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The stress-activated kinase p38MAPK contributes to ATF6 activation and resistance to ER stress in cancer cells with mutant p53

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1) Abstract

Endoplasmic Reticulum Stress (ERS) is one hallmark of cancer cells: tumor hypoxia, glucose reduction and genome instability all promote accumulation of misfolded proteins in the endoplasmic reticulum. ER stress triggers the Unfolded Protein Response (UPR), a conserved pathway orchestrated by three ER-resident receptors, IRE1α, PERK, and ATF6, that activate specific and overlapping transcriptional programs aimed to overcome the stress or induce cell death. Accumulating evidence suggest a role for UPR in cancer progression, therefore uncovering functional interactions of this pathway with the oncogenic circuits that drive various tumors may be relevant for therapy.

The tumor suppressor p53 is one of the most frequently mutated genes in cancer and missense mutant p53 proteins (mutp53) can acquire powerful oncogenic properties. We recently reported that mutant p53 can modulate the UPR, specifically sustaining activation of the ATF6 branch. This molecular axis may contribute to cancer aggressiveness and resistance to therapy. However, the mechanisms by which mutant p53 can modulate the UPR in cancer cells remained unexplored.

In this Thesis, I describe one of the possible mechanisms exploited by mutp53 to sustain ATF6. Using breast, prostate and pancreatic cancer cell lines, I found that mutant p53 enhances ERS-induced activation of the stress kinase p38MAPK. I also found that inhibition of p38MAPK reduces ERS-induced proteolytic cleavage of ATF6 and its transcriptional activity. These data suggest that p38MAPK may have a pro-survival role in the context of ER stress. Indeed, pharmacologic inhibition of p38MAPK increased the sensitivity to Thapsigargin in cancer cells with mutant p53.

Regarding the possible action of p38, I measured the turnover of the active ATF6 fragment, and found that inhibition of p38MAPK caused a perceptible reduction in ATF6f stability. Therefore, one mechanism by which mutp53 can reshape the UPR is by increasing the stability of the active ATF6f protein via enhanced activation of p38MAPK.

2) Introduction

2.1) The Endoplasmic Reticulum (ER)

The Endoplasmic Reticulum (ER) is a dynamic organelle that plays a pivotal role in many cellular functions, such as synthesis of lipid and protein and regulation of intracellular Ca2+ levels. It spreads through the cytoplasm, and it is divided in three major areas: Nuclear Envelope (NE), peripheral cisternae and tubular network (English and Voeltz, 2013).

NE surrounds the nucleus and acts like a barrier in order to control the transport in and out of the region (English and Voeltz, 2013). The peripheral ER is formed by an interconnected network of cisternae, localized closer to the NE and tubules, mainly found in the periphery (Puhka et al., 2012; Terasaki et al.,1987). ER cisternae present a big amount of ribosomes, the reason why it is known as “rough” ER; on the contrary ER tubules are known as “smooth” ER because of the lack of ribosomes. Cisternae also have a larger luminal volume to surface area than tubules rendering them the preferred site for luminal processes like protein folding (Shibata et al., 2010; Puhka et al., 2012; West et al., 2011). ER tubules have lower volume suggesting that they could be the main site for the accumulation of integral membrane proteins and for processes connected to lipid synthesis: proteins and phospholipids are indeed transferred and biochemically modified in a region of the ER close to the Golgi apparatus called ER-Golgi Intermediate Compartment (ERGIC), where they are distributed throughout the cell through organelle contacts or secretory vesicles. (West et al., 2011;Appenzeller-Herzog and Hauri, 2006; Fagone and Jackowski, 2009). ER is also important as the major store of intracellular Ca2+. Several calcium channels, inositol 1,4,5-trisphosphate receptors and ryanodine receptors reside in the ER membrane being responsible for releasing Ca2+ from the ER into the cytosol when intracellular levels are low. Furthermore, Ca2+ can move from cytoplasm into the ER via sarcoendoplasmic reticular Ca2+ ATPases (SERCAs) (Clapham, 2007).

2.2) Endoplasmic Reticulum Stress and the Unfolded Protein Response (UPR)

Conditions that change cellular homeostasis, like unbalanced Ca2+ concentration, redox stress, hypoxia, nutrient deprivation or conditions that force cell having high biosynthetic demand followed by hyperaccumulation of proteins or by a huge amount of unfolded proteins cause perturbations in the lumen of the ER leading to the condition of Endoplasmic Reticulum Stress (ERS). Cells can respond to stress since in the ER there are sensors able not only to detect changes in cellular equilibrium, but also to activate downstream signaling cascades to alleviate the stress. With these mechanisms cells can increase the capacity of the ER, accelerate degradation of proteins or upregulate chaperones and folding enzymes (Smith and Wilkinson, 2017). Defects or lack of activation in sensing and signaling pathways during ER stress are associated with numerous pathologies, including Parkinson's and Alzheimer's diseases and cancers (Wang and Kaufman, 2016 **;**Lindholm et al., 2017)

To overcome the disruption of ER homeostasis, cells rely on different mechanisms. One of these is ER-Overload Response (EOR) which is triggered by the hyperaccumulation of proteins. Ca2+ released by the ER stimulates reactive oxygen species production leading to the activation of NF-κB signaling (Pahl1 and Baeuerle, 1995) (Heike L. Pahl, 1997).

Another conserved pathway that allows cells to recover the homeostasis is ER Associated protein Degradation (ERAD). Unfolded ER substrates are retro-translocated into the cytosol, polyubiquitinated and finally degraded by the proteasome. ERAD is a complex mechanism that comprises different pathways (ERAD-L, M, and C) based on the localization of the misfolded domain of the proteins, in particular if it is in the lumen, in the membrane, or on the cytosolic side of the ER. Each pathway is in turn regulated by different ubiquitin ligases. There is also an additional pathway responsible for the degradation of misfolded inner nuclear membrane proteins (Wu and Rapoport, 2018). Proteins with misfolded luminal or intramembrane domains are moved across the ER membrane through a channel created by ubiquitin ligase Hrd1. Polyubiquitinated substrates are extracted from the membrane by the Cdc48/p97 ATPase complex and forwarded to the proteasome (Lemberg and Strisovsky, 2021; Wu and Rapoport, 2018) . Noteworthy is the role of HERPUD1 (homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1), an ER resident protein that can

act as a shuttle in order to target the ubiquitinated substrate to the proteasome (Huang et al., 2014; Okuda-Shimizu and Hendershot, 2007). The protein can also regulate the ubiquitination of Hrd1 (Kny et al., 2011). One of the most important proteins involved in the degradation of misfolded N-glycosilated protein is ER Degradation Enhancing a-Mannosidase I-like protein (EDEM1). In the lumen of the ER, calnexin and calreticulin are two chaperones that recognize mono-glucosylated N-glycans in proximity to unfolded regions and promote the protein folding together with uridine diphosphate (UDP)-glucose, which ensures that incompletely folded proteins stay mono-glucosylated and remain in the calreticulin-calnexin- cycle (Lemberg and Strisovsky, 2021). When a protein cannot be correctly folded, the interaction between the protein and the chaperone is disrupted by the action of EDEM1 which extracts and targets the misfolded glycoproteins for degradation (Maurizio Molinari et al., 2003). In particular, Cormier and colleagues showed that EDEM1 can discriminate unfolded and folded proteins during their maturation and quality control relying on its mannosidase-like domain in a glycan-independent manner to target them to the ER membrane dislocation and ubiquitination complex (Cormier et al., 2009).

Another important mechanism to overcome the disruption of ER homeostasis is the Unfolded Protein Response (UPR) (Hetz et al., 2011). Through this mechanism, cells activate adaptive responses such as decrease in mRNA translation, degradation of misfolded/unfolded proteins, expansion of the ER membrane, and increase in the ER folding capacity, by augmented production of components of the folding and quality control machinery. If the homeostasis cannot be restored, ER-stress prolongs and the response moves from pro-adaptive to proapoptotic thus inducing cell death (Walter and Ron, 2011). The UPR program is orchestrated by three ER resident proteins: IRE1α (inositol-requiring protein 1a), ATF6 (activating transcription factor 6a), and PERK (PKR-like endoplasmic reticulum kinase) (fig.1A). These receptors are organized in three main regions: a cytosolic domain, an ER luminal domain, and a single transmembrane helix. Through the ER luminal domain these proteins are able to sense misfolded proteins and translate the signals to the cytosol to activate different downstream pathways. The simplest mechanism of activation relies on the binding with key ER chaperone GRP78/BiP. In unstressed condition, these sensor molecules are inactive and associated with the 78 kDa glucose-regulated protein (GRP78) (Schröder and Kaufman, 2004).

Upon ER stress, GRP78 is released from the sensors allowing them to oligomerize and activate, triggering downstream signaling events. The signaling pathways triggered by the three different receptors are also referred to as three arms of the UPR.

2.2.1) The IRE1α arm

Ire1 is the most conserved arm of UPR since it's present in all eukaryotes (Mori, 2009). In specific, is a type I transmembrane protein able to sense misfolded proteins in the ER with its luminal domain and triggers the UPR through a cytoplasmic kinase domain and an RNase domain (Tirasophon et al., 2000). Upon ER stress, the protein dimerizes and auto-transphosphorylates, thus allowing a conformational change that activates its endoribonuclease domain. When activated, the protein can activate different downstream signaling events; one of the most relevant is the excision of a 26-nucleotide intron in the mRNA encoding the transcriptional factor X- box binding protein-1 (XBP1) (Walter and Ron, 2011). XBP1 spliced protein (XBP1s) acts as a transcription factor controlling the expression of several UPR genes involved in protein folding, protein entry into the ER, and protein degradation via ERAD. In addition, XBP1s can also regulate the biogenesis of the ER and Golgi increasing the activity of enzymes related to phospholipid biosynthesis. (Hetz et al., 2011).
During a prolonged ER stress condition, the hyperactivation of IRE1 can trigger a process called Regulated IRE1-Dependent Decay (RIDD) that is able to orchestrate a wide spectrum of biological processes by the regulation of different miRNA or mRNA balancing the expression of membrane and secretory proteins through the endonucleolytic cleavage mediated by the RNase domain (Maurel et al., 2014). In addition to its endonuclease activity, IRE1 regulates different signaling pathways that strongly impact cell survival by enhancing the activation of NF-κB by interacting with TRAF2 and the inhibitor of nuclear factor κβ kinase (IKK) (fig.2). This interaction also triggers the activation of the apoptosis signal-regulating kinase 1 (ASK1) and Jun-N terminal kinase (JNK) pathway (Urra et al., 2020). Binding of IRE1 to SH2/SH3-containing adaptor protein Nck can modulate p38 MAPK family and ERK1 (MAPK3) signaling, also impacting cell survival (Hetz et al., 2011).

2.2.2) The PERK arm

PERK is a type I transmembrane protein which presents luminal domain homology with IRE1 (Chuan Yin Liu et al., 2000). The activation of PERK is similar to that of IRE1. In the presence of ER stress, GRP78 releases the luminal domains of the kinase because of its increased affinity for unfolded proteins, thus leading to the oligomerization and trans-phosphorylation of PERK monomers (Schröder and Kaufman, 2005). Once activated, the protein phosphorylates the α-subunit of the Eukaryotic Initiation Factor 2 alpha (eIF2α) to prevent the formation of a ternary complex with GTP and tRNAmet, responsible for translational initiation. In this way, PERK reduces cap-dependent protein synthesis in order to attenuate the secretory protein flux and to allow the release of ribosomes for selective translation of specific UPR response genes. In fact, despite the general attenuation of protein synthesis, the translation of mRNAs containing short open reading frames in their 5′-UTR (uORF) is still possible. Among these mRNAs, Activating Transcription Factor 4 (ATF4) is selectively induced (Harding et al., 1999). ATF4 induces the expression of two other transcriptional regulators, ATF3 and CHOP. ATF3 together with ATF4 bind to the promoter region of GADD34, a regulatory subunit of protein phosphatase 1, which targets protein phosphatase 1 to phosphorylated eIF2 promoting its dephosphorylation, thus generating a negative feedback regulatory loop. CHOP is able to induce apoptosis since it can regulate different pro-apoptotic molecules, such as death receptor 5 (DR5) and tribbles homologue 3 (TRB3) (Jiang et al., 2004) (Read and Schröder, 2021)

Another protein activated by PERK is NF-E2-related factor-2 (Nrf2). Nrf2 releases from its negative regulator KEAP1, allowing it to accumulate and translocate to the nucleus in order to activate the transcription of genes encoding antioxidant proteins. In this way PERK contributes to the maintenance of glutathione levels in cells undergoing ER stress (Cullinan and Diehl, 2004).

Interestingly, PERK signaling may be involved in ATF6 expression and its transport from ER to Golgi. In fact, Teske et colleagues found that loss of PERK in liver cell lines impaired ER stress responses, and in particular ATF6 transcriptional activity. They proposed that ATF4 activity facilitates trafficking of ATF6 from the ER to the Golgi, thus enhancing ATF6 processing and

ATF6-dependent transcription of genes involved in regulation of ERAD, chaperones, and vesicular trafficking (Teske et al., 2011). PERK signaling is also important for mitochondrial pro-survival signaling. The activated kinase can be found at mitochondria-associated ER membranes (MAMs), suggesting that PERK's activation and downstream signaling pathways can influence mitochondrial mediated cell survival (Verfaillie et al., 2012).

2.2.3) The ATF6 arm

The third branch of UPR is regulated by ATF6, a type II ER transmembrane protein. It is present in two isoforms, ATF6α and ATF6β, with high sequence homology. However, ATF6β has low transcriptional ability, and can inhibit the transcriptional activity of ATF6α (Thuerauf et al., 2004; Walter & Ron, 2011).

As shown in figure 1B, ATF6 can be divided in three principal domains: N-terminal cytosolic domain consisting of a Transcriptional Activation Domain (TAD), a bZIP domain and a Nuclear Localization Sequence (NLS), transmembrane domain (TM) and a luminal domain consisting of two conserved regions, CD1 and CD2, involved in sensing of misfolded protein, Golgi translocation, and proteolytic cleavage (Chen et al., 2002). Without stress, ATF6 can be found as a monomer, dimer or oligomer through formation of intra/inter-molecular disulfide bonds due to the presence of two conserved cysteines within its luminal domain. In this region there are also two Golgi localization sequences that are covered by the chaperone GRP78. During ER stress, the accumulation of misfolded proteins induces BiP to dissociate from ATF6. This is followed by the reduction of disulphide bridges which monitor the ER environment acting as redox sensors. As proved by Schindler and Schekman, during stress ATF6 forms an interaction with the protein complex COPII, required for vesicular traffic of cargo proteins from the ER to Golgi (Schindler and Schekman, 2009).

Oka and colleagues identified an ER oxidoreductase, ERp18, that exerts an important regulatory role during the UPR associating with ATF6 only after ER stress. They demonstrate that in ERp18 KO cells, ATF6 is rapidly trafficked to the Golgi, suggesting that the oxidoreductase may retain ATF6 in the ER after BiP release to allow the packaging of the protein into the COPII vesicles, preventing premature exit from the ER (Oka et al., 2019).

