cells (Vogiatzi et al., 2016; Walerych et al., 2016). Similarly, by binding and inhibiting DAB2IP, mutant p53 is able to enhance activation of survival and metastatic pathways AKT and NF-kB in response to inflammatory cytokines or hyperinsulinemia (Di Minin et al., 2014; Valentino et al., 2017). In the nucleus, mutant p53 is able to boost basic properties of transcription factors, such as NF-Y, SREBP1 and 2 or ETS2, leading to aberrant activation of their downstream programs and intersection with other key oncogenic pathways such as YAP/TAZ (Huang et al., 2013; Martinez, 2016; Sorrentino et al., 2014). Finally, mutant p53 can significantly reprogram the metabolism of cancer cells (Zhou et al., 2014), supporting adaptation to the tumor microenvironment.

Interestingly, analysis of TNBC cell lines with different p53 mutations indicated the Unfolded Protein Response as a biological process affected by mutp53 in all lines (Fig 1;Walerych et al., 2016). This suggested that mutant p53 may exert its oncogenic functions, at least in part, by controlling protein folding and by inhibiting UPR-related pro-apoptotic factors, in order to sustain cancer cell survival under ER stress conditions.

In this Thesis, using metastatic cell lines as model, and inducing ER stress with two different drugs (Tm, tunicamycin and Tg, thapsigargin), we uncovered an involvement of mutant p53 in the regulation of UPR. Specifically, we demonstrated that mutant p53 promotes cancer cells survival upon ER stress, and that mutant p53 depletion dramatically sensitizes cells to ER stress-induced apoptosis.

We also explored the mechanisms by which cells depleted for mutant p53 become more sensitive to Tg and Tm treatments, and found that mutant p53 acts by inhibiting IRE1 and PERK activities, while it sustains ATF6 functions.

Notably, IRE1 and PERK control two of the major pro-apoptotic effectors of ER stress, JNK and CHOP, so mutant p53, by dampening activation of these receptors, can inhibit apoptosis. In fact, we found that induction of DR5, a common target of JNK and CHOP, is important for ER stress-induced apoptosis upon depletion of mutant p53. Our data confirm that mutant p53 can inhibit IRE1 and PERK to alleviate pro-apoptotic responses to ER stress.

The mechanism by which mutp53 dampens IRE1- and PERK-dependent pro-apoptotic responses remains to be defined. We observed that mutp53 depletion increases IRE1 and PERK mRNA and protein levels, suggesting that mutant p53 inhibits expression of these receptors. This might in part contribute to their reduced activity. However, it is plausible that other actions of mutant p53 could be involved in CHOP and JNK inhibition. For instance, mutant p53 interaction with DAB2IP – a positive modulator of ASK1 activity – might indirectly impinge on IRE1-mediated JNK activation. In fact, DAB2IP has been reported to interact with IRE1 and mediate TRAF2 recruitment and ASK1/JNK signaling upon ER stress (Luo et al., 2008). In this respect, preliminary experiments indicate that Tg-induced early JNK phosphorylation is indeed dampened upon co-depletion of DAB2IP and mutp53. However, we see no reduction in apoptosis after longer treatments (data not shown). These findings may suggest that DAB2IP is important for IRE1/TRAF2/ASK1-dependent JNK phosphorylation during early steps of ER stress, but other apoptotic mechanisms are activated upon prolonged drug treatments. Additional experiments will be required to better define these results.

Another possibility, is that cytoplasmic mutant p53, which accumulates abundantly in cancer cells, might directly interact with UPR sensors such as IRE1 and/or PERK, or their downstream effectors, modulating their functions.

Finally, it is possible that mutant p53 indirectly dampens activation of pro-apoptotic UPR effectors, by sustaining the adaptive, homeostatic, and pro-survival activities of ATF6 – de facto

reducing ER stress. Indeed, an intriguing observation emerging from loss of function and gain of function experiments described in this Thesis is the positive action of mutp53 in sustaining ATF6 processing and transcriptional activity.

ATF6 protects cells from chronic stress by activating multiple transcriptional targets (Adachi et al., 2008; Wu et al., 2007), and we observed that mutant p53 depletion reduced ATF6 transcriptional activity. Strikingly, ATF6 depletion – or inhibition – reduced viability and invasion of cancer cells. In particular, ATF6 depletion in one hand induced JNK phosphorylation and in the other reduced mRNA and protein levels of the ER chaperone GRP78/BiP (official gene symbol HSPA5), a key UPR regulator that maintains ER homeostasis, suppresses stress-induced apoptosis, and is significantly elevated in malignant cells (Yao et al., 2015). Moreover, we observed a reduction in GRP78/BiP mRNA and protein also upon depletion of mutant p53 in our cancer cell line model, thus suggesting that mutp53 might increase BiP expression via ATF6.

