Review

Proteotoxic stress and cell death in cancer cells

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Abstract: To maintain proteostasis, cells must integrate information and activities that supervise protein synthesis, protein folding, conformational stability, and also protein degradation. Extrinsic and intrinsic conditions can both impact on normal proteostasis, causing the appearance of proteotoxic stress. Initially, proteotoxic stress elicits adaptive responses aimed to restore proteostasis, allowing cells to survival the stress condition. However, if the proteostasis restoration fails, a permanent and sustained proteotoxic stress can be deleterious and cell death ensues. Many cancer cells convive with high levels of proteotoxic stress and this condition could be exploited in a therapeutic perspective. Understanding the cell death pathways engaged by proteotoxic stress is instrumental to better hijack the proliferative fate of cancer cells.

Keywords: UPR; NOXA; DR5; BCL2; apoptosis; necroptosis; ferroptosis; proteotoxic

1. Proteotoxic stress: an introduction

Proteins are key macromolecules that play fundamental roles in almost every cellular process from gene expression to cell/tissue protection [1]. The important and relentless actions of proteins oblige cells to supervise and guarantee their correct folding and assembling. Protein homeostasis or proteostasis is the fundamental cellular effort aimed to reach this goal. Proteostasis is governed through a complex network of regulative mechanisms and is an essential task for cell survival [2]. The vast majority of proteins need to assume a peculiar thermodynamically stable three-dimensional structure that depends on their amino acid sequence [3]. During the folding process, proteins, particularly those presenting complex domains, can often produce folded intermediates. These intermediates can expose hydrophobic amino-acid residues, thus becoming more susceptible to stack into a misfolded condition. A risk for the formation of misfolded aggregates [4].

Cells use a complex network, called Proteostasis Network (PN) in order to monitor protein homeostasis. The PN includes molecular chaperones and proteolytic machineries. These genes families promptly cooperate to guarantee the regular proteostasis. In this manner the PN coordinates protein synthesis with folding and, if necessary, it can trigger protein degradation [5-7]. The importance of proteostasis maintenance become evident in the presence of PN dysfunctions. Inefficiency in these monitoring activities are responsible of several pathologies, including neurodegenerative diseases. Frequently, these deficiencies are age-dependent, with imponent social and economic costs [7-10]. Within the PN the control of protein folding is supervised by chaperones, which require ATP hydrolysis and a high cost in terms of energy. Particularly, the chaperons of the Heat Shock Protein (HSP) family help protein folding and are also fundamental when critical conditions, able to alter proteostasis, such as heat stress, oxidative stress or hypoxia emerge [1]. These particular proteins were defined as HSP because their expression is dramatically up-regulated when cells are exposed to high temperature or other forms of stress. The human genome can encode for about 330 chaperones and co-chaperones [11]. The most known classes of chaperones include: the ATP-dependent HSP70s, HSP90s, HSP60s (also called chaperonins) and HSP100s, and the ATP-independent small HSPs (sHSPs) [11]. In many cases chaperones are helped in their activities, by regulatory proteins called co-chaperones. A large protein family, which includes 244 different

members. Some examples of co-chaperones are: HSP40s (49 proteins) as regulators of the HSP70s and the tetratricopeptide repeat proteins (TPR) (114 proteins) as regulators of the HSP90s. In general, co-chaperones assist the functions of the chaperones by providing more selectivity and specificity toward the substrate [11,12].

Chaperones function as the main players in the maintenance of proteostasis, by assisting the folding of the proteins. They usually bind to the hydrophobic polypeptide segments exposed by unfolded or not completely folded proteins, thus avoiding their aggregation throughout the folding process [1]. HSP70s and the HSP90s are the most important members of the ATP-dependent chaperones. They work through ATP-regulated cycles of binding and release from the proteins during the folding. This process end when proteins are finally able to obtain their correct structure [2,13]. Moreover, some proteins are uncapable to fold without the presence of chaperones and this event determines the limit of the Anfinsen dogma. An example of this type of proteins is the cytoskeletal actin [14].

Chaperones can do their duties either in cooperation with the ribosome, for example the mammalian Ribosome-Associated Complex (RAC) and some specialized HSP70s (HSP70L1) [15,16], or alone, once the polypeptide is released. This is the case of the HSP70s, the HSP90s, and the TRiC/CCT chaperonin. In particular TRiC are complexes, structured as double-ring, that encircle, for a short time the unfolded protein, in a structure similar to a cage. In this manner TRiC allow both the correct folding and avoid the formation of aggregates [2,17]. In addition, the ATP-independent sHSPs work as a support in the maintenance of the proteins in a stable state. Through this strategy, proteins will not go under aggregation processes [18].