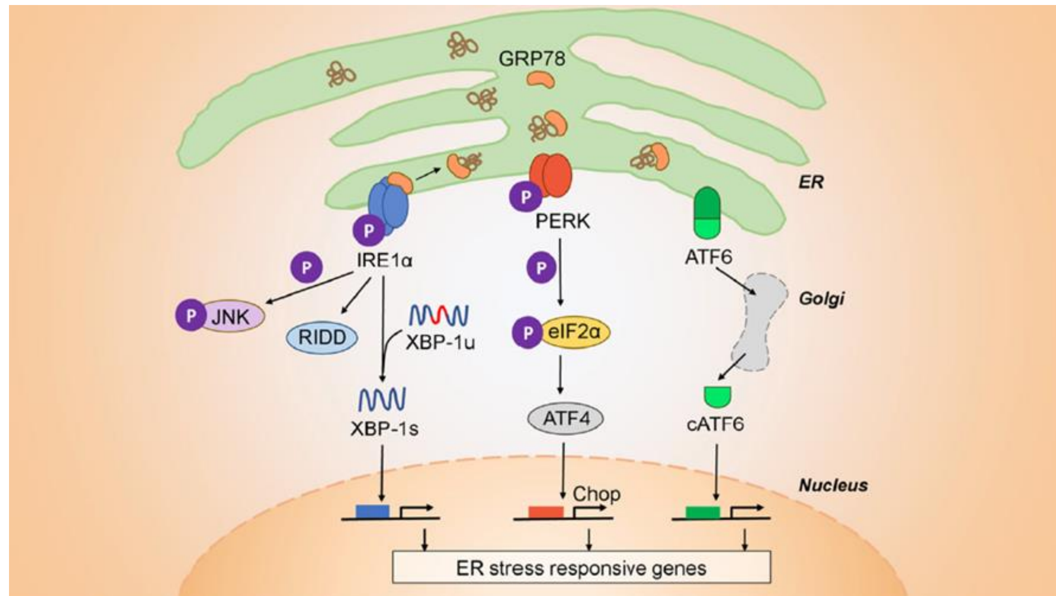
To be transported from ER to Golgi, ATF6 requires the protein disulfide isomerase A5 (PDIA5). Higa and colleagues proved that this protein plays a pivotal role to disulfide bond rearrangement in ATF6 upon ER stress, thus allowing not only ATF6 export from ER to Golgi, but also the activation of its target genes. They also proved that PDIA5/ATF6 activation loop increase the chemoresistance of leukemia cancer cells to imatinib (Higa et al., 2014). With a more recent work, Oka and collegues give further light on the mechanism, showing that during the early stage of UPR, ATF6 dimers are preferentially translocated to Golgi and cleaved. In cells overexpressing ERp18 they observed a dramatic decrease in dimer formation (Oka et al., 2022).

In the Golgi, ATF6 is activated through a process of regulated intramembrane proteolysis. The protein is cleaved by S1P/S2P proteases to release its N-terminal cytoplasmic domain (ATF6f), in a similar way to SREBP proteins (Haze et al., 1999). Within the nucleus, ATF6f binds specific consensus sequences, ER Stress Response Elements CCAAT-N-CCACG (ERSE-I), ATTGG-N-CCACG (ERSE-II) and Unfolded Protein Response Element TGACGTGG/A (UPRE) promoting transcription of a variety of target genes (Li et al., 2000; Wang et al., 2000). ATF6f binds DNA as part of a complex by binding to the CCACG part of the ERSE with the Nuclear transcription Factor Y (NF-Y), which in turn binds to the CCAAT part (Yamamoto et al., 2004; Yoshida et al., 2000).

The transcriptional target genes regulated by ATF6f promote ER adaptive response, like ERAD activity or the increase of ER folding capacity. One example are genes encoding for GRP78/BiP and Protein Disulfide Isomerase (PDI). Interestingly, ATF6 also induces the transcription of genes involved in cholesterol and lipid biosynthesis (Maiuolo et al., 2011; Maruyama et al., 2013). As proved by Wu et al., ATF6 deletion in mice impairs the correct function of the secretory pathway disabling the adaptation during chronic ERS, thus proving that the protein is essential for tissues to optimize protein folding, secretion, and degradation during chronic ER stress (Wu et al., 2007). The pro-adaptive function of ATF6 is relevant also in Parkinson disease, a neurodegenerative disorder linked to aberrant accumulation of α-synuclein. Credle and collegue demonstrated that the aberrant accumulation of α-synuclein is able to impairs ATF6’s processing due to the restricted incorporation in COPII vescicles, thus leading to an increased pro-apoptotic signaling (Credle et al., 2015).

ATF6 can also promote pro-apoptotic functions, indeed the activated fragment is able to transactivates CHOP (Gotoh et al. 2002) together with PERK arm of UPR.
Post-translational modifications of ATF6 can influence its function. The protein contains three evolutionarily conserved N-linked glycosylation sites within its carboxyl luminal domain, but their functional significance is not fully understood. They might be a monitoring mechanism for ER homeostasis (Hong et al., 2004).
Another relevant post translational modification of ATF6 is revealed by Hou and collegues. In specific, they discovered that the active fragment of the protein can be subjected to SUMOylation, which impairs its transcriptional activity, due to the presence of a conserved sumoylation site at the N-terminal part of the protein (Hou et al., 2017).
ATF6 can also be phosphorylated by the MAP kinase p38 and the implications of this interaction will be discussed in the paragraph related to p38 (see page 25).

A



B

Figure 1. A) The three sensors regulating the Unfolded Protein Response (UPR). UPR is orchestrated by IRE1a, PERK and ATF6. Each branch is able to activate different transcriptional programs in order to restore cellular homeostasis or to induce cell death (Zanetti et al., 2022)**. B) Schematic representation of ATF6α structure.** The protein presents a N-terminal cytosolic domain where lays the Transcriptional Activation Domain (TAD), a bZIP domain and a Nuclear Localization Sequence (NLS); a transmembrane domain (TM); a luminal domain consisting of two conserved regions, CD1 and CD2 through which ATF6 is able to sense misfolded proteins. (Chen et al., 2002).

3.1) UPR and disease: focus on cancer

Altered ER proteostasis and abnormal UPR signaling have been implicated in a wide range of human diseases, including cancer. In fact, highly proliferative tumors are exposed to several intrinsic and extrinsic factors that force cell to overcome stress conditions. ER stress in transformed cells is caused by intrinsic factors, such as genomic instability, excessive secretory activity and redox imbalance. Genetic alterations can promote persistent activation of UPR arms, since the loss of tumor suppressors and hyperactivation of oncogenes readily increase protein synthesis to cope with the increased metabolic demand during tumorigenesis (Chen and Cubillos-Ruiz, 2021)**.** Extrinsic factors, like acidosis, hypoxic conditions and nutrient deprivation may also alter the normal function of the endoplasmic reticulum (ER) (fig.2). *Sustained activation of UPR is present in different human cancers and the aberrant activation of its branches allows cells avoiding the ERS induced cell death taking advantage* to the different activation of that mechanisms (Yu et al., 2021; Urra et al., 2016)**.**
Oncogenic transformation is one of the main features of tumors. One example is MYC hyperactivation, which induce proteotoxic stress. Zhao et al., proved that oncogenic Myc is able to directly regulate the expression of IRE1 by the binding to its promoter and enhancer. The hyperactivated protein is also able to form transcriptional complex with XBP1, thus increasing its transcriptional activity, as proved by the increased expression of ER chaperones, folding and modification enzymes. Interestingly, they also found that MYC-hyperactivated cells are sensitive to the pharmacological inhibition of IRE1, thus leading to a reduction in tumor growth in vivo (Zhao et al., 2018).
Once activated, PERK induces ATF4, that allows tumor cells to overcome hypoxia by the up regulation of the genes involved in protein synthesis and antioxidant responses for survival. In fact, cells PERK−/− under the condition of hypoxia, have lower viability and lose their capability of angiogenesis (Blais et al., 2006)**.** In addition, it has been demonstrated that PERK induces the activation of Nrf2 which in turn inactivates CHOP, thus blocking the apoptotic signaling (Cullinan and Diehl, 2004). Another example that strongly proves the impact of deregulated UPR on cancer cells' destiny is the activation of the Phosphoinositide 3-Kinases/ Protein Kinase B (PI3K/PKB) pathway and the upregulation of Induced Myeloid Leukemia Cell

Differentiation Protein (Mcl-1) mediated by IRE1α arm: the activation of the sensor is indeed able to enhance the survival of melanoma cells (Dong et al., 2011).

Tay et al., revealed that sustained activation of IRE1α and ATF6 signaling plays a critical role in melanoma cells, protecting them from ER stress-induced apoptosis. They found that damping of these pathways renders cancer cells sensitive to ER stress. In addition, they found that the activity of MEK/ERK signaling is required for sustained activation of IRE1α and ATF6 signaling branches upon ER stress (Tay et al., 2014).

The reduced oxygenation of solid tumors induces hypoxia, nutrient deprivation and subsequently, the decrease of ATP production. These factors trigger neo-angiogenesis, a commonly used way by which cancer cells are able to adapt to nutrient starvation and low oxygen conditions, thus facilitating the survival and the rapid proliferation of cells. Hypoxia and reduced ATP also cause ER stress; several observations, indeed, indicate that the UPR can promote angiogenesis by regulating the transcription of different pro-angiogenic factors or through the modulation of the expression and the mRNA stability of Vascular Endothelial Growth Factor A (VEGFA), Fibroblast Growth Factor 2 (FGF2), IL-1ß, IL-6 and IL-8(Pereira et al., 2010). PERK-mediated ATF4 induction up-regulates VEGF expression through the direct binding of the transcription factor to VEGF promoter (Binet and Sapieha, 2015; Wang et al., 2012). In parallel, the IRE1 arm contributes to neo-angiogenesis by both the up-regulation of VEGFA and IL-6, and the suppression of different anti-angiogenic factors (Auf et al., 2010; Ghosh et al., 2010). Interestingly, Karali and colleagues proved that VEGF is able to induce the UPR in endothelial cells through an unconventional mechanism that requires PLC-gamma and mTORC1 in the absence of ER stress, thereby promoting endothelial cells survival and angiogenesis (Karali et al., 2014).

Of note, the UPR is also involved in cell invasion and metastasis. In this context, PERK's arm of UPR plays a pivotal role. Feng et al. demonstrated that cells undergoing Epithelial to Mesenchymal Transition (EMT) are able to constitutively activate the kinase. This results in both a reduction of E-cadherin and an increase in Twist levels, thus allowing cells to invade, metastasize, and form tumorspheres (Feng et al., 2014). ATF4 is also upregulated in esophageal squamous carcinoma, where it augments cell invasion and metastasis in cells with low metastatic potential by the upregulation of matrix metalloproteinases (Zhu et al., 2014).

Interestingly, Nalgerkeke and colleagues reported that in breast cancer, hypoxic conditions promote the activation of a PERK/ATF4/LAMP3 (Lysosomal-Associated Membrane Protein 3) molecular axis by the regulation of UPR, thus allowing cell migration (Nagelkerke et al., 2013). IRE1a is also implicated in regulating metastasis by at least two mechanisms. Chen et al. revealed that the protein may modulate the adhesion and migration properties of triple negative breast cancer xenografts via increased Xbp1s activity, which promotes more efficient lung metastasis (Chen et al., 2014). Dejeans et al found that the IRE1α pathway in glioma cells negatively regulates cell adhesion and migration through the RIDD degradation of SPARC mRNA, that encodes for a matrix-associated protein that drives changes in cell shape, inhibits cell-cycle progression and influences the synthesis of ExtraCellular Matrix (ECM)(Dejeans et al., 2012).

Recent data revealed a direct link between UPR and tumor dormancy. Cancer cell "dormancy" is characterized by a state of quiescence in which tumor cells block their abnormal proliferation arresting the cell cycle in the G0/G1phase. Dormant cells are therefore more resistant to chemotherapeutic drugs that preferentially target proliferating cells. Dormant cells, then, can reactivate when they find optimal conditions for cell proliferation, resuming their metabolic activity (Urra et al., 2016). From a molecular point of view, cell dormancy is characterized by the balance of p38 MAPK and ERK activity: in particular, it occurs when p38 MAPK suppress crucial elements involved in ERK activation, such as FoxM1 and c-Jun(Hsu et al., 2019). In this context, Schewe and Ghiso revealed that ATF6, in dormant squamous carcinoma cells, can transduce survival signals through a ATF6-Rheb-mTOR pathway with the strong contribution of p38a (Schewe and Aguirre-Ghiso, 2008). Intriguingly, in a model of dormant human epidermoid carcinoma, Rangatan et al., showed an increased p38 dependent splicing of XBP1. In addition, p38 dependent activation of PERK and the regulation of Bi protected dormant cells from drug induced cell death, as compared to tumorigenic cells. (Ranganathan et al., 2006).

Several studies have also demonstrated the interplay between the UPR and the DNA Damage Response (DDR)(González-Quiroz et al., 2020). In a recent work, Benedetti et colleagues have found that ATF6 plays a pivotal role in DNA repair in colon cancer cells treated with the ER stressors 3,4-dihydroxyphenyl ethanol (DPE) and Thapsigargin, by sustaining BRCA-1

expression through mTOR activity. They also proved that ATF6 inhibition sensitized cancer cells to the cytotoxic effect of Adriamycin, demonstrating that the inhibition one of the UPR arms may reflect and potentiate the effect of DNA damaging agent (Benedetti et al., 2022). Another interesting study performed by Dufey et colleagues revealed an unexpected role of IRE1α in presence of DNA damage. In specific, they demonstrated that during DNA injury and in absence of ER stress, the protein physically interacts with c-Abl, which in turn is activated by DNA damage. After this, IRE1α becomes active and, exerting no effect on XBP1, is able to induce the RIDD response through which it controls the stability of such mRNA regulating the DDR, DNA repair, cell cycle and apoptosis (Dufey et al., 2020)

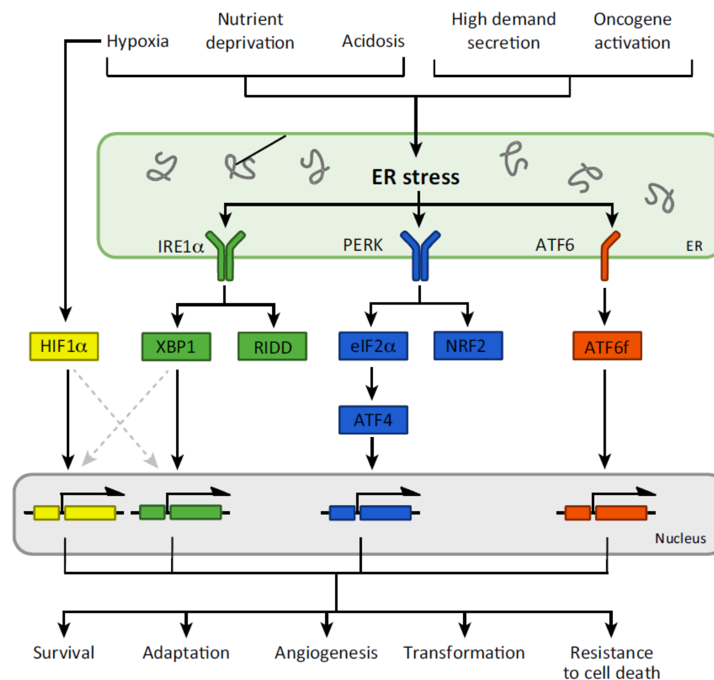


Fig. 2. The contribution of UPR in cancer. In cancer cells, ER stress is triggered by a wide range of stimuli. To cope with these perturbation UPR branches activate different pathways which can promote adaptation, survival, transformation, angiogenesis, and resistance to cell death (Urra et al., 2016).