Regarding the mechanism by which mutant p53 sustains ATF6 activity, we found that mutant p53 depletion reduces ATF6 processing to ATF6f, both in basal conditions and upon ER tress. This correlates with impaired transactivation of an ATF6-specific reporter and ATF6 target genes such as GRP78, suggesting that mutant p53 expression in cancer cells sustains a constant condition of ATF6 activation. We hypothesize that mutant p53, by sustaining ATF6, can maintain the status of chronic ER stress under the threshold of toxicity, thus protecting cancer cells from apoptosis.

The biochemical mechanism by which mutp53 promotes ATF6 processing and transcriptional activity remains to be defined. One intriguing possibility is that mutant p53 controls ATF6 transport from the ER to the Golgi. ATF6 needs to be bound by COPII vesicles components sec23 and sec24 to be translocated to the Golgi apparatus (Schindler and Schekman, 2009), so we tested expression of sec24 upon mutp35 depletion. Notably, preliminary results shown a reduction in sec24 mRNA and protein levels in the absence of mutant p53, supporting the hypothesis that mutp53 might be facilitating ATF6 translocation to the Golgi by upregulating components of COPII vesicles. However, we cannot exclude that other mechanisms are involved in this process. For instance, mutant p53 could affect the glycosylation pattern of ATF6, since it was demonstrated that glycosylation is crucial for ATF6 ER-to-Golgi trafficking (Hong et al., 2004). Similarly, it is possible that mutant p53 might increase ER-to-Golgi vesicular trafficking via some indirect mechanism, thus promoting a constant delivery of ATF6 to the cis-Golgi even in the absence of acute ER stress. Clearly, all these hypotheses await experimental confirmation.

Another interesting question regards the possibility that mutant p53-dependent activation of ATF6 might induce a specific gene expression profile, with preferential transcription of pro-survival ATF6 target genes. In fact, current evidence indicates that mutant p53 does not bind directly to DNA, but rather interacts with several transcription factors to modify their transactivation program. It would be very interesting to test whether ATF6-f binding to target promoters is affected by the presence of mutp53, or even if mutp53 might interact with ATF6 to sustain transcription of specific target genes. The potential identification of target genes regulated by mutant p53 and ATF6, could also give an advantage in better understanding the oncogenic role of ATF6.

The complex interconnections between the three branches of UPR, including the fact that several target genes are regulated by the different UPR branches, makes it difficult to define whether ATF6 is indeed the key mediator of mutant p53 gain-of-functions in this context. However, we wanted to use these novel discoveries to set a strategy for cancer therapy. The idea is to attack tumors by targeting several different pro-survival pathways which are used by cancer cells to resist to stress during tumor progression.

In this perspective, ATF6 is an appealing candidate for cancer treatment because of its protective role from ER stress, a condition that is chronic in most tumors. ATF6 is required for full induction of genes that preserve the protein processing capacity of the ER, and ATF6-depleted cells are more sensitive to long term stress (Wu et al., 2007). Our data suggest that mutant p53 sustains ATF6 activation to augment the protective functions of UPR, so mutp53-bearing cancer cells might be particularly sensitive to ATF6 inhibition.

In fact, the combination of drugs directly targeting mutant p53 with drugs inhibiting mutant p53-related pathways (ATF6 or proteasome) is emerging as a promising strategy, as it might favor the decrease of compensatory responses and dosage toxicity, and thus an increase in therapeutic efficacy. Additional experiments will have to be performed to verify the additive effect of these drugs, and test whether they may impact on cancer cell migration and invasion capacities, eventually also in in vivo models.

Despite the recent availability of a highly specific ATF6 inhibitor Ceapin-A7 (Gallagher and Walter, 2016), we have chosen to use Nelfinavir in these experiments, since NFV is already approved for clinical use. However, we are aware that NFV acts non-specifically and inhibits various pathways in addition to ATF6, so these results need to be carefully considered.

Development of less toxic and more bio-available versions of Ceapins is currently ongoing at the Walter laboratory (UCSF), and amelioration of these molecules may represent an optimal strategy to improve specificity of treatments.

In conclusion, TP53 is one of the most frequently mutated genes in human cancer and is emerging as a major oncoprotein which controls a multitude of tumor-promoting activities. Therefore, not only mutant p53 itself but also its many downstream pathways and cooperators, represent important potential drug targets. We have uncovered a novel functional axis that links mutant p53 to the UPR, and may contribute to protect cancer cells from basal or therapy-induced ER stress. Targeting this axis could provide an additional opportunity for treatment.