In general, proteins can be divided into proteins that fold easily and quickly after the interaction with the upstream chaperones, like the HSP70s, and proteins that require more help during the process. The first group of proteins do not need downstream chaperones, instead the second group of proteins are not able to complete correctly the folding and need more specialized chaperones, like the HSP90s or the chaperonins, to achieve the proper structure [19]. These chaperone-dependent proteins are usually larger than the average and comprehend multiple domains or domains which have complex topologies of folding. For these reasons they need a strong interaction with the chaperones and also with the co-chaperones, generating an interconnected network called “chaperome” [20].

The human PN has not yet been completely characterized in all its parts. However, investigations aimed to dissect the network of proteins interacting with HSP90s, revealed the presence of E3 ligases (enzymes involved in the last step of ubiquitin-conjugation). This finding highlights the close relationship between the folding and protein degradation processes [21]. A detailed study, which involved about 70 chaperones, co-chaperones and proteins of the quality-control compart, has illustrated that there is a hierarchical organization of the chaperones network. This organization is centered on the interconnected chaperone systems of HSP70 and HSP90 [22]. Another study revealed that the chaperones network can be rewired after oncogenic transformation, in a new network of interactions “epichaperome” that can favor cancer cell survival [23].

Importantly, the chaperones systems evolved several mechanisms to compensate when a single chaperone fails or is disabled. This aspect is important also in a therapeutic perspective when inhibitors against a chaperone are evaluated [24]. For example, the inhibition of HSP90 can promote the binding of the unfolded proteins to Hsc70, the constitutively expressed HSP70 [22]. Furthermore, between members of the BAG family of co-chaperones, which act as a nucleotide exchange factors of HSP70, BAG2 is the only one that has similar substrate range compared to Hsc70. This evidence permits to conclude that BAG2 could be a general co-factor, which is important in the folding mechanism of Hsc70 substrate proteins. Finally, among the interactors of Hsc70 has been identified

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the E3 ligase CHIP (Carboxy Terminus of HSP70-Interacting Protein), thus further confirming the correlation between the chaperones and the Ubiquitin–Proteasome System (UPS) [25,26].

2. The protein quality system

In order to maintain the proteostasis eukaryotic cells have evolved several systems monitoring the quality control. These systems are different from the folding/re-folding actions of chaperones and are involved in the disruption of damaged and misfolded proteins. The most important system is represented by the UPS. It works in cooperation with the lysosomal system [27,28] and plays a crucial role in a several cellular processes, by controlling the physiological turn-over of proteins [29]. The degradation of the proteins through the UPS is due to the presence of an ubiquitin tag, which is conjugated though a multistep process called ubiquitylation. An ubiquitin moiety is initially covalently linked onto Lys residues of target proteins (isopeptide bound) and next elongated through the use of specific Lys of the ubiquitin itself, most frequently Lys 48. The ubiquitylation requires the coordinated action of three enzymes. The E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugation enzymes and E3 ubiquitin ligases enzymes [30-32]. After the ubiquitylation, the tagged proteins are translocated to the 26S proteasome, an ATP-dependent protease complex found in the cytosol and in the nucleus of all eukaryotic cells. The proteasome is composed by about 50 different subunits, but it is possible to define two critical subcomplexes: the 20S catalytic core and one or two 19S regulatory subunits. The 19S particles are bound to one or both ends of the 20S component [33-35]. The ubiquitin tag is recognized by the 19S regulatory subunits and here, it is recycled by the action of Deubiquitinases (DUBs). Three DUBs are associated with the 19S regulatory subunits: RPN11/POH1, USP14 and UCH-L5 [36-38]. This process is crucial for the degradation, since the presence of the ubiquitin chain would impact sterically on the translocation from the 19S regulatory subunits to the 20S catalytic core. In fact small molecules that inhibit these DUBs triggers cell death and cellular responses, similarly to inhibitors of the catalytic portion, such as bortezomib, carfilzomib and ixazomib [36,39-41].