3.2) Targeting the UPR for cancer therapy

As previously described, due to extrinsic and intrinsic stimuli, UPR is activated in cancer cells. The identification of new targets with a clinical relevance appears a crucial point in order to discover new therapeutic opportunities. For this reason, targeting molecular pathways that allow adaptation to stress in cancer cells may represent a good strategy to selectively induce cell death in tumors, limiting the negative side effects to normal cells.

3.2.1) Targeting IRE1

Upon ER stress, the activation of IRE-1 can either promote adaptive signals through the splicing of XBP1 mRNA or, in contrast, trigger cell death signals via RIDD, or TRAF2 /ASK1 mediated activation of JNK. There are two main classes of IRE1 inhibitors which either interact with the catalytic core of the RNAse domain or bind to the ATP binding pocket of the kinase domain (Ali et al., 2011; Wang et al., 2018)**.**

The first class comprises molecules that have similar chemical mechanisms of action. The inhibitors share a common hydroxy-aryl-aldehyde (HAA) moiety which reacts with a specific lysine in the RNase domain, resulting in the formation of stable imine via Schiff base, thus preventing ER stress-induced site-specific mRNA splicing (Raymundo et al., 2020)**.** In this way, the drugs disrupt the catalytic capacity of the protein without affecting its phosphorylation and oligomerization (Tomasio et al., 2013; Wang et al., 2018). These inhibitors do not affect the other arms of the UPR. One of the compounds is STF- 083010. The molecule was identified as an inhibitor of XBP1 mRNA splicing activated by thapsigargin, tunicamycin, glucose deprivation, or severe hypoxia. The inhibitor is able to block IRE1 endonuclease activity with no effect on its kinase activity. Another compound presenting the same function is 4μ8C which compared to STF-083010, shows the capability of inhibiting IRE1 autophosphorylation via Schiff base formation with Lys599 located in the kinase pocket of the receptor (Raymundo et al., 2020).

Notable is MKC-3946. The molecule, applied in a context of Multiple Myeloma (MM), was able to block the splicing of XBP1 enhancing cytotoxicity of bortezomib or 17-AAG via ER stress-mediated apoptosis, as proved by up-regulation of CHOP (Mimura et al., 2012).

The second class is composed by molecules that bind to the ATP binding pocket within the kinase domain of IRE1, thus inhibiting its phosphorylation. These drugs are divided into two further subgroups based on their ability of blocking the RNAse domain of IRE1. APY29 and Sunitinib are type I IRE1 kinase inhibitors that stabilize the active ATP-binding pocket conformation, and activate its RNAse activity (Wang et al., 2018). In specific, APY29 completely inhibited the expression of GRP78 in BEAS-2B cells stressed with As_2O_3 treatment. Yuan et colleagues also proved that the inhibitor not only induced a reduction in Bip expression, but impacted on the phosphorylation of JNK, ERK and p38 MAPK (Yuan et al., 2021). Kinase-inhibiting RNase-attenuators (KIRAs) compounds are able to alter the oligomerization process on the DFG-motif for stabilizing the enzyme in a helix-αC-out conformation, with effects reflecting on both kinase and RNase activities depending on the dose used (Raymundo et al., 2020). Quercetin and Compound 3 are type II kinase inhibitors that stabilize the inactive conformation of the ATP-binding pocket and suppress the RNAse activity of IRE1 (Wang et al., 2018). The imidazo[1,5-a] pyrazine motif of Compound 3 forms two hydrogen bonds with the hinge region and occupies the adenine pocket of IRE1. The inhibition induces movements of the DFG-motif into the activation loop thus resulting in the inhibition of XBP1 mRNA splicing (Wang et al., 2012). Liu et al., proved that quercetin was efficiently able to prevent the phosphorylation of IRE1 in rats' hepatocytes exposed to Pb and in general, they observed that ER stress induced by lead was significantly inhibited by quercetin (Liu et al., 2013). GSK2850163 was identified as a new IRE1 selective inhibitors which exerts both RNase and kinase inhibition in a dose-dependent manner in myeloma cell lines (Concha, N.O. et al., 2015)

3.2.2) Targeting PERK

GSK2606414 was the first described PERK inhibitor (Axten et al., 2012)**.** One work that showed the efficacy of the molecule was performed by Rozpędek and colleagues, who reported that

GSK2606414 could induce a significant decrease in eIF2a phosphorylation in cancer cell lines SH-SY5Y (human neuroblastoma) and HT-29 (human colorectal adenocarcinoma) (Rozpędek et al., 2017). In support of this notion, Moreno *et al.,* reported that GSK2606414 can inhibit neurodegeneration in prion-infected mice (Moreno et al., 2013).

Another ATP competitive inhibitor of PERK enzymatic activity is GSK2656157. Encouraging data showed that the drug was able to reduce ER-stress induced PERK autophosphorylation, together with eIF2a phosphorylation and subsequent activation of ATF4 and CHOP in multiple cell lines (Atkins et al., 2013). However, a subsequent study contradicted some of the previous assumptions: apparently, GSK2656157-mediated inhibition of PERK, does not correlate with the reduced phosphorylation of eIF2a. In addition, the effect of the molecule does not mimic the biological effect of PERK inactivation, since cells increase the phosphorylation of the protein in order to offset the reduction of PERK activation. Moreover, GSK2656157 can induce ERS-related cell death with a mechanism of action that does not depend on eIF2a block (Krishnamoorthy et al., 2014).

Within this UPR branch, another druggable target is eIF2a since its prolonged phosphorylation induces the upregulation of ATF4 and CHOP that can in turn induce apoptosis. Salubrinal and Guanabenz are two compounds able to inhibit eIF2α dephosphorylation. In particular, Hamamura *et al* reported that in breast cancer cell lines, the inhibitors suppressed cell proliferation and invasion both in vitro and in vivo (Hamamura et al., 2014).

3.2.3) Targeting ATF6

ATF6 is the only UPR sensor with the capability to act as a transcription factor. So, the protein can directly regulate the expression of genes involved in ER stress response, without the involvement of other effectors in the cascade between the perception of the stimuli and the transcriptional outcome. In contrast, ATF6 lacks any enzymatic activity and for this reason it is difficult to directly target the protein. A group of compounds that indirectly affect ATF6 signaling are Protein Disulfide Isomerase (PDI) inhibitors, since the protein contains a disulfide bond in its luminal domain that requires the catalytic activity of that enzyme. Among these,

PACMA31 and 16F16 were reported to inhibit ATF6 signaling (Wang et al., 2018**;** (Nadanaka et al., 2007; Xu et al., 2012)**.** In particular, Higa et collegues proved that in leukemia cells, ATF6 silencing and its inhibition through the PDI inhibitor 16 F16 restores sensitivity to Imatinib (Higa et al., 2014)**.**

ATF6 signaling can be blocked by inhibiting the proteases S1P and S2P that cleave the protein in the Golgi. One example is the compound 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF). The drug is a serine protease inhibitor that efficiently blocks the formation and nuclear localization of the active ATF6 fragment, with little or no effect on ER to Golgi protein transportation (Okada et al., 2003). Conceptually, any other S1P/S2P inhibitor would inhibit ATF6 activation. However, the above-described drugs are not selective for ATF6, because their targets are shared among multiple pathways, and they have important side effect.

Nowadays, the most selective ATF6 inhibitors are Ceapins, a class of pyrazole amides discovered by Peter Walter and his group (Gallagher and Walter, 2016). The compounds present high specificity for ATF6a in response to ER stress, trapping the protein into the ER and allowing the formation of foci. Strikingly, the activation of ATF6b or SREBP, two ER-localized proteins that undergo similar trafficking and processing by S1P and S2P in the Golgi apparatus, is not affected. Ceapins also do not significantly alter the other branches of the UPR, indicating that ER stress sensing is not affected by their mechanism of action. By selectively inhibiting ATF6, Ceapins are able to sensitize cells to ER stress without strongly affecting viability of unstressed counterpart (Gallagher et al., 2016)**.**

Recently, Torres et colleagues discovered a molecular mechanism that explains the high selectivity of Ceapins for ATF6. In specific, the inhibitor induces neomorphic inter-organelle junctions between endoplasmic reticulum and the peroxisome, directing the cytosolic domain of ATF6a to the transmembrane region of the peroxisomal ABC transporter ABCD3. Normally, these proteins don’t interact with each other, and localize in different cellular regions. Creating this new link, ceapins act as a chemical misdirector preventing translocation of ATF6 to the Golgi, and this could explain the high selectivity for ATF6 (Torres et al., 2019)**.**

Unfortunately, it seems that Ceapins may be toxic when administered in vivo, and this is probably the reason why there are still no studies reporting their applications in preclinical animal models.

One of the primary transcriptional targets of ATF6 is GRP78/BiP. This chaperone could also be another important druggable target since it plays a significant role in cancer cell survival, metastasis, and drug resistance. Some compounds have an inhibitory activity against the protein. One example is Epigallocatechin gallate (EGCG), a molecule extract from a green tea plant that binds the ATP-binding site. EGCG blocks the formation of the GRP78/Caspase 7 complex which is formed after overexpression of BiP, thus preventing the anti-apoptotic effects due to the inhibition of the caspase (Ermakova et al., 2006)**.**

In conclusion, the UPR plays a pivotal role in tumors, since it can induce both survival and apoptotic responses. It’s easy to imagine that this represents a mixed blessing for the development of cancer strategies targeting specific UPR components. For this reason, a deeper knowledge of the underlying mechanism is necessary. In particular, it would be important to establish the “driving branch” of UPR in different types of cancer in order to develop or improve the appropriate therapy. This conserved pathway could be targeted in order to actively induce cell death or, in contrast, to block its cell survival and adaptation responses. These approaches can be combined with other drugs with a different mechanism of action to greatly improve the efficacy of treatment.

4.1) p53 and cancer

TP53 is a tumor suppressor gene that plays a pivotal role in regulating different cellular functions. It was initially discovered in 1979 associated with simian virus 40 (SV-40) large T antigen in virally transformed cancer cells (Deleo et al., 1979). Several years after its discovery, the scientific community understood that the protein is a powerful tumor suppressor and not a proto-oncogene as researchers had initially assumed.

The p53 protein can be activated by many different cellular stresses, including replicative stress, DNA damage, aberrant oncogene expression, hypoxia, metabolic unbalance. These are all factors that can promote tumor initiation; unsurprisingly, the protein is one of the most mutated genes in human cancers. Once activated, the p53 protein acts as a transcription factor that forms homotetramers which bind to specific responsive elements in the DNA and controls expression of a large set of target genes (Cho et al.; El-Deiry et al., 1992; Friedman et al., 1993). Its activation results in wide range of cellular responses, such as apoptosis, senescence, cell-cycle arrest, DNA repair, and metabolic adaptations in order to restore homeostasis.

The protein activity is finely regulated by a complex system of post-translational modifications. Among these, the most important is mediated by the E3 ubiquitin ligase MDM2 which regulates p53 stability through a negative feedback loop. In specific, p53 activates the transcription of the mdm2 gene, then mdm2 targets p53 for ubiquitin dependent degradation (Freedman et al., 1999). In addition, phosphorylation, acetylation and sumoylation affect the protein in different ways, for example favoring the binding to specific sites in the DNA, changing the protein turnover, and altering interaction with other proteins (Kruse and Gu, 2009).

P53 post-translational modifications are modulated by a variety of pathways, in response to a variety of stress conditions, but all this complexity can be simplified by the concept that upon activation p53 has essentially a dual role: to trigger preliminary protective responses in order to restore cellular homeostasis, or to induce a definitive anti-proliferative response that includes cellular senescence or cell death by apoptosis.

4.1.1) Mutant p53: a Jedi that switched to the dark side of the force

As mentioned before, TP53 is one of the most mutated genes in cancer. Mutations are associated with adverse prognosis in many tumors, and germline TP53 mutations cause the Li Fraumeni syndrome, a rare but highly penetrant familial cancer predisposition disease (Kandoth et al., 2013; Malkin et al., 1990).

In the large majority of tumors TP53 undergoes missense mutations consisting in single residue substitutions. The most frequent (hotspot) mutations are within the DNA binding domain, blocking the ability to activate canonical target genes and thus inducing a loss-of-function phenotype. However, in many cases, the mutant protein (mutp53) also acquires new oncogenic properties, referred to as gain-of-function (or GOF). Hotspot mutations can be divided in two types: contact (e.g. R273H, R248Q, and R248W), that produce structural changes that directly alter DNA binding; and conformational (e.g. R175H, G245S, R249S, and R282H), that generate structural changes related with protein folding (Kim and Lozano, 2018).

There are many evidence that mutant p53 proteins not only lose their tumor suppressor role, but under various circumstances can act as oncogenes, promoting different hallmarks of cancer, such as cell proliferation, survival, reconversion of metabolism, invasion, metastasis, and chemoresistance.

The mechanisms of this GOF, its implications for cancer aggressiveness, and its potential relevance for therapy are currently the subject of intense research. Concerning mechanism, one relevant point is that mutant p53 is a very stable protein, and this certainly helps its new oncogenic activity. One of the mechanisms that contribute to its stabilization is the loss of the capability of binding the DNA. In this way, mutp53 does not induce transcription of MDM2, and is therefore released from the feedback mechanism controlling its turnover, resulting in its accumulation.

Another mechanism is the stable association of mutp53 with other proteins. One example is its interaction with components of the HSP90 chaperone machinery, involved in the resolution of proteotoxic stress by the elimination of the toxic accumulation of misfolded proteins (Terzian et al., 2008).

Despite losing the capability to bind DNA in a sequence-specific manner, mutant p53 proteins still maintain a strong impact on gene expression, typically by interacting with other transcription factors, modifying their activity or promoting the regulation of specific target genes.

One example that demonstrates how mutp53 can orchestrate and hijack gene transcription in order to promote cancer progression, proliferation and survival is the work of Gurtner et al. They found that mutp53, together with NF-kB and NFY, binds to the Mitogen-Activated Protein (MAP) Kinase Kinase 3 (MAP2K3) regulatory region in order to up-regulate its expression in different cancer cell lines. Since MAP2K3 is an upstream activator of p38 MAPK, they monitored the phosphorylation of its downstream target ATF2 and found that MAP2K3 knockdown inhibited cell proliferation and survival, thus indicating that the GOF of mutp53 can act also via MAP2K3 (Gurtner et al., 2010).

Because of its stability, mutant p53 is present in the nucleus but also in the cytoplasm of many cancer cells. Such cytosolic localization facilitates the formation of protein-protein interactions that normally do not occur, and this may contribute to its oncogenic GOF.

For instance, the Collavin group found that mutp53 proteins can bind and inactivate the tumor suppressor DAB2IP in the cytosol. DAB2IP is a RasGAP and adaptor protein that modulates various singnaling pathways, including those triggered by TNF, Interferon, and various growth factors (Bellazzo et al., 2016 review). Interaction with mutp53 leads to a block of JNK phosphorylation and the activation of NF-kB pathway upon TNFa treatment, promoting an aggressive response of cancer cells to inflammation (di Minin et al., 2014)**.** Also, the inhibition of DAB2IP by mutant p53 can increase AKT phosphorylation upon insulin treatment, thus sustaining tumor invasion and proliferation in conditions of hyperinsulinemia (Valentino et al., 2017).