7. Experimental Procedures

Cell Culture. H1299 were cultured in RPMI medium (Sigma) supplemented with 10% FBS (ECS0180L, Euroclone), and antibiotics (ECB3001D, Euroclone). MDA-MB-231, MEF and HBL-100 were cultured in DMEM medium (Sigma) supplemented with 10% FBS, and antibiotics. SUM-159PT cells were maintained in DMEM:F12 Ham's medium 1:1, supplemented with 10% FBS. All human cell lines were subjected to STR genotyping with PowerPlex 18D System and confirmed in their identity comparing the results to reference cell databases (DMSZ, ATCC, and JCRB databases). MEFs were generated by crossing mice of the appropriate genotype, and collecting cells from 13.5 d.p.c. embryos. MEF p53KO and MEF KI p53R172H were immortalized through retroviral transduction of H-RasV12 (Girardini et al., 2011).

*Transfections, plasmids and siRNAs***.** Plasmid transfections were performed using Lipofectamine® LTX with Plus™ Reagent (ThermoFisher), following manufacturer's instructions. 24 hours post transfection, cells were washed with PBS for two times and new fresh culture medium was added. p5xATF6-GL3 and peGFP-ATF6 were purchased from Addgene (#11976, #32955). pLPC-p53R175H and pLPC-p53R280K were obtained by cloning respectively p53R175H and p53R280K coding region into pLPC empty vector. pLPCp53*R175H and -p53*R280K were generated introducing silent mutations in the region targeted by p53 siRNA by site directed mutagenesis in pLPC-p53R175H and -p53R280K respectively. pSR-shp53 plasmid was generated by cloning in the pRS retroviral vector a double stranded oligo corresponding to sip53-ORF (described above).
For siRNA transfections, cells were plated and transfected the day after with 50 nM siRNA oligonucleotides using Lipofectamine RNAiMax (Invitrogen), following manufacturer's instructions. After 48 hours of silencing, cells were processed.
siRNAs, used in this Thesis are listed in the following table:

siRNA	Sequence	Purchase from/ References
Control siRNA (siC)	Unknown	All star negative control (1027281, Qiagen)
SiRNA p53	GACUCCAGUGGUAAUCUAC	Eurofins MWG
siRNA ATF6 (smart pool)	1.CAUGAGAAAUGUCGGUUCA 2.GGAGGCACCUUCUAGGAUU 3.GAGGAUGGGUUCAUAGACA 4.UGGAUGAAGUUGUGUCAGA	Darmacon
siRNA CHOP	AAGGAAGUGUAUCUUCAUA	Eurofins MWG
siRNA JNK	AGAAGGUAGGACAUUCUUU	Eurofins MWG
siRNA DR5	AUGAGAUAAAGGUGGCUAA	Eurofins MWG

Viral Transduction. For Retrovirus and Lentivirus production HEK-293GP and HEK-293T cells were transfected with the packaging plasmids and the plasmid construct of interest, using standard calcium-phosphate method or Fugene reagent (Promega), respectively. After 8 hours, medium was changed and cells were incubated at 37°C. After 48 hours, the supernatants containing viral particles were filtered (0.45 µm filter), supplemented with 10% FBS and polybrene (8ug/ml). The culture medium of target cells growing at low confluence (≈30-40%) was replaced by the appropriate viral supernatant and incubated at 37°C for 24 hours. Cells were selected with puromycin (0,5 µg/ml) and kept under selection for the entire experiment.

Treatments. Thapsigargin (Tg) was purchased from Sigma (T9033) and dissolved in DMSO. Cells were treated with Tg [1uM] for the indicated times. Tunicamycin (Tm) was purchased from Sigma (T7765) and dissolved in DMSO. Cells were treated with Tm [5 ug/ml] for the indicated times. IRE1 inhibitor (STF-083010) was purchased from Calbiochem (412510) and dissolved in DMSO. Cells were treated with IREi [60uM] for 48hours. PERK inhibitor (GSK-2606414) was purchased from Tocris (PF 429242) and dissolved in Ethanol. Cells were treated with PERKi [100 uM] for 48 hours. Nelfinavir mesylate hydrate (NFV) was purchased from Sigma (PZ0013) and

dissolved in DMSO. Cells were treated with NFV [25uM] for 48 hours. Ceapins-A7 were a gift from Peter Walter's lab and dissolved in DMSO. Cells were treated with Ceapins-A7 [5uM] for 48hours. SAHA was purchased from CAYMAN (149647-78-9) and dissolved in DMSO. Cells were treated with SAHA [5uM] for 48 hours. Cerivastatin (CER) was purchased from Sigma (SML0005) and dissolved in DMSO. Cells were treated with two doses [0,1uM] and [0,001uM] for 48 hours.