The 20S catalytic core is constituted by two heptameric β-subunits and two heptameric α-subunits. The α-subunits have a structural role and instead the β-subunits have a catalytic role. In particular, β1 has a caspase-like activity, β2 has a trypsin-like activity and β5 has a chymotrypsin-like activity [42-44]. A recent analysis of the proteasome and of its substrates, through cryoelectron microscopy, offers a new intriguing sight into this complex process [45-47]. Since the necessity for substrate unfolding, the proteasome is uncapable to degrade the aggregated proteins directly. Normally, once bound to the proteasome, the substrate is unfolded by the action of six ATPase subunits (Rpt1–6) [48]. Hence, an obligatory pre-requisite is the disaggregation through the chaperones network. A second example of such cooperation is the direct interaction between the E3 ligase CHIP and HSP70. CHIP can thus ubiquitylate chaperone-client proteins. However, this modification may still be inverted by DUBs [49]. Understanding how these conflicting mechanisms are controlled will be important for the knowledge of the protein quality control.

In summary, even in the presence of the UPS, protein misfolding can induce the creation of insoluble aggregates, particularly under stress situations. Differently, autophagy can directly eliminate these aggregates via lysosomal degradation [50]. The complex molecular system involved in this task is defined as Autophagy Lysosomal Pathway (ALP) system. It includes core ATGs products and additional factors, with a total of about 500 components [51]. The aggregated proteins, therefore, can be accumulated in ubiquitin-positive regions, in which the autophagic system is recruited by chaperones in a process known as Chaperone-Assisted Selective Autophagy (CASA) [52-54]. In normal unstressed conditions, the soluble proteins that need to be degraded can also be eliminated by a different type of autophagy, such as the Chaperone-Mediated Autophagy (CMA). This response involves first the action of Hsc70, which can recognize the substrate and then the lysosomal translocation, operated through the lysosomal receptor LAMP2A (Lysosome-Associated Membrane Protein 2A). CMA avoid the formation of the autophagosome [55]. Although both the UPS

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and the ALP display an important grade of specificity toward their variety of substrates, they are connected each other. They often compensate themselves when one of these two pathways is not working properly [56-59].

3. Cellular responses to the unfolded proteins

Despite the presence of the chaperone systems, some errors occur during folding and many stressors such as heat, heavy metal ions, oxygen radicals and mutations can hamper the correct folding. A misfolded or unfolded protein is not functional and can elicit a pathological condition, derived from its aggregation [1,60-63]. Folding maturation in the ER is a difficult task. Proteins of the secretory pathway if unable to fold correctly are retained in the Endoplasmic Reticulum (ER) and then retro-translocated to the proteasome for their degradation. The process is called ER-Associated Degradation (ERAD) [64]. A fundamental role in ERAD is played by the cytosolic ATPase p97 (VCP/Cdc48). This ATPase is involved in delivering the ubiquitylated unfolded proteins from the ER to the proteasome, through ATP hydrolysis [65]. If this system is overloaded, accumulation of incorrect folded proteins occurs in the ER, thus leading to ER dysfunctions, including an altered redox equilibrium. Conditions that trigger the induction of the ER-stress [66-70]. In response to ER-stress, cells activate the UPR (Unfolded Protein Response) [71,72]. This adaptive response is important for sustaining cell survival. To this end, the UPR blocks protein translation, increases the activation of chaperones and potentiates the ERAD pathway. Through the UPR, cells avoid the accumulation of misfolded proteins and restore the physiological condition of proteostasis. As explained above the UPR allows cells to survive to the stress condition [64, 70-73]. The UPR is governed by three sensors: PERK (Protein kinase RNA-like ER Kinase), IRE1 (Inositol-Requiring Enzyme 1) and ATF6 (Activating Transcription Factor 6) [64,74,75]. All these sensors work in parallel to decrease the ER stress. PERK and IRE1 activation can decrease protein synthesis with the consequent reduction in the amount of proteins that can enter the ER. The activation of ATF6 can upregulate the transcription of different chaperones involved in controlling protein folding [64].