One of the hallmarks of cancer cells is metabolic reprogramming, such as aerobic glycolysis and mitochondrial alterations. There are some evidences that mutp53 is also involved in these cellular deviations. Zhou et al., for example, found that the protein binds and inhibits Adenosine Monophosphate (AMP)-activated Protein Kinase (AMPK), leading to an increased lipid production, aerobic glycolysis, resulting in invasive cell growth (Zhou et al., 2014).

In the cytoplasm, mutant p53 extend its hegemony also to other cellular compartments. A recent work by Capaci et al., reported that mutp53 is able to modify the function and, more deeply, the structure of the Golgi Apparatus, by promoting HIF1a-induced activation of miR-30d. The consequences of this Golgi modification are the enhanced vesicular trafficking and secretory activity of cancer cells that, in this way, reinforce the malignant phenotype by changing the tumor microenvironment with the accumulation of soluble factors and elements that remodel the Extra-Cellular Matrix (ECM) (Capaci et al., 2020).

Indirectly, mutp53 can also modulate protein secretion through the transcription of the ER localized enzyme ectonucleside triphosphate diphosphohydrolase (ENTPD5). This event leads to stimulation of the calnexin/calreticulin-dependent quality control that could help cells to survive in proteotoxic conditions. By the upregulation of ENTPD5, mutant p53 promotes N-glycosylation, folding, and maturation of secreted proteins, augmenting cancer cell proliferation and survival (Vogiatzi et al., 2016).

As already mentioned, accumulating evidence suggest a fundamental role of mutp53 in the adaptation of cancer cells to proteotoxic stress. For example, Li et al., discovered a mechanism by which mutp53 promotes activation and stabilization of Heat Shock Factor 1 (HSF1) by stimulation of the EGFR/ErbB2/MAPK/PI3K signaling cascades. Once activated, HSF1 is also bound by mutp53 and this interaction allows the recruitment of both proteins to the promoter of HSF1 target genes, enhancing a transcriptional program that improves the resistance of cancer cells to proteotoxic stress (Li et al., 2014).

More recently, mutp53 was reported to upregulate genes involved in proteasome machinery. In particular, the protein directly interacts with the transcription factor NRF2 stimulating the expression of proteasome subunit genes. Enhanced proteasome activity in cancer cells augments their aggressiveness because of the increased turnover of tumor suppressors, such as CDK inhibitors or pro-apoptotic proteins(Walerych et al., 2016). In addition, enhanced proteasome activity also contributes to alleviate proteotoxic stress, favoring cancer cells' adaptation and survival (Mantovani et al., 2019).

Finally, mutant p53 is capable to modulate adaptation to proteotoxic stress by regulation of the UPR. Specifically, our group discovered that mutp53 increases cell survival upon ER stress by modulating the UPR. The mechanism is dual: on one hand mutp53 enhances activation of

the pro-survival effector ATF6; on the other hand, mutp53 dampens the pro-apoptotic signaling of IRE1a and PERK, as demonstrated by the reduction of the downstream targets JNK and CHOP.

Interestingly, our experiments also evidenced that ATF6 promotes two striking mutant p53-related oncogenic phenotypes: resistance to ERS-inducing drugs, and cell invasion. So, according to our model, mutp53-bearing cancer cells not only rely on ATF6 to resolve ER stress, but they also make use of this UPR branch to exert their aggressiveness (Sicari et al., 2019)**.**

The mechanism by which mutp53 sustains ATF6 remains unknown and is currently under investigation. However, some evidence in the literature suggests a link between ATF6 and p38 MAPK**.** In particular, Thuerauf and colleagues found that in cultured cardiomyocytes ATF6 can be phosphorylated by p38 MAPK, resulting in augmented transcriptional activity and the induction of several cardiac-specific genes, like Atrial Natriuretic Factor Gene (ANF). This p38-dependent phosphorylation of ATF6 could allow it to bind Serum Response Factor (SRF), which is able to bind Serum Response Elements (SREs) within the promoter of the ANF gene (Thuerauf et al., 1998)**.** Another work reported that p38-mediated phosphorylation of ATF6 in cells treated with Azacitidine, or with a constitutively active mitogen-activated protein kinase kinase (MKK6), another p38 activator, enhanced the ability of ATF6 to transactivate the GRP78/BiP promoter (Luo and Lee, 2002).

Interestingly, as already mentioned, there is some evidence that p38MAPK may be indirectly under the control of mutp53 via MAP2K3 (Gurtner et al., 2010).

Together, these studies suggest the existence of a functional link between three powerful cellular players: mutant p53, that is able to sense various different stimuli and generates appropriate responses in order to completely subvert normal cellular behavior (fig 3); ATF6, that plays a pivotal role in cell adaptation to ER stress, and possibly in cancer aggressiveness; and p38MAPK, that presides over a wide variety of cellular functions in the presence of stress.

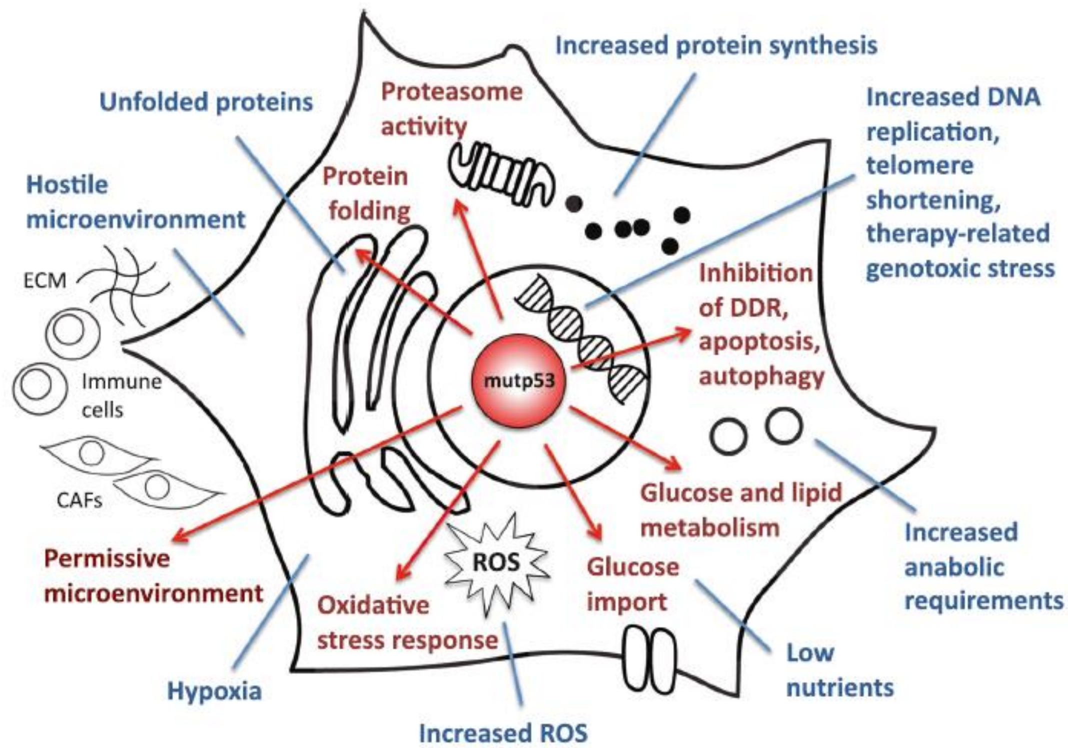


Fig. 3. Mutant p53 promotes adaptive responses to different stress conditions to support cancer progression. Cancer cells are constantly exposed to different stress stimuli (in blue). Mutp53 is sensitive to these intrinsic and extrinsic perturbations and is able to induce adaptive mechanisms that enhance cancer aggressiveness (in red) (Mantovani et al., 2019).

5.1) p38 MAPK: the multifaced protein

p38 is a protein ser/thr kinase that belongs to the superfamily of Mitogen Activated Protein Kinase (MAPK) and plays a pivotal role in converting cellular stimuli into a wide range of cellular processes, such as proliferation, stress response, apoptosis, migration, and survival. As all MAP kinases, p38 is activated by a cascade of upstream regulators, from MAPK kinase kinases (also known as MKKKs or MAP3Ks), to MAPK kinases (MKKs, MEKs, or MAP2Ks), to finally MAPKs. In humans there are four p38 kinases, encoded by different genes; of theses, p38alpha (official gene symbol MAPK14) is the most abundant and ubiquitously expressed (Martínez-Limón et al., 2020)**.**

p38MAPK is activated by a wide range of stimuli that trigger a cascade of phosphorylation events (fig.4). In particular, the activation occurs when the protein is phosphorylated on Tyr and Thr residues in its kinase subdomain (Doza et al., 1995)**.**The phosphorylation allows the protein to assume an open conformation useful for recognition of the substrate. As mentioned in a previous paragraph, MAP2K3 is one of the upstream activators of p38, together with MAP2K6 (Alonso et al., 2000; Derijard et al.1995) which in turn are activated by phosphorylation mediated by various upstream kinases, including ASK1 and TAK1 (Chen Wang et al., 2001)**.** P38 activity is also regulated by phosphatases, such as PP2A and PP2C, that remove the phosphate group on Thr residues in the activation motif, rendering the protein less active (Zhang et al., 2008)**.**

The fact that p38MAPK has a role in cancer development and progression is undisputable; however, it is not completely understood whether p38 acts as an oncogene or a tumor suppressor, because there are some evidences that it may belong to both categories.

For example, Gubern and colleagues demonstrated that upon stress p38 acts as a tumor suppressor, phosphorylating the N-terminal region of the Retinoblastoma (RB) protein and thus rendering it insensitive to the CDKs, which normally inactivate the protein through phosphorylation in a different region. In this way, p38 delays the entrance of cancer cells in S phase by reducing E2F-dependent transcriptional regulation. Indeed, through the expression of RB mutants that mimic p38-mediated phosphorylation, they observed a reduction of proliferation in several cancer cell lines (Gubern et al., 2016).

Intriguingly, p38 may have a role in sustaining tumor dormancy; in fact, in HNSCC, the inhibition of the kinase was able to reactivate the proliferation of quiescent metastases (Sosa et al., 2014).

Despite numerous studies describing the oncosuppressive role of p38, the scientific landscape is scattered with many others works indicating that the kinase may act as an oncogene, by enhancing cancer cell survival, migration, or resistance to stress and chemotherapeutic agents (Limon et al., 2020). In fact, compared to classic tumor suppressor genes that are frequently mutated in several cancer types, the p38 family does not show significant frequency of loss-of-function mutations in human malignancies. Results obtained from the sequencing of about 1500 samples have revealed that the frequency of p38 mutation in human cancers is lower than 1% (Pritchard and Hayward, 2013).

In contrast, there are evidence that p38 is often activated in cancer. For instance,when compared to the normal tissue, the activation of the p38 protein was found to be higher in a panel of 18 lung-resected tumors (Greenberg et al., 2002).

Several works reported a close relationship between p38 activation and metastasis. For example, Kumar et al., showed that p38 regulates the expression of various matrix metalloproteinases, including MMP2 and MMP9, and inhibition of the kinase reduced the invasion of bladder cancer cells in vitro (Kumar et al., 2010). Also, Demuth et al., found that in glioma cells p38 was strongly activated by MAP2K3, and this promoted tumor invasion, progression, and poor patient survival. They underlined the importance of this functional interaction by inhibition of the kinases, which led to a dramatic reduction of glioma invasiveness in vitro (Demuth et al., 2007). In addition, it has been shown that p38 activity is able to induce the expression of two strong pro-angiogenic molecules, Vascular Endothelial Growth Factor A (VEGFA) and Hypoxia Inducible Factor 1 (HIF1) in order to promote tumor vascularization and metastasis (Wagner and Nebreda, 2009).

In summary, these reports underline how unclear is the role of p38 in a tumor context, providing evidence to support both an oncosuppressive and an oncogenic role. Importantly, the pro-oncogenic activities of p38MAPK would make it a potentially interesting “druggable" target, at least in certain tumors and under certain conditions; however, to date, all clinical trials on p38 have given negative results, with no compound approved because of systemic

side effects in the heart, liver, and nervous system (Limon et al., 2020). Nonetheless, a better understanding of the role of p38MAPK in the cell response to various stress conditions may reveal actionable opportunities for combining p38 inhibition with other targeted therapies in the context of very specific tumor types.

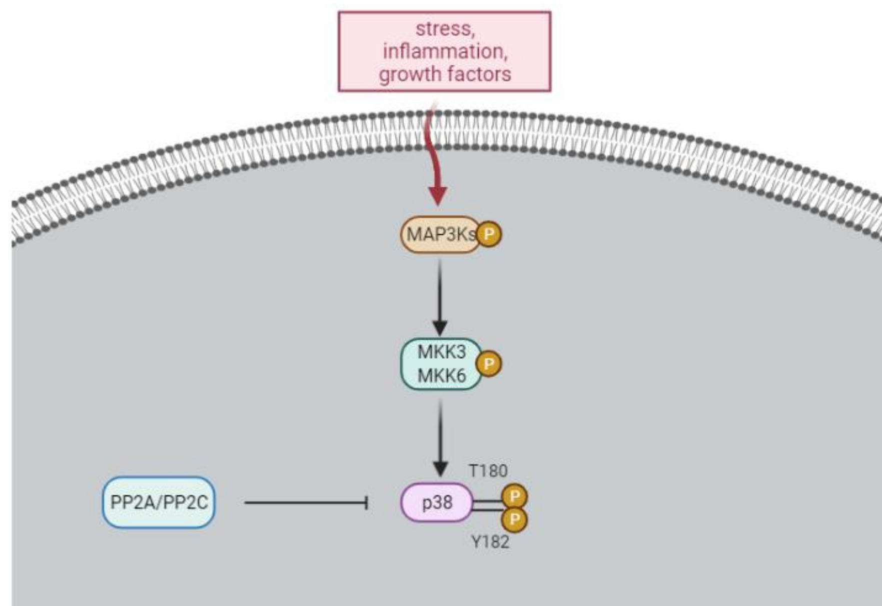


Fig. 4. Schematic representation of p38 activation. Following different stress stimuli, p38 is activated after dual phosphorylation on Thr and Tyr residues. PP2A and PP2C remove the phosphate group on Thr residues rendering the protein less active (Image create with Biorender).

3) Aim of the thesis

ER stress (ERS) is a condition that occurs when ER homeostasis is unbalanced. To cope with ERS, cells trigger a specific conservative program known as Unfolded Protein Response (UPR). The UPR is often deregulated in cancer cells that make use of it as a survival strategy to overcome proteotoxic stress and promote tumor progression (Wang & Kaufman, 2014). Clearly, the UPR intercepts other pathways activated in cancer cells, and a detailed understanding of the genetic determinants that favor adaptation to ER stress may uncover useful targets for cancer therapy.

One of the most mutated genes in cancer is TP53, and the great majority of tumors express a mutant p53 protein that acquires "new" oncogenic functions and may promote cancer progression, aggressiveness and resistance to chemotherapeutics; interestingly, mutp53 may also contribute to promote adaptation to all the stresses to which cancer cells are subjected within a growing tumor (Mantovani et al., 2019). In a recent work, we found that mutp53 is able to modulate the UPR, rendering cancer cells more resistant to ER stress. We observed that mutp53 dampens IRE1α and PERK signaling, with reduced activation of proapoptotic effectors, but promotes activation of ATF6, that mediates a pro-survival and potentially pro-aggressive response to ER stress. Accordingly, we showed that ATF6 inhibition, alone or in combination with mutp53 inhibitors, could increase ERS-induced cell death in a TNBC cell line (Sicari et al., 2019). We therefore concluded that by sustaining ATF6 activation, mutp53 can render cancer cells able to endure chronic ER stress, thus contributing to cancer aggressiveness. However, the underlying mechanism that allows mutp53 to reshape the UPR is still an open question.