Cell manipulation and Western blot analysis. Total cell extracts were prepared in RIPA buffer without SDS (150mM NaCl, 50mM Tris-HCl pH8, 1mM EDTA, 1% NP-40, 0.5% Na-deoxycholate) supplemented with 1 mM PMSF, 5 mM NaF, 1 mM Na3VO4, 10µg/ml CLAP, 1µM TSA and 5µM nicotinamide. Protein concentration was determined with Bio-Rad Protein Assay Reagent (#500-0006, Bio-Rad). Lysates were resolved by SDS/PAGE and transferred to nitrocellulose (Millipore). Western blot analysis were performed according to standard procedures using the following primary antibodies.

Antibody	**Diluition**	**Purchase from/ References**
Anti-ATF6	1:1000	Abcam (ab 122897)
Anti-PERK	1:4000	Cell signaling (#3192)
Anti-IRE1a	1:2000	Cell signaling (#3294)
Anti-pJNK	1:500	Cell signaling (#9251)
Anti- pan JNK	1:1000	Cell signaling (#9252)
Anti-DO1	1:8000	**Santa cruz (sc-126)**
Anti-PARP p85 fragment	1:1000	TB273 (Promega)
Anti-GRP78	1:8000	GeneTex (GTX113340)
Anti-actin	1:1000	Sigma (#A9718)

Anti-GFP	1:1000	home-made rabbit polyclonal
HSP90	1:5000	sc-13119 (Santa Cruz)

Anti-mouse and anti-rabbit HRPO-conjugated (Sigma), were used as secondary antibodies.

RNA extraction and RT-qPCR. Total RNA was extracted with QIAzol (Qiagen) following manufacturer's instructions. For mRNA expression analysis, 5ug of total RNA were reverse-transcribed with QuantiTect Reverse Transcription (Qiagen). Analyzed genes were amplified using SsoAdvancedTMSYBR® Green Master Mix (Biorad) on a CFX96™ Real-Time PCR System (Biorad).

List of primers used:

Target	**Primers**
H3	Fw: 5'GAAGAAACCTCATCGTTACAGGCCTGGT3' Rw: 5'CTGCAAAGCACCAATAGCTGCACTCTGGAA 3'
IRE 1	Fw: 5'- ACGCCCACTCTGTATGTTGG -3' Rw: 5'- CAAACTTGACGTCCGTGCTG -3'
PERK	Fw: 5'- ATCCCCCATGGAACGACCTG -3' Rw: 5'- ACCCGCCAGGGACAAAAATG -3'
ATF6	Fw: 5'- TGAACTTCGAGGATGGGTTG -3' Rw: 5'- TCACTCCCTGAGTTCCTGCT -3'
XBP1s	Fw: 5'- TGCTGAGTCCGCAGCAGGTG -3' Rw: 5'- GCTGGCAGGCTCTGGGGAAG -3'
CHOP	Fw: 5'- CAGAACCAGCAGAGGTCACA -3' Rw: 5'- AGCTGTGCCACTTTCCTTTC -3'
GRP78	Fw: 5'- TGTTCAACCAATTATCAGCAAACTC -3'

	Rw: 5'- TTCTGCTGTATCCTCTTCACCAGT -3'
DR5	Fw: 5’ - CTCTCTCAGGCATCATCATAGG - 3’ Rw: 5'-GCAAGATACTCACGATCTCATT-3

*Cell viability assay and FACS analysis***.** For cell viability assay, we used ATP-lite Luminescence Assay System (PerkinElmer, 6016943). Cells were plated in 96 well for 24 hours before further treatments. ATPlite reactions were done according to manufacturer instructions, and measured using an Enspire plate-reader.

For FACS analysis, adherent and floating cells were harvested, permeabilized with 0.1% NP-40 in PBS containing RNase A (200 µg/ml) and then stained with 50 µg/ml Propidium Iodide (#P4865, Sigma). At least $2x10^4$ cells were counted in each experiment, using a FACSCalibur flow cytometer (Becton-Dickinson). Cell cycle analysis was performed with FlowJo software (http://www.flowjo.com/).

*Luciferase assay***.** Luciferase assays were performed using p5xATF6-GL3, bearing five repetitions of a specific binding sequence for ATF6-f. Luciferase reporters were transfected together with CMV-Renilla to normalize for transfection efficiency. Luciferase was measured on a Promega luminometer. For luciferase assays in siRNA-transfected cells, cells were first transfected with the indicated siRNAs and, after 24 h, washed from transfection media, transfected with plasmid DNA, and collected 24 h later.

Statistical Analysis. In all graphs data are expressed as mean ± SD of three independent experiments, except when otherwise indicated. Differences were analyzed by Student’s t test using Prism 6 (GraphPad), except when otherwise indicated. P-values < 0.05 were considered significant.

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