PERK is a serine/threonine kinase that has several substrates. The best characterized is the eukaryotic translation Initiation Factor-2 alpha (eIF2α). PERK is able to phosphorylate eIF2α at serine 51 [70,71,76], thus blocking the CAP-dependent translation and diminishing the ER stress [64]. Another notable substrate is NRF2 (Nuclear factor erythroid-derived 2), a master regulator of the redox homeostasis [77]. PERK can phosphorylate NRF2 on Thr 80, localized within the Neh2 domain [78]. This favors the activation of NRF2 and its nuclear import. From the nucleus NRF2 coordinates the expression of the anti-oxidant response by binding the Antioxidant Response Elements (ARE) in regulatory regions of several genes [75]. Additional studies have revealed that also FOXO transcription factors [80,81] and Diacylglycerol (DAG) [82,83] can be phosphorylated by PERK in order to reduce ER stress. Other PERK-related kinases exist that supervise different stress conditions. Protein kinase R (PKR) is involved in the antiviral response, GCN2 is involved in sensing the aminoacid pool depletion and finally, HRI that is activated by heavy metals, heat shock, and proteasome inhibition [84]. All kinases phosphorylate eIF2α, reduce translation and reduce proteotoxic stress. Interestingly, HRI confers resistance to UPS inhibitors such as bortezomib [85].

BiP/GRP78, an HSP70 family member localized into the ER, is a master regulator of the UPR in response to ER stress. It monitors the release and activation of the three sensors PERK, IRE1, and ATF6 [86,87]. PERK, IRE1, and ATF6 are constitutive clients of Bip/GRP78. Increasing protein unfolding, by incessantly confiscating BiP/GRP78, unleashes the three sensors and activates the UPR. In particular, after the disassociation from BiP/GRP78, PERK can dimerize and favor its autophosphorylation and activation [88]. The activated form of IRE1, after BiP/GRP78 release, has an endoribonuclease activity that can splice a 26-base intron of the mRNA of X-box Binding Protein 1 (XBP-1) [89], which is a TF that supervises the transcription of genes involved in ERAD and protein folding [90]. Finally, the dissociation of ATF6 from BiP/GRP78 permits its translocation from ER to Golgi where it is processed. The cleaved ATF6 can enter the nucleus where it acts as TF to transcribe

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genes that can favor the ER folding potential such as GRP78 and GRP94 [91]. This sophisticated adaptive response allows cells to survival the stress conditions. However, if the proteostasis restoration fails, a permanent and sustained activation of the UPR can be deleterious. Engaged to permit cell survival, the UPR can switch to trigger the induction of cell death [64,74,92].

4. Cell death pathways activated by proteotoxic stress

The induction of proteotoxic stress through the use of small compounds/drugs is achieving therapeutic interest, particularly in an anti-tumor perspective [93]. In order to better synergize the induction of proteotoxic stress with the available therapies is fundamental to understand the molecular mechanisms controlling cell death in response to proteotoxic stress.

- *The extrinsic pathway of caspase activation*

It is well established that proteotoxic stress engages the mitochondrial pathway of caspase activation [94]. Indeed, proteotoxic stress is a broad pro-death insult and, for example, also the extrinsic pathway contributes [95]. This role was suggested by early studies, reporting the up-regulation of TNFRSF10B/DR5, the TRAIL receptor, in response to ER-stressors/PERK activation, UPS inhibitors and by the influences of caspase-8 inhibitors on the proteotoxic-induced cell death [39,95-101]. More recently, it has been proposed that the UPR not only up-regulates DR5 expression but, misfolded proteins can directly engage with DR5 in the ER-Golgi intermediate compartment, to drive the assembly of DR5 in complexes competent for caspase-8 activation (Fig. 1). An activation that occurs independently from its canonical extracellular ligand Apo2L/TRAIL [102]. Although the mechanism involved in such activation is unknown, a plausible hypothesis points to the release of an autoinhibitory activity that normally prevents spontaneous activation of the receptor. The increased levels of expression, the trapping in a particular membrane domains and the priming effect of misfolded proteins could be the culprits [102,103].