Aim of this Thesis is to shed light on the mechanism by which mutant p53 selectively sustains activation of the ATF6 arm of the UPR upon ER stress. Starting from previous studies suggesting that ATF6 may be activated by p38MAPK, and that p38MAPK may be activated by mutant p53, I set out to test whether mutant p53 can support ATF6 activation through enhanced p38 activity, and eventually define the molecular circuitry involved in such regulation.

4) Results

We previously found that depletion of mutant p53 in cancer cells reduced both the basal processing and ERS-induced cleavage of the UPR sensor ATF6. We also observed that depletion of ATF6 reduced the cellular viability and invasion and sensitized cells to ER stress, suggesting that its activation confers a pro-oncogenic phenotype to cancer cells. Therefore, the increased activation of ATF6 represents a relevant mutp53 gain-of function and may be targeted for therapy – as we already showed (Sicari et al 2019). Since mutp53 does not induce changes in ATF6 transcription, I supposed that the action of mutp53 could be post-translational. I decided to focus on p38 MAPK, a protein that belongs to the family of Mitogen-Activated Protein Kinases, which is able to respond to different stress stimuli. As discussed in the introducion section, there are two main evidences linking ATF6 to p38 in the literature: I) ATF6 can be phosphorylated in its N-terminal region by a member of the MAPK family (Thuerauf et al., 1998); II) MAP2K3, an upstream activator of p38, is transcriptionally regulated by several p53 mutants that bind to its regulatory regions in a complex with NFY and NF-κB (Gurtner et al., 2010). Starting from these observations I hypothesized that mutp53 may regulate ATF6 via p38.

1) Mutp53 sustains the activation of p38 upon ER stress

In order to understand the molecular mechanism by which mutp53 sustains ATF6 arm of UPR, in particular if the effect is exerted via p38 MAPK, we started to assess if mutp53 depletion could impact on p38 activation during ERS. We used MDA-MB-231, a triple-negative breast cancer cell line bearing p53 R280K mutation and used a retroviral vector expressing a p53-specific shRNA in order to stably silence p53. We also transfected a p53-specific siRNA already tested and validated (Sicari et al., 2019) in two other aggressive cancer cell lines: PANC-1, a pancreatic cancer cell line with p53 R273H, and DU145, a prostatic cancer cell line with p53 F223L and V274F mutations. To mimic stress conditions, cells were treated for 2, 4 and 6 hours with 1µM Thapsigargin (Tg), a specific inhibitor of sarcoplasmic/endoplasmic reticulum Ca2+-ATPase (SERCA) able to induce a decrease in ER calcium levels. In this way calcium-dependent

ER chaperones, such as calnexin, lose their activity leading to the accumulation of unfolded proteins. To measure p38 phosphorylation, western blot experiments were performed. As shown in figure 1A, the knockdown of mutp53 reduced ERS-induced phosphorylation of p38α in all cancer cell lines, suggesting that mutp53 is required for efficient p38 activation. To further confirm the data, we asked if overexpression of mutant p53 could increase p38 phosphorylation even without induction of ER stress. We transfected p53-null H1299 lung cancer cell line for 48 hours using expression vectors bearing two different p53 mutants, R280K and R175H. As shown in figure 1B, the ectopic expression of mutp53 increased the basal phosphorylation of p38. We also noticed that the R280K mutant seems to be more efficient than the other. Together, these data confirm that mutp53 stimulates p38MAPK activation.

2) Exploring a possible mechanism linking mutant p53 to enhanced p38 activation

We asked if mutant p53 might enhance p38 activation by increasing the expression levels of upstream positive regulators of p38, such as the MAP kinase-kinase MAP2K3, as reported by Gurtner (A. Gurtner et al., 2010) or other upstream kinases such as MAP2K6 or MAP3K5 (ASK1) and MAP3K7 (TAK1). As a first approach, we analyzed public data reporting gene expression changes induced by mutant p53 depletion in five TNBC cell lines (Walerych et al., 2016; (Girardini et al., 2011). We found no major common effects, however knockdown of mutp53 variably reduced expression levels of at least one p38-activating upstream kinase in all cell lines, with ASK1 and/or TAK1 being affected in four out of five cell lines (Table 1).

Next, we performed qRT-PCR analysis to monitor expression of the main p38-activating kinase (MAP2K3) in PANC-1 and MDA-MB-231 cells after 48 hours of mutp53 silencing. As reported in figure 2, the knockdown of mutp53 resulted in a decrease of MAP2K3 in both cell lines.

Although not conclusive, these data suggest that mutp53 may facilitate p38 activation in different cellular backgrounds by exerting a positive effect on the expression levels of some of its upstream kinases, in particular MAP3K5 and MAP2K3 (MKK3).

3) p38 affects ATF6f levels upon ER stress

To validate the hypothesis that mutp53 promotes ATF6 activity during ER stress via p38 MAPK, we examined the effects of p38 knockdown in breast cancer cells MDA-MB-231 treated with Tg at different time points using a p38α specific siRNA already tested and validated in the lab. The results shown in figure 3A confirm that depletion of the protein reduced the levels of the active fragment of ATF6 as compared to the control, especially at longer times of treatment (i.e. 4-6h). Importantly, the same results were obtained treating pancreatic, breast, and prostatic cancer cells with the specific p38α and p38β inhibitor SB203580 at the concentration of 10 uM (fig. 3B). Since chemical inhibitors may have nonspecific off-target effects, we treated MDA-MB-231 with SB202190, another p38 inhibitory drug, confirming the same ATF6f reduction (fig. 3C). The inhibitors of p38α and p38β block the catalytic activity of the proteins by occupying the ATP binding pocket of the kinase, that can still be phosphorylated by upstream kinases; as a consequence, we observe accumulation of catalytically inactive but phosphorylated p38α, that represent a positive control of efficient inhibition.

To complement these data, we decided to test the effects of stimulating p38α activation by overexpression of its upstream kinase, with or without ER stress. We transiently overexpressed a constitutively active form of the upstream p38α activators MAP2K3 or MAP2K6 for 48 hours in PANC1 cells and MDA-MB-231 cells. To induce ER stress, we treated cells with Tg for 4 hours. In line with the other experiments, overexpression of upstream MAP Kinase kinases increased p38α phosphorylation and production of the active cleaved fragment of ATF6 as compared to the empty vector. Noticeably, this was better appreciated in the absence of ER stress (fig. 3D).

These data strongly support the notion of a possible link between the activity of p38 and the levels of active ATF6f. To further test this hypothesis, we analyzed the transcriptional activity of ATF6f using a specific luciferase (LUC) reporter construct which harbors five repetitions of the ATF6 binding sites (Shen et al., 2002). MDA-MB231 cells were transfected with the ATF6-LUC plasmid and treated with Tg to induce ER-stress in the presence or absence of the p38 inhibitor SB203580. As shown in figure 3E, inhibition of p38 significantly reduced the activity of the ATF6 reporter.

To reinforce this observation, we assessed the transcriptional activity of ATF6 by measuring the mRNA levels of endogenous ATF6 target genes in cells treated with Tg in the absence or in the presence of p38 inhibitor. In particular, I analyzed by qRT PCR the ATF6 targets XBP1, CHOP, Bip, EDEM, ERO1, HERPUD and EDEM. As reported in Figure 3F, under these conditions, none of the genes analyzed was significantly affected by p38 inhibition, with the exception of XBP1 that in some experiments showed a clear repression.

Taken together, these results support the hypothesis that p38α contributes to positively regulate ATF6f levels and activity, although its impact on ERS-induced expression of endogenous target genes remains to be established.

4) Evidence that p38 activity modulates ATF6f stability

The data we previously collected suggest that p38 MAPK contributes to regulate ATF6f levels and activity. To understand the biochemical basis of this action, we asked whether p38 might influence the stability of the cleaved ATF6f fragment. We therefore aimed to measure the half-life of the protein in presence or absence of p38 inhibition.

Upon ER stress conditions, ATF6 moves from ER to Golgi where it is sequentially cleaved by Site-1 protease (S1P) and the metalloprotease Site-2 protease (S2P) to release the active ATF6f fragment (Ye et al., 2000). To monitor ATF6f turnover it is necessary to efficiently block ATF6 cleavage, and this should be done under conditions that minimally affect other biological pathways and do not further increase ER stress. We tested various approaches, and eventually developed a specific protocol (fig. 4A) where we first triggered ATF6 cleavage by inducing moderate ER stress, and then blocked further ATF6 maturation using the S1P inhibitor 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF). AEBSF efficiently prevents cleavage of ATF6 (Okada et al., 2003), so we could estimate the turnover of ATF6f by immunoblotting at different times after treatment.

In a series of preliminary experiments, we set the conditions that would minimize residual ATF6 cleavage, without significant cell toxicity, in a time frame of 4 hours. Specifically, we first treated cells for 90 min with 0,1 μM Tg in order to induce ATF6 cleavage. Next, cells were washed to remove the ERS-inducing drug. Finally, we added fresh medium containing 300 μM

AEBSF to block further ATF6 cleavage. Levels of ATF6f fragment were evaluated by immunoblotting at time 0, and after 2 and 4 hours.

When necessary, we pre-treated cells with 10 µM of SB202190 for 1hour in order to block p38MAPK activation. To keep the kinase blocked, the drug was maintained in the medium for all the experiment.

Using this protocol, we monitored ATF6f turnover rate in MDA-MB-231, PANC1 and DU145 cell lines (fig. 4B) with or without SB202190. In all cell lines, p38MAPK inhibition caused a reduction in ATF6f half-life, particularly evident at 4 hours. This strongly indicates that p38 MPAK contributes to the stability of the active ATF6f fragment.

We then asked if depletion of mutant p53 would have the same effect on ATF6f stability. Using the same protocol, we measured ATF6f half-life in MDA-MB-231 cells in which mutp53 was silenced by stable expression of a specific shRNA (fig 4C). Preliminary results indicate increased turnover of ATF6f in p53 knockdown cells as compared to cells transduced with a control shRNA, in line to what observed with p38 inhibitor.

Taken together, these observations suggest that p38MAPK stabilizes ATF6f during ER stress, thus increasing the adaptation potential of the cells and this effect may be amplified in cancer where expression of mutant p53 proteins fosters p38 activation.

5) Inhibition of p38MAPK sensitizes cancer cells to ERS

Chemotherapeutic drugs directly or indirectly can induce ER stress, and cancer cells chronically activate the UPR to avoid ERS-induced death. In this perspective, UPR can sustain not only survival, but also chemoresistance of tumor cells (Chen and Cubillos-Ruiz, 2021b; Yu et al., 2021). Accordingly, inhibition of adaptive responses to ER stress represents a promising strategy to improve chemotherapy.

p38 MAPK has a controversial role in cancer. As discussed in the introduction, p38 can behave as a tumor suppressor, but it can also support cancer progression by increasing migration, survival, and resistance to stress and chemotherapy drugs (Limon et al., 2020; Wagner et al.,

2009; Han et al., 2022). Given the availability of efficient inhibitors, the oncogenic role of the protein may be exploited to develop new anticancer strategies.

Based on our results on ATF6 activation, we asked whether the inhibition of p38 could specifically sensitize cancer cells to ER stress.

At first, we wanted to assess if during ER stress SB203580 exerted a cytotoxic effect on cells bearing p53 mutations. To measure cell viability, we used ATP-lite, a luminescence assay that can assess the effects of a wide range of drugs, by monitoring intracellular ATP.

We treated cells with different concentration of p38 inhibitor SB203580, alone or in combination with 1 µM of Tg for 24 hours. As shown in figure 5A, the treatment of cells with SB203580 at concentrations up to 10 µM has no cytotoxic effect in all cell lines tested, although it’s possible to see a decrease in cell viability at the highest concentration of 100 µM. In contrast, the combined treatment with Tg increased the cytotoxicity of SB203580, evident also at relatively low concentrations of SB. Under these conditions, pancreatic cancer PANC1 cells displayed the highest sensitivity to p38 inhibition.

Then, in order to evaluate whether p38 inhibition would sensitize cells to lower doses of ER stress, we treated MDA-MB-231, PANC1 and DU145 cancer cells for 24 hours with various concentrations of Tg, with or without another p38 inhibitor, SB202190, at the maximal non-toxic concentration of 10 µM. As shown in figure 5B, the combined treatment with p38 inhibitor increased sensitivity to 1 µM Tg in all three cell lines. The decrease in viability correlated with evident signs of cellular distress (Fig 5C). Under these conditions, a moderate although not significant decrease in viability was observed already at 0.5 µM Tg. Notably, PANC1 cells showed great sensitivity to p38 inhibition, with significant reduction in viability at the lowest Tg concentration of 0.1 µM (Fig. 5B). This observation confirms that cancer cells with mutant p53 can rely, to various extent, on p38MAPK to survive under ER stress, and this is particularly evident in PANC1.

To understand the relevance of mutant p53 in this circuit, we tested the impact of p38 inhibition in the p53-null prostate cancer cell line PC3. Interestingly, we did not observe increased sensitivity to Tg-induced ER stress in PC3 treated with SB203580 (Fig. 5D), in striking contrast with DU145, a prostate cancer cell line with mutant p53. We then tested another p53-null cell line, lung cancer H1299, and observed yet another pattern of response: in these

cells, treatment with SB203580 alone significantly increased cell viability, as measured by ATPlite. Upon ER stress, viability was reduced to levels comparable to SB-untreated controls (Fig. 5C).

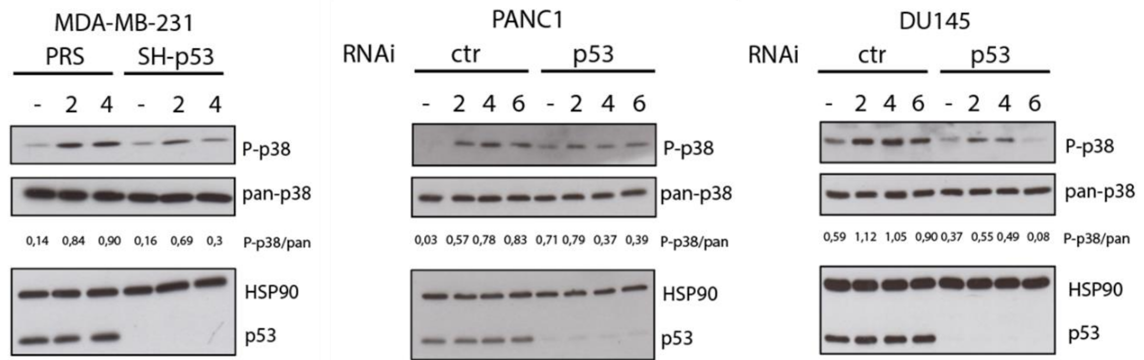
Although not definitive, these observations confirm that p38 plays an important role in the response of cancer cells to ER stress, in particular in cells bearing mutation in p53, protecting them from ERS-induced cell death, possibly by increasing the stability of ATF6f.

These data also suggest that p38 inhibitors may be considered for pharmacological approaches aimed to sensitize cancer cells to endogenous or therapy-induced ER stress.

5) Figures

Figure 1

A



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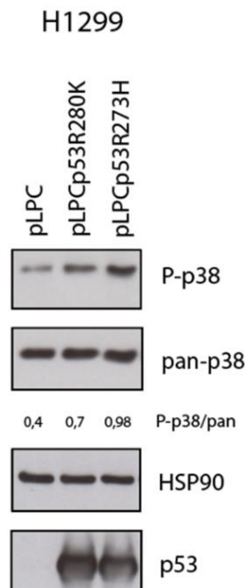


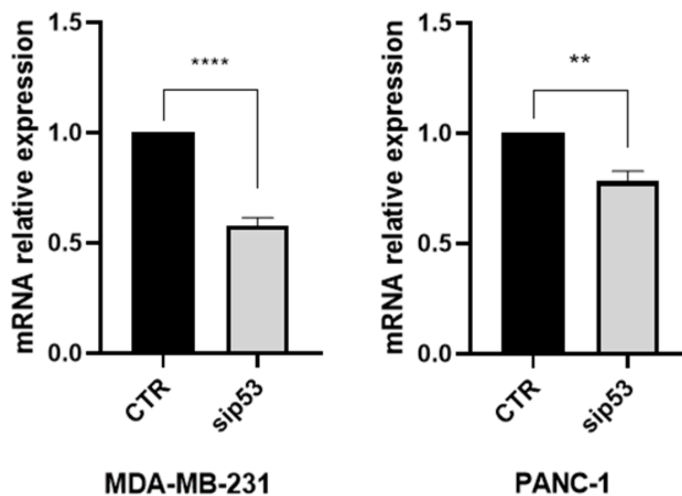
Figure 1. Mutp53 exerts a positive effect on p38 MAPK.

A. Depletion of mutp53 reduces p38 phosphorylation. MDA-MB-231 stably deplete for mutp53, were treated for Tg 1 µM as indicated. PANC1 and DU145 cells were transfected with p53 or control siRNA. After 48 hours, cells were treated with Tg 1 µM for 2, 4 and 6 hours as indicated. Phosphorylated and total p38α were detected by immunoblotting with HSP90 as loading control and p53 as silencing control. **B. Ectopic expression of mutp53 increased p38 phosphorylation in null p53 cancer cells.** H1299 cancer cells were plated in 6mm dishes and transiently transfected with pLPC-p53R280K, pLPC-p53R273H or pLPC empty vector as a control. Phosphorylated and total p38α were detected by immunoblotting with HSP90 as loading control and p53 as transfection control.

	BT549	HCC1395	MB468	SUM149	MB231	MB231 (*)
MAPK14 (p38)						
MAP2K3						
MAP2K6						
MAP2K4						
MAP3K1-4 (MEKK)						
MAP3K5 (ASK1)						
MAP3K7 (TAK1)						
MAP3K9-11 (MLK1-3)						
TAOK1-3						
DLK1-2						

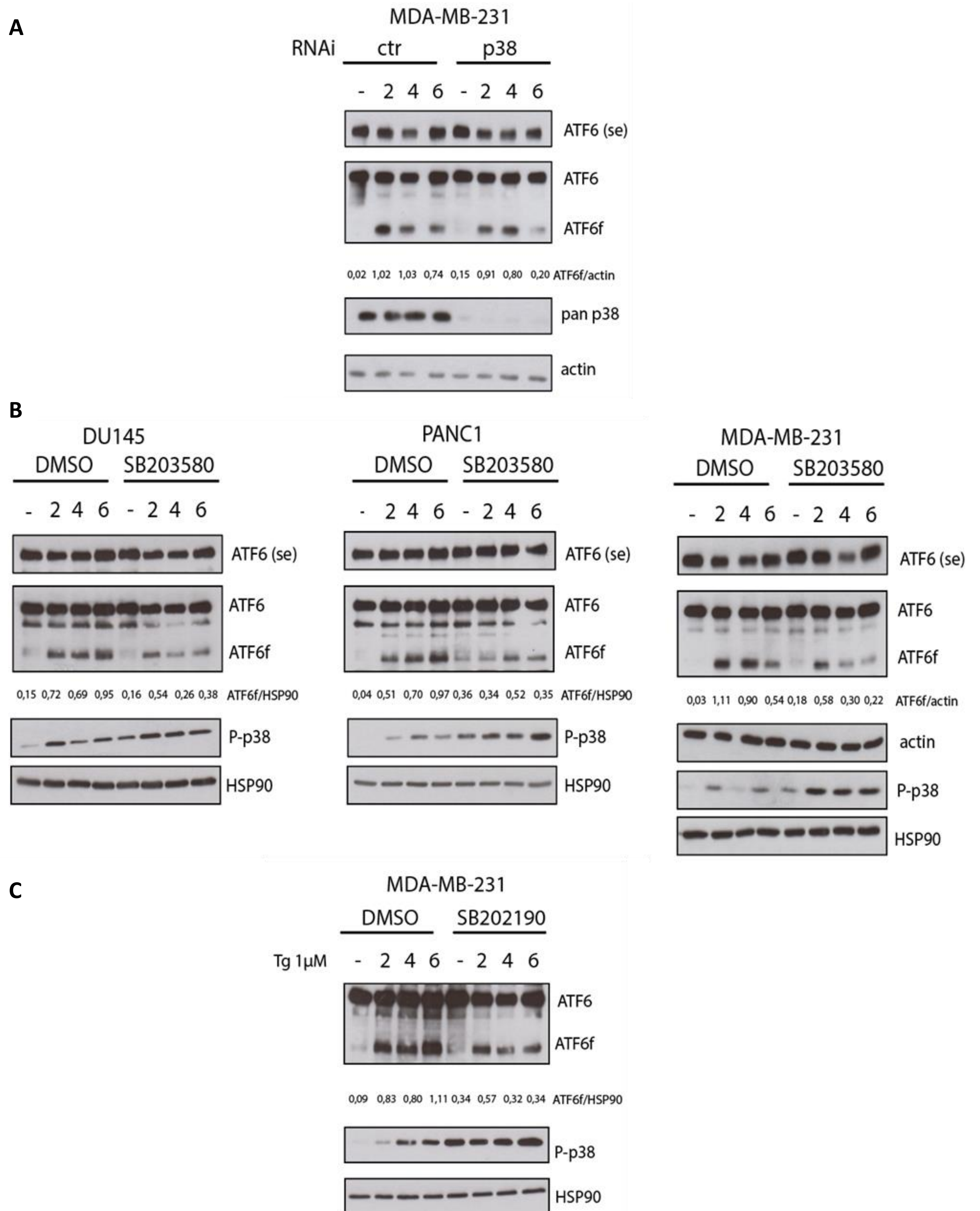
Table 1. A survey of mutant p53-dependent expression of p38 activating kinases in TNBC cell lines. Colored boxes indicate genes downregulated upon mutp53 knockdown (Orange, logFC <0; red, logFC <-0.6; Adjusted P.val <0.05). All data from GSE68249 (Walerych et al., 2016), except (*) from GSE26262 (Girardini et al., 2011).

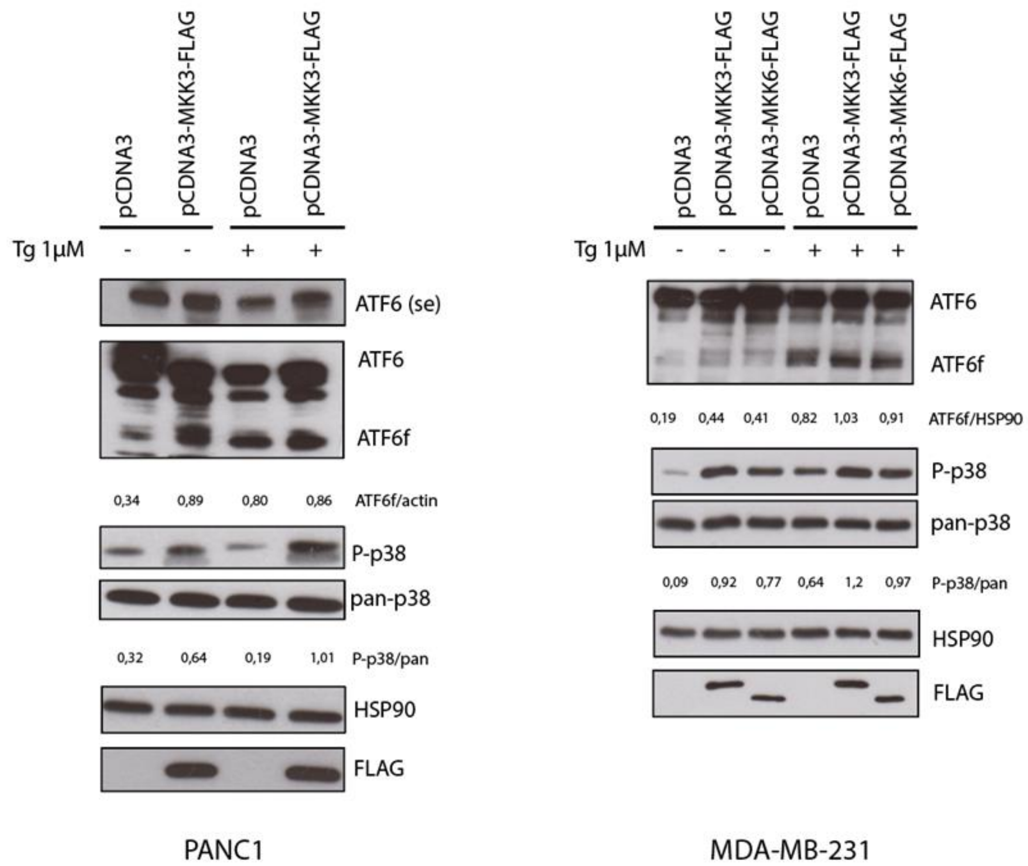
Figure 2



A. Mutp53 depletion alters MKK3 mRNA levels. PANC1 and MDA-MB-231 cells were plated in 6 mm dishes and transiently silenced for mutp53 (sip53) or with a control siRNA (CTR). After 48 hours cells were harvested for RNA extraction. RT-qPCR data are normalized to H3 and compared to the control (mean ± SD; n = 3;*p < 0.01).

Figure 3





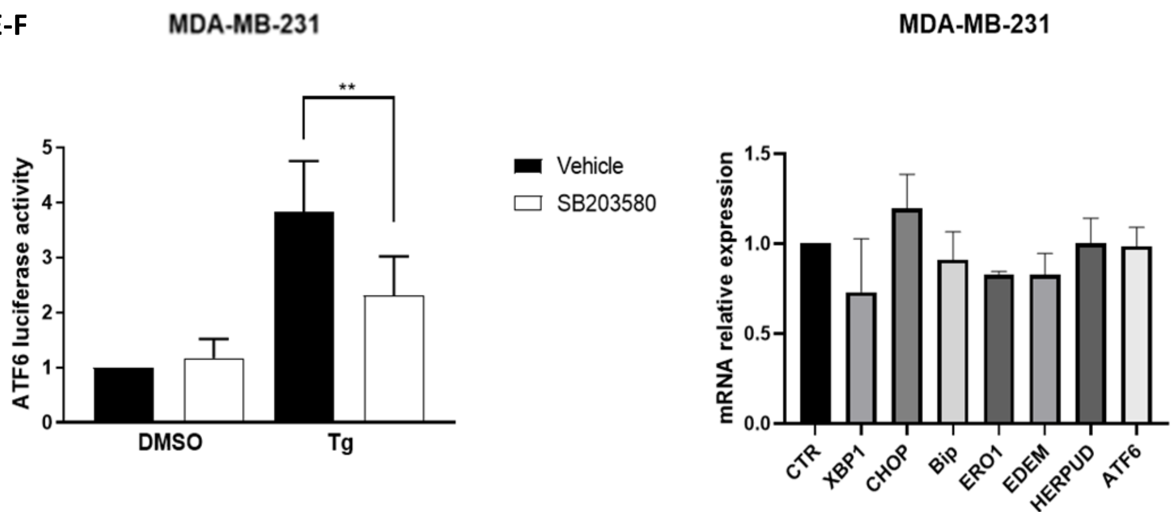


Figure 3. p38 impact on ATF6f levels and activity upon ER.

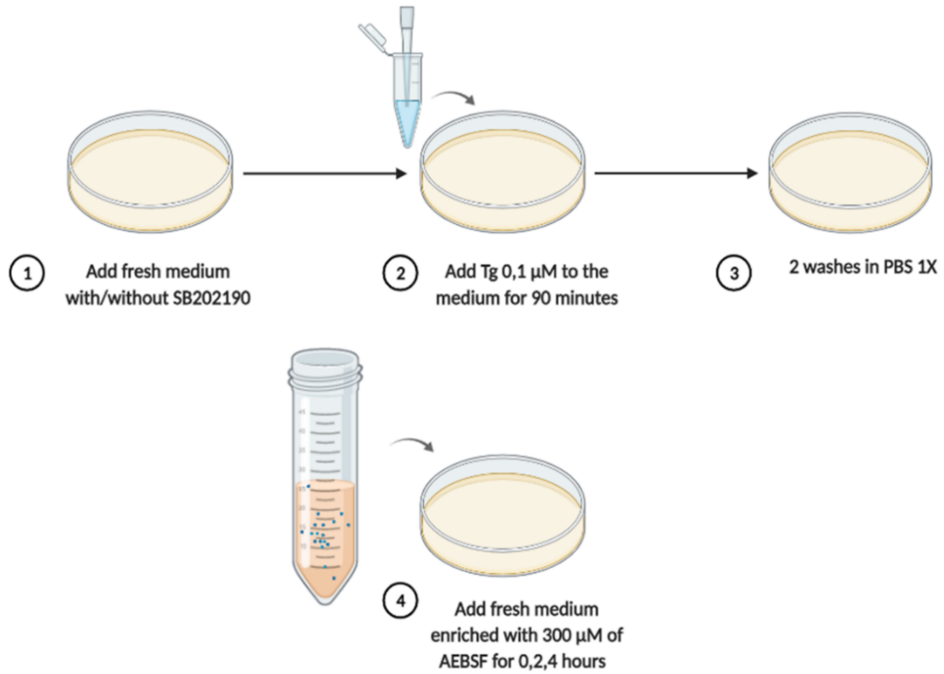
A. Depletion of p38α reduced ATF6f levels in breast cancer cell line MDA-MB231. Cells were transiently silenced for p38α (sip38) or with control siRNA (CTR). After 48hours cells are treated with 1μM ER-stress inductor

Thapsigargin (Tg) for 2, 4 and 6 hours. p38α, cleaved and total ATF6 were detected by immunoblotting using actin as loading control. **B/C. p38 inhibitors SB203580 and SB202190 reduce ATF6 cleavage in cancer cell lines.** DU145, PANC1 (B) MDA-MB-231 (B and C) cells were pre-treated for 1 hour with SB203580 or vheicle before the treatment with Tg as in A. Phosphorylated and total p38α, cleaved and total ATF6 were detected by immunoblotting with actin or HSP90 as loading control.