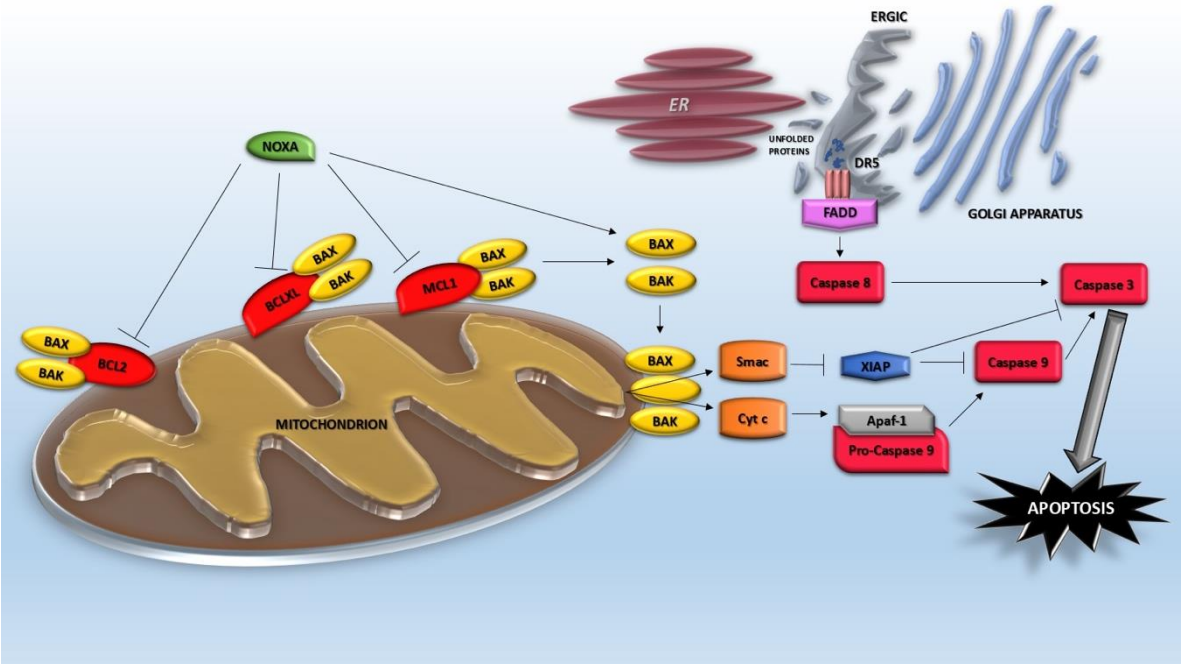

Figure 1. Apoptotic pathways engaged by proteotoxic stress

In the receptor-independent activation of caspase-8 in response to ER-stress, a contribution of RIPK1 has also been proposed. The contribution appears indirect and is sustained by the use of Ripk1-deficient murine cells. The involvement of Ripk-1 in ER stressors-induced apoptosis is still mysterious. It is independent from the kinase activity, from cIAP1/2-mediated ubiquitylation, and

does not involve the direct regulation of JNK or CHOP [104]. ER-stress can also promote inflammatory responses in the presence of chemotherapeutic regiments. Here again, ER-stress elicits TRAIL receptors up-regulation, which results in a caspase-8/FADD/RIPK1-dependent activation of NF-κB. Similarly to cell death, inflammatory cytokines production occurs in a ligand-independent manner. The importance of this response is testified by the protection observed in DR5−/− mice from taxol-induced inflammation. [103]. These studies confirm that, similarly to other observations, DR5 engagement can result in different cellular responses, which are context dependent [105].

- *The ATFs network*

A huge plethora of studies indicate that cell death induced by proteotoxic stress can follow different routes. Certainly, the foremost investigated signaling pathway linking proteotoxic stress to apoptosis regards the ER-stress and the consequent UPR. A key element of this pathway is represented by ATF4 (Activating Transcription Factor 4) a TF that belongs to the cAMP response element-binding protein (CREB)-2 family of proteins [106]. As explained above, eIF2α phosphorylation results in the attenuation of the cap-dependent protein translation, as well as, in the translation of selected mRNAs, including ATF4 itself. Normally, ATF4 protein is almost undetectable, due to its very short half-life and low translation efficiency [107,108]. In fact, ATF4 levels dramatically increase in response to proteasome inhibitors, because of the double effect exerted by the UPR activation and by the suppression of its degradation [109]. ATF4 is structured into different domains and comprises a basic/leucine zipper domain (bZIP domain) that binds the DNA. ATF4 interacts with several partners that influence its variegated transactivation activities and its stability [106,107]. As a consequence, ATF4 controls the expression of a wide range of genes, which play different roles in resolving proteotoxic stress. Some of these genes are directly transcribed by ATF4, others, indirectly, through the action of other TFs (Fig. 2). An example of a TF regulated by ATF4 is CHOP/GADD153 (CCAAT-enhancer-binding protein homologous protein), an important player of the apoptotic response [110]. Again, translation of CHOP mRNA is sustained by eIF2α phosphorylation that allows the escape from a poor translation initiation sequence [111]. Interestingly, this signaling arm is also involved in controlling ferroptosis, through both GCN2-dependent and independent mechanisms, which convey on cysteine depletion [112,113].

ATF4 can trigger cell death also independently from CHOP. It can promote the down-regulation of the IAP (Inhibitors of apoptosis) family member XIAP, in a still undefined manner. These proteins can bind and block caspase activities but can also, through a RING zinc finger domain with E3 ubiquitin ligase activity, promote ubiquitylation and the subsequent proteasomal degradation of their substrates, including caspases [114].

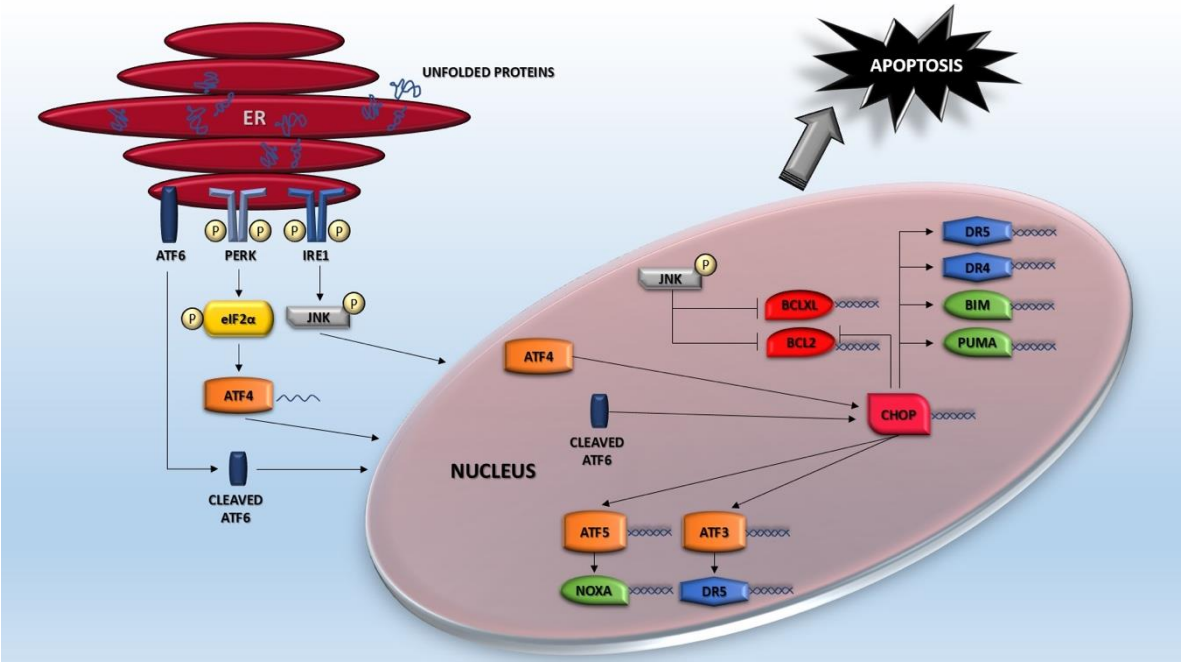

Figure 2. The ATFs network in the response to proteotoxic stress

CHOP enhances the expression of a collection of genes. Interestingly, some these genes are shared with ATF4, thus suggesting the existence of a feed-forward mechanism to sustain proteotoxic-dependent gene expression [115]. Similarly, the control operated by ATF6 on CHOP transcription can be viewed as a cooperative mechanisms to resolve the proteotoxic stress [116]. A gene under the direct transcriptional control of CHOP is DR5 [103,117,118]. A CHOP-binding site is present in the 5'-flanking region (position –281 and –216 from TSS) of the DR5 gene [117]. Moreover ATF3, another ATF/CREB family TF that facilitates apoptotic cell death, has been shown to be involved in the ER stress-mediated DR5 induction in human p53-deficient colorectal cancer cells [119,120]. TRAIL-R1/DR4 was also shown to be involved with ER stress, although with less relevance. CHOP/ATF4 can promote also DR4 up-regulation, although with differences among models and cell lines and via both transcriptional and post-transcriptional mechanisms [121,122].