D. Over-expression of p38's upstream activators enhance its phosphorylation and production of ATF6f. Cancer cell line are transiently transfected with pCDNA3-MKK3-FLAG and pCDNA3 empty vector (PANC1) and with pCDNA3-MKK3-FLAG, pCDNA3-MKK6-FLAG and pCDNA3 empty vector as a control (MDA-MB-231) for 48 hours. Cells are subsequently treated with Tg 1µM or DMSO as a control for 4hours. Phosphorylated and total p38α, total and cleaved ATF6 were detected by immunoblotting with HSP90 and actin as loading control and anti-Flag as transfection control. **E) p38α inhibition dampens ATF6 transcriptional activity.** MDA-MB-231 cells are transfected for 48 hours with p5xATF6-GL3 reporter plasmid and pCMV-Renilla reporter plasmid to normalize for transfection efficiency. Cells were then pre-treated with SB203580 for 1 hour and then with Tg or DMSO as a control for 6 hour before processing for luciferase assay (mean ± SD; n = 3;**p < 0.01). **F) p38 inhibition reduces ATF6 transcriptional target.** MDA-MB-231 cells were plated in 6 mm dishes and treated with SB203580 for 6 hours. At the end of the indicated time, cells were harvested for RNA extraction. RT-qPCR data are normalized to H3 and compared to the control (mean ± SD; n = 3;*p < 0.01).

Figure 4

A



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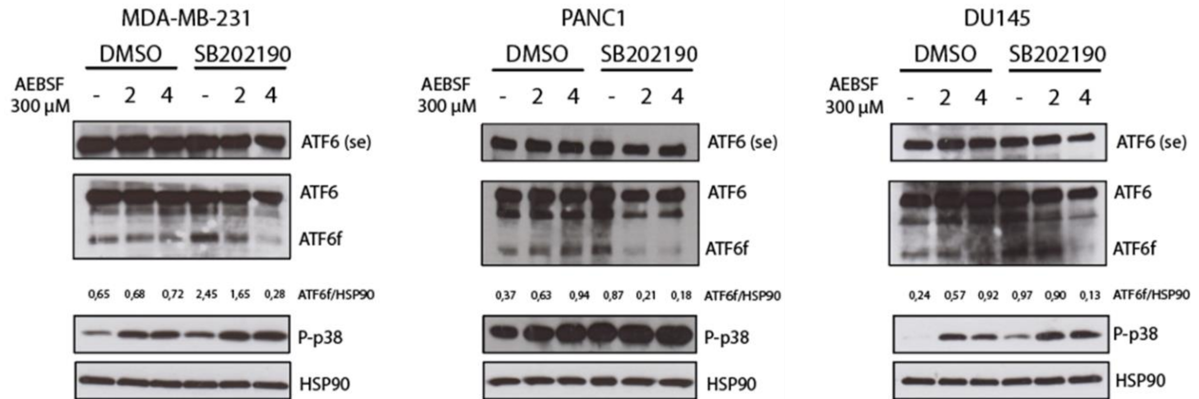

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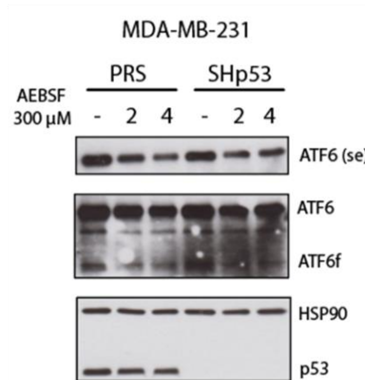
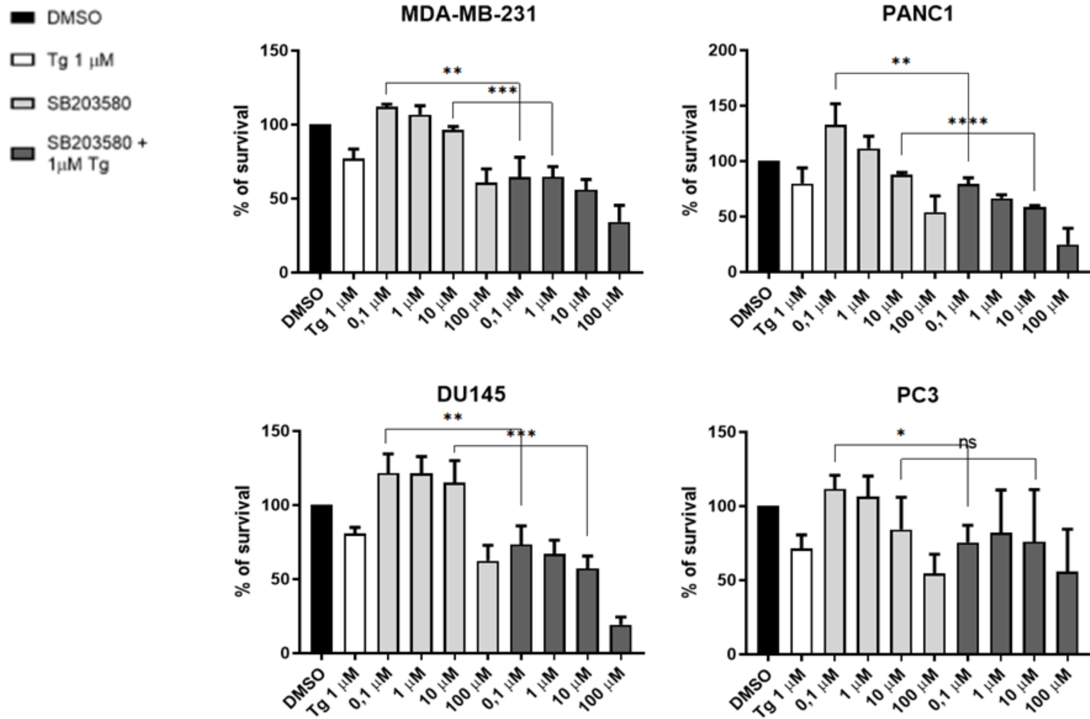
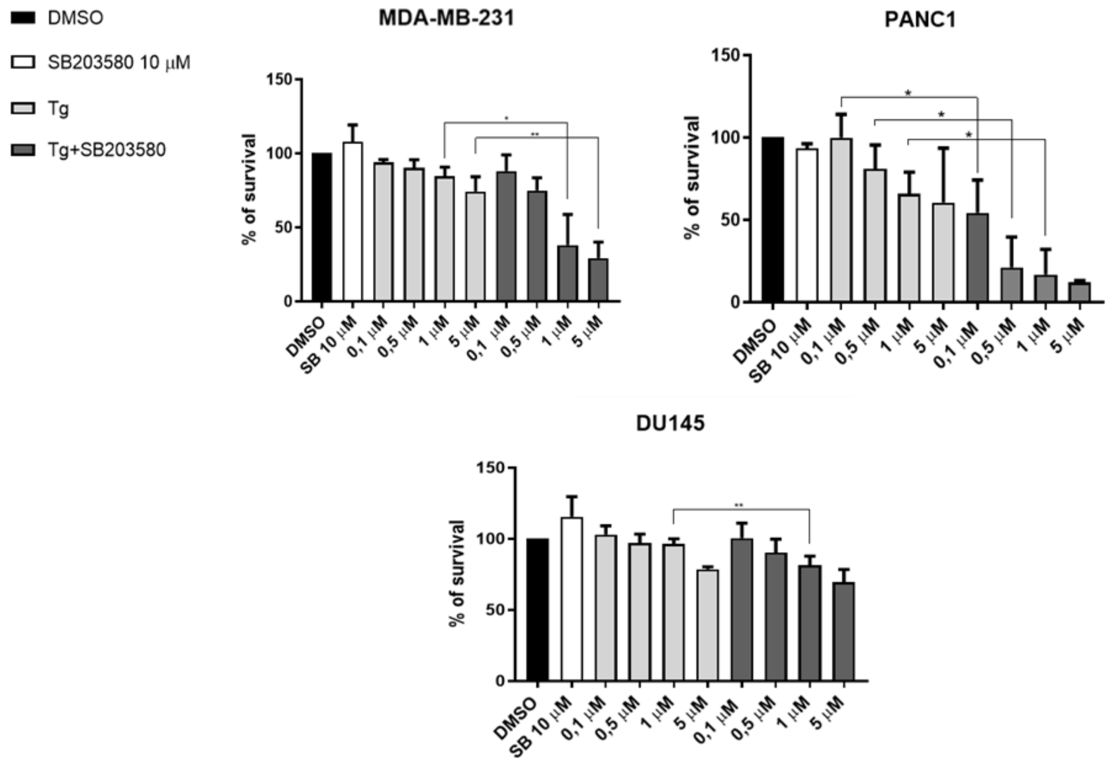


Figure 4. p38 stabilizes ATF6 fragment upon ERS.

A. ATF6f half-life assay. Schematic representation of the assay to detect ATF6f half-life during ERS**. B. p38 inhibition reduces ATF6f half-life.** MDA-MB-231, PANC1 and DU145 cell lines were treated following the assay previously described. Cleaved and total ATF6 were detected by immunoblotting with HSP90 as loading control and phosphorylated p38 as control of its inhibition**. C. Depletion of mutp53 shorten ATF6f half-life**. MDA-MB-231 cells stably depleted for p53 were treated as B. Cleaved and total ATF6 were detected by immunoblotting using HSP90 as loading control and p53 as control of depletion.

Figure 5





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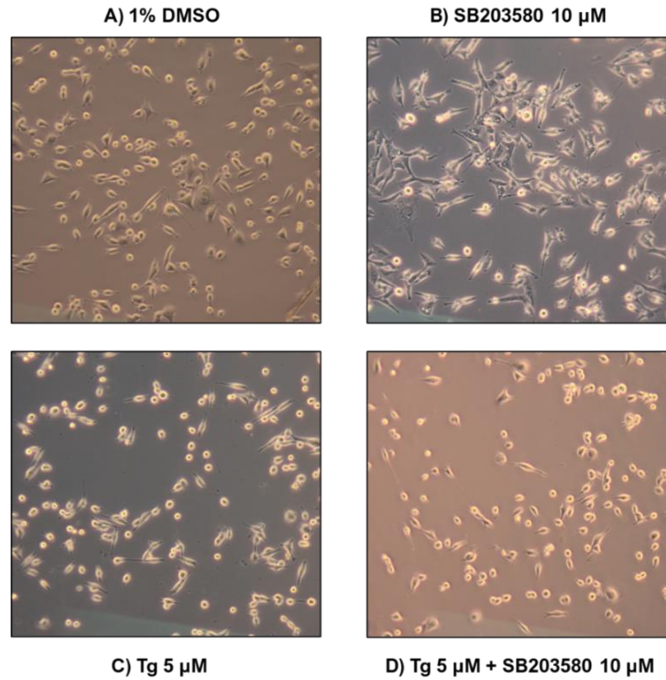

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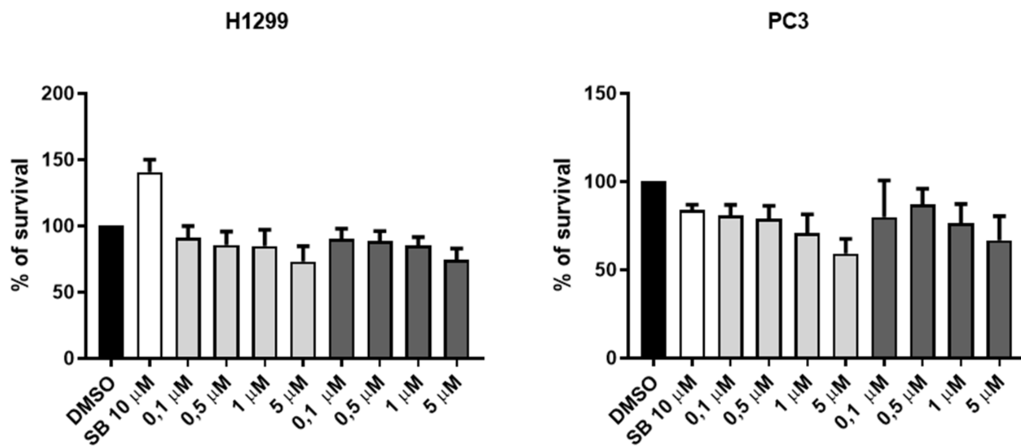

Figure 5. Inactivation of p38 increase cancer cells ERS induced cell death. A) MDA-MB-231, PANC1, DU145 and PC3 cells were plated in 96 wells. After 24 hours, cells were treated with SB203580 at the indicated concentration and with Tg 1 µM alone or in combination. After 24 hours, ATP-lite viability assays were performed. Graphs represent cells viability normalized to DMSO (mean ± SD; n=3; ***P<0.001, **P<0.01). **B-D)** DU145, MDA-MB-231, PANC1, H1299 and PC3 cells were plated in 96 wells. After 24 hours, cells were treated with Tg at the indicated concentrations and with 10 µM SB202190 alone or in combination. After 24 hours ATP-lite viability assays were performed. Graphs represent cells viability normalized to DMSO (mean ± SD; n=3; ***P<0.001, **P<0.01). **C)** Effect of the treatment with 1% DMSO (A), 10 µM SB203580 (B), 5 µM Tg (C), 10 µM SB202190 + 5 µM Tg (D). MDA-MB-231 cells were plated in 6 wells plated and treated as B.

6) Discussion and conclusion

Altered Endoplasmic Reticulum (ER) homeostasis leads to the accumulation of unfolded or misfolded proteins, generating a condition known as ER stress. Highly proliferative tumors are exposed to several intrinsic and extrinsic factors that may alter the normal function of the endoplasmic reticulum, generating chronic stress conditions. To cope with this, cancer cells can take advantage of the Unfolded Protein Response (UPR) to better tolerate persistent ER stress. Unlike normal cells, the cancerous counterpart develops mechanisms to bypass the cell death mechanisms triggered by sustained UPR; under various conditions, activation of this pathway can actually sustain and promote malignant transformation and progression. In this scenario, the specific role of each of the UPR sensors IRE1a, PERK, and ATF6 is currently under investigation because of their capability to promote the activation of pathways which can both lead to survival or induce apoptosis.

In this Thesis, I focused on ATF6, that, unlike the other sensors, acts directly as a transcription factor in response to ER stress. The regulatory mechanism or the specific interactions that control ATF6 activation and function are still incompletely understood, as well as the conditions that render the protein able to promote cell survival and cancer progression rather than cell death and cancer suppression. A deeper knowledge of these mechanisms is needed in order to clarify how activation of the UPR can sustain cancer.

One of the most mutated genes in human cancer is TP53. Through its missense mutation, the now "ex" guardian of the genome loses its tumor-suppressive activity and acquires new oncogenic properties. This gain of "new" function is exerted through a wide range of mechanisms (Mantovani et al., 2019). For example, mutp53 can bind to other transcription factor in order to subvert the normal cellular activity in favor of responses aimed at promoting a pro-oncogenic phenotype. To stay on the subject, Gurtner et al., revealed that the increased MAP2K3 expression driven by mutp53's golpe with NF-kB and NFY is responsible for the increased cell proliferation and survival (Gurtner et al., 2010). Mutp53 may extend its oncogenic activity even modulating the UPR. We recently described a "new" gain-of-function of the protein in response to ER stress, exerted by dampening IRE1a and PERK activation and by selectively sustaining the activity of ATF6, thus promoting two striking oncogenic phenotypes: the resistance to ERS-inducing drugs, and cell invasion (Sicari et al., 2019).

In this Thesis, I aimed to explore one of the possible mechanisms by which mutp53 could sustain ATF6 branch of the UPR, focusing on the role of the stress-kinase p38MAPK.

We based our work on previous studies reporting that p38MAPK could phosphorylate and activate ATF6fincreasing is transcriptional activity (Thuerauf et al., 1998; Luo and Lee., 2002), and on more recent observations that linked mutp53 to MAP2K3 (Gurtner et al., 2010), an upstream activator of p38MAPK. We also analyzed public microarray data and confirmed that the knockdown of mutp53 could reduce the expression levels of some p38-activating upstream kinases in breast cancer cell lines (Table 1).

On these premises, we started by analyzing the effects of mutp53 on ERS-induced p38 activation in different cancer cell lines bearing different missense TP53 mutations. We found that the knockdown of mutp53 reduced ERS-induced phosphorylation of p38α in all cell lines. This observation is in line with the concept that mutp53 and p38 are both sensitive to stress stimuli and confirm the existence of a regulatory interaction. Accordingly, overexpression of different mutants of p53 augmented the activation of the protein, as demonstrated by the increased phosphorylation of p38. Notably, this was observed even in the absence of drugs that would induce ER stress, confirming that high levels of mutant p53 protein can facilitate/promote/enhance activation of p38MAPK.

These encouraging results induced us to start thinking that these proteins may be elements of an axis that plays an important role for cancer cells survival in a context of ER stress. For this reason, we decided to assess the effect of p38 inhibition on the cleaved fragment of ATF6 (ATF6f). Using different strategies, such as RNA interference and drug-induced p38 inhibition, we proved that upon ER stress, the depletion or inactivation of p38 induces a reduction of the amount of ATF6f. In line with other works present in literature, this reduction in ATF6f was accompanied by decreased transcriptional activity. The results obtained with p38 inhibition are very similar to those we previously obtained by inhibiting mutp53 (Sicari et al., 2019), supporting the hypothesis that p38MAPK may be one of the ways by which mutp53 exert its oncogenic role in the UPR.

To reinforce this concept, we tested the impact of p38MAPK inhibition on ERS-induced expression of a panel of ATF6 target genes in TNBC cells. Despite the clear results with the luciferase reporter (Fig. 3E), we did not observe a significant reduction in the endogenous

mRNA levels of any of these targets. Perhaps this apparently disappointing result was to be expected, due to the high redundancy of responsive elements in the promoters of UPR target genes, that are regulated by additional UPR-responsive and stress-responsive transcription factors. Perhaps a ChIP-seq assay for promoters bound by ATF6f, with or without p38MAPK inhibition, would better address this point.

Nevertheless, the contribution of ATF6 to the response to ER stress cannot be ignored, and our data indicate that p38MAPK can regulate not only ATF6f activity, but also its levels. This is further strengthened by our observation that overexpression of p38MAPK activators MAP2K3 and MAP2K6 was sufficient to increase in ATF6f levels, an effect particularly evident in PANC1 cells.

So, we started to reason that p38 may act on ATF6f by affecting its stability during ER stress. To test this hypothesis, we set up a specific assay in order to measure ATF6f protein turnover with or without p38MAPK inhibition. Despite the conceptual simplicity of the experiment, setting this assay proved to be difficult, because ATF6 is strongly sensitive to durance and intensity of the stress. After testing various conditions, we obtained a satisfactory result by inducing a moderate ER stress to trigger the production of cleaved ATF6f fragment, and subsequently removing the ERS-inducing drug while blocking any further ATF6f production with the S1P/S2P inhibitor AEBSF. This allowed us to estimate the half-life of ATF6f by monitoring the cleaved protein at different times by immunoblotting. Using this approach, we found that p38MAPK inhibition reduced ATF6f stability in all cell lines tested, most evident at the longest time point of 4h (Fig. 4). Although we have not proved that ATF6f is phosphorylated by p38MAPK under these conditions, the simplest explanation for this observation is that in the absence of p38-dependent phosphorylation the cleaved fragment of the protein may not be stable and is rapidly degraded. The mechanism of ATF6f turnover is still an open question and the literature is very limited. We thus cannot exclude that p38MAPK modulates ATF6f stability indirectly, regulating the activity of unknown factors that control ATF6f ubiquitination, or perhaps its ubiquitin-independent degradation. For instance, it is possible that p38 inactivates ATF6f-specific E3 ubiquitin ligases, or that p38-mediated phosphorylation prevents the interaction of E3 ligases with ATF6f. All these hypotheses await investigation.

It is also possible that p38 inhibition affects more general stress-response mechanisms that intersect with the UPR and could modulate ATF6f stability. For instance, it has been suggested that p38 inhibition can induce autophagy under certain conditions. Specifically, Quiang et al. observed that in keratinocytes and MEFs treated with UVB, autophagy was induced via AMPK, thus resulting in the blocking of apoptosis. In this case, they observed a reduction in p38 phosphorylation. Under same conditions, inhibition of autophagy resulted in UVB-induced apoptosis, correlating with increased p38 activation (Qiang et al., 2013).
Similarly, it has been shown that under vaiorus conditions ER stress can induce autophagy as a homeostatic response (Deegan et al., 2013). Moreover, inhibition of p38MAPK, with consequent reduction of ATF6f activity, could reduce the expression of genes involved in proteostasis, such as ERAD genes and chaperones, causing further accumulation of proteins no longer manageable; in order to ensure survival, cancer cells could activate other pro-survival mechanism, such as autophagy. Therefore, it cannot be excluded that ATF6f may be degraded by autophagy, and this hypothesis shall be tested in the future.
A certain ambiguity of p38's role in the response to ER stress is also evident from our survival experiments. In p53-null cancer cells, the inhibition of the kinase did not enhance ERS-induced cell death. In H1299 we actually observed a little increase in cell survival. It is possible that in these cell lines p38 may act as a tumor suppressor; for this reason, its inactivation implies an increased viability or an increased metabolic activity. In cells with mutp53, instead, p38 may act as an oncogene sustaining ATF6f stability and thus its inactivation reduces viability. This is a further confirmation of the molecular axis linking p38 with mutp53.
Mutant p53 is involved in a wide range of cellular pathways, and it would be naïve to think that p38MAPK activity is the only mechanism through which mutp53 promotes ATF6 activity in cancer cells. For instance, in addition to promoting p38-mediated stabilization, mutp53 could impact on ATF6 trafficking from the ER to the Golgi apparatus. Indeed, it has been recently shown that mutant p53 causes a structural reorganization of the Golgi and promotes an increased secretory phenotype thus reshaping the extracellular tumor microenvironment (Capaci et al., 2021). It cannot be excluded that under these circumstances, the translocation of ATF6 from the ER to Golgi may occur more quickly or more frequently, increasing ATF6f production.

Another mechanism by which mutp53 may support the oncogenic role of ATF6 is by regulating its glycosylation, a post translational modification that affects the production and stability of the transcriptionally active ATF6f fragment (Hong et al., 2004). Vogiatzi et al., demonstrated that mutant p53 upregulates the ER enzyme UDPase Ectonucleoside Triphosphate Diphosphohydrolase 5 (ENTPD5), important for the folding and secretion of N-glycosylated proteins (Vogiatzi et al., 2016). Currently it is not known if ENTPD5 may also affect ATF6 glycosylation and/or processing, but this could be another mechanism by which mutp53 exert is oncogenic role on ATF6. On the same line of thinking, since the glycosylation of ATF6 is a crucial step for its regulation and function, it is tempting to speculate that mutp53 may reinforce this post-translational modification through upregulating other enzymes involved in the secretory pathway, for instance the calnexin-calreticulin mechanism.

Regardless all legitimate speculations above, we found that one of the mechanisms by which mutp53 can sustain ATF6 activity is through stabilization of the ATF6f fragment via p38MAPK. We could not clarify the mechanism by which mutp53 enhances ERS-induced activation of p38MAPK. One possibility is via increased transcription of upstream activator kinases such as MAP2K3 (Gurtner et al., 2010) or ASK1/TAK1 (Table 1). Clearly, other mechanisms cannot be excluded.

In any case, we found that increased p38 activation leads to an increase in ATF6f protein, contributing to protect cancer cells from ER stress. In fact, inhibition of p38 with two different drugs sensitized three different cancer cell lines with mutant p53 to thapsigargin-induced cell death (Fig. 5). In summary, these observations reveal that not only ATF6 is a good candidate target for cancer treatment, but also p38 may be considered as a potential target, at least under certain conditions. For this reason, it will be important to define the molecular mechanism dictating p38 activation and p38 effects under specific conditions in different cancer types. It will also be important to identify genetic or non-genetic biomarkers of the potential effectiveness of p38 pharmacological inhibition in specific tumors. One of these could be the mutation status of p53, and this hypothesis is worth further investigation.

7) Experimental procedures

Cell culture. MDA-MB-231 (bearing p53 R280K) and PANC-1 (bearing p53 R273H) were cultured in DMEM medium (Sigma) supplemented with 10% FBS (ECS0180L, Euroclone), and 1% antibiotics (Penicillin/streptomycin ECB3001D0, Euroclone). PC-3 (p53 null), DU-145 (bearing p53 F223L and p53 V274F), and H1299 (p53 null) cells were maintained in RPMI medium (Sigma) supplemented with 10% FBS and 1% antibiotics as above.

Transfections, plasmids and siRNAs. Plasmid transfections were carried out 24h after cell plating, using Lipofectamine® LTX with Plus™ Reagent (ThermoFisher) or Lipofectamine® 3000 (Invitrogen), following manufacturer's instructions. After 6 hours, cells were washed with PBS for two times and fresh culture medium was added. For siRNA transfections, cells were transfected with 40nM siRNA oligonucleotides, using Lipofectamine® RNAiMax (Invitrogen), following manufacturer's instructions. pLPCp53R280K and pLPCp53R273H were obtained by cloning p53R280K/p53R273H coding region into pLPC empty vector. pCDNA3-MAP2K3b FLAG and p5xATF6-GL3 were obtained from Addgene. pRS-shp53 plasmid was generated by cloning in the pRS retroviral vector a double stranded oligonucleotide corresponding to the sequence of the p53 siRNA.
siRNAs used in this work are listed in the following table:

siRNA	SEQUENCE	PURCHASED FROM
control siRNA	unknown	All star negative control (1027281, Qiagen)
p53 siRNA	GACUCCAGUGGUAAUCUAC	Eurofins MWG
p38α siRNA	GAAGCTCTCCAGACCATTT	Eurofins MWG

Drug treatments. Thapsigargin (Tg) was purchased from Sigma (T9033) and dissolved in DMSO at the stock concentration of 1 mM. Tg is a non-competitive inhibitor of the sarco/endoplasmic reticulum Ca2+ ATPase (SERCA). Cells were treated with Tg at the concentrations of 0,1, 0,5, 1, 5 µM. SB203580 and SB202190 were purchased from Sigma and dissolved in DMSO at the

stock concentration of 10 mM. They are inhibitors of p38α and p38β catalytic activity by binding to the ATP binding pocket. Cells were treated with SB203580 at the concentrations of 0,1, 1, 10, 100 µM. SB202190 was used at the concentration of 10 µM. AEBSF readymade solution was purchased from Sigma (SBR00015) and used at the concentration of 300 µM. AEBSF is an efficient inhibitor of S1P protease.

Viral infection. For Lentivirus production, HEK-293GP and HEK-293T cells were transfected with the packaging plasmids and the plasmid construct of interest using Polyethylenimine purchased from Sigma (#408727). After 6 hours, medium was replaced and cells were incubated at 37°C. After 48 hours, the supernatants containing viral particles were filtered (0.45 µm) and supplemented with 10% FBS and polybrene (8ug/ml). The culture medium of target cells growing at 40% confluence was replaced by the appropriate viral supernatant and incubated at 37°C for 24 hours. Cells were then selected with puromycin (0,5 µg/ml) and kept under selection for the entire experiment.

*ATP lite assay***.** Cells were seeded in 96 wells plate with transparent bottom purchased from Perkin Elmer (#6005181). After 24 hours, cells were treated with drugs at the concentration and the time indicated in the results. At the end of the treatment, cell medium was removed and replaced with 100 µl of ATP lite 1-STEP buffer(Perkin Elmer #6016731) following manufacturer’s instructions. Luminescence was measured using Synergie BioTeck plate reader.

Protein extraction and Western blot analysis. Cells were lysed in RIPA buffer without SDS (150mM NaCl, 50mM Tris-HCl pH8, 1mM EDTA, 1% NP-40, 0.5% Na-deoxycholate) supplemented with protease and phosphatase inhibitors (1mM PMSF, 5mMNaF, 1 mMNa3VO4, 10µg/ml CLAP). Protein concentration was determined with Bio Rad Protein Assay Reagent (#500-0006). Total lysates were separated by SDS/PAGE and transferred to nitrocellulose membranes (Millipore). Western blot analysis was performed according to standard procedures using primary and secondary antibodies indicated in the following table.

Antibody	Diluition	Purchased from
α-ATF6	1:1000	Abcam #122897
α-p53 (α-DO1)	1:5000	Santa Cruz #sc-126
α-p-p38	1:1000	Cell signaling #9216
α-pan-p38	1:1000	Cell signaling #8690
α-flag	1:1000	Sigma #F1804
α-actin	1:4000	Sigma #A9718
α-HSP90	1:5000	Santa Cruz #sc-13119
Anti-mouse and anti-rabbit HRPO-conjugated	1:2000	Bethyl, #A90-516P, #A120-201P

RNA extraction and RT-qPCR. Total RNA was extracted with Eurogold TRIfast following manufacturer's instructions. For mRNA expression analysis, 500ng 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). The primer used were the following:

Target	Primer
H3	Fw: 5'- GAAGAAACCTCATCGTTACAGGCCTGGT - 3' Rev: 5'- CTGCAAAGCACCAATAGCTGCACTCTGGAA - 3'
MAP2K3	Fw: 5'- AGGAAGAACCCCGCAGAGCGTA - 3' Rev: 5' - TCACGAAGGCAGCAATGTCCGT - 3'
XBP1	Fw: 5'- GCAGCACTCAGACTACGTGC -3' Rev: 5'- GACTGGGTCCAAGTTGTCCA -3'
EDEM	Fw: 5'- CAAGTGTGGGTACGCCACG -3' Rev: 5'- AAAGAAGCTCTCCATCCGGTC -3'
CHOP	Fw: 5'- CAGAACCAGCAGAGGTCACA -3' Rev: 5'- AGCTGTGCCACTTTCCTTTC -3'
GRP78	Fw: 5'- TGTTCAACCAATTATCAGCAAACTC -3' Rev: 5'- TTCTGCTGTATCCTCTTCACCAGT -3'

ERO1	Fw: 5'- AATCTGAAGCGACCTTGTCC -3' Rev: 5'- GCCCAGCTTTTATTCCAACC -3'
HERPUD1	Fw: 5'- CAGAAATCAACGCCAAGGTG -3' Rev: 5'- GAACTTCCCTTTGCCTTAAACC -3'

Luciferase assay. Luciferase assays were performed using the reporter plasmid p5xATF6-GL3, bearing five repetitions of a specific binding sequence for ATF6f. The luciferase reporter was transfected together with CMV-Renilla to normalize for transfection efficiency. After 24 hours, cells were treated with or without indicated drugs. Luciferase was measured on a Promega luminometer.

Statistical Analysis. In all graphs data are expressed as mean ± SD of three independent experiments, except when indicated. Differences between samples were analyzed by Student's t test using Prism 8 (GraphPad). P-values < 0.05 were considered significant.

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