ATF5 is another ATF/CREB family member under the CHOP/ATF4 control (Fig. 2). Transcription up-regulation occurs via the direct binding of CARE elements in the ATF5 promoter [115,123]. Similarly to ATF4 and CHOP, ATF5 is preferentially translated once eIF2 is phosphorylated. Among the ATF5-dependent genes involved in apoptosis can be found the BH3-only protein NOXA/PMAIP1 [123]. Experimental downregulation of each of these TFs (ATF3, ATF4, ATF5 and CHOP) results in abrogation of NOXA induction in response to proteotoxic stress. Hence, they all contribute to sustain the feedforward loop that drives to apoptosis [115,123,124].

- *The BCL2 family members*

NOXA/PMAIP1 is a BCL-2 pro-apoptotic family member that plays important role in different apoptotic responses. NOXA is the smallest of BH3-only proteins (54 residues) and its expression is dramatically up-regulated after proteotoxic stress [125]. Initially identified as TP53 target gene [126], further studies have demonstrated that its transcription can be potently up-regulated by TP53-independent mechanisms, under different stress conditions including oncogenic transformation and proteotoxic stress [127-130]. NOXA depletion impairs apoptosis in response to proteotoxic stress. NOXA can act either as sensitizer and activator, by virtue of its BH3 domain that is inserted into the hydrophobic binding groove of multi-domains pro-apoptotic or anti-apoptotic BCL2 family members. As sensitizer it interacts with MCL1, BCLXL, and BCL2A1 (Fig. 1). In this manner, NOXA interrupts the sequestration operated by these anti-apoptotic proteins against multi-domains pro-apoptotic

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proteins, such as BAX and BAK. As consequence, NOXA unleashes the pro-death activities (oligomerization and channel formation) of BAX/BAK. Differently as activator, NOXA directly binds and activates BAX/BAK [131-134]. Curiously, murine Noxa contains two BH3 domains (A and B encoded by exons 2 and 3) with only the BH3 domain B conserved in humans [126].

Additional mechanisms are used by the proteotoxic stress to engage the mitochondrial pathway of caspase activation. BIM/BCL2L11 and PUMA/BBC3 are other BH3-only proteins, which up-regulation was reported in several models of proteotoxic stress and particularly during the ER-stress. Ablation of these proteins influence the death response to proteotoxic stress [84,93,94]. BIM was reported being a transcriptional target of CHOP [135]. Similarly, PUMA expression is induced through transcriptional up-regulation in a variety of human cell lines in response to an ER stress stimulus. [136,137]. In addition to the action on BH3-only proteins, proteotoxic stress can downregulate BCL2 at a transcriptional level, by CHOP [138] (Fig. 1). Moreover JNK activation, via the IRE1 pathway triggers BCL2 and BCLXL phosphorylation and their subsequent inactivation [139,140]. Among the different routes that proteotoxic stress can engage to trigger apoptosis, must be included also the regulation of BOK. This pro-apoptotic BCL2 family member is normally expressed at low levels, because it is constitutively degraded with a short half-life of 15 min. During proteotoxic stress, E3 ligases, such as gp78 that mediates BOK degradation, become saturated because of the accumulation of misfolded proteins. Hence, BOK can accumulate to favor mitochondrial outer membrane permeabilization [141]. Normally, DNAJB12 (JB12) contributes to maintain low levels of BAK and to sustain the survival of cancer cells. This chaperon is an endoplasmic reticulum (ER)-associated Hsp40 family protein that recruits Hsp70 to the ER surface in the protein quality control system [142].

- *Additional cell death responses*

When proteotoxic stress advances the UPS become clogged by the accumulation of polyubiquitylated proteins. Blocking the proteasome affects the expression of unstable signaling proteins and therefore signaling pathways controlling cell survival and cell death are modulated. Two important UPS-targets, controlling the survival/death switch, are the inhibitor of NF-kB, IkBα [143] and TP53 [144]. Furthermore, also elements of the apoptotic machinery both pro and anti-apoptotic, such NOXA, BIM and MCL1 accumulate in response to UPS saturation [29,145,146]. MCL1 stabilization represents the dark side in the anti-cancer effect engaged by UPS inhibitors. Interestingly, multiple kinase inhibitors such as erlotinib, rapidly enhance UPS-dependent degradation of MCL1. Erlotinib upregulates NOXA expression, which in turn, through the action of the mitochondria-associated ubiquitin ligase MARCH5, supervises MCL1 degradation [147,148]. Similarly to MCL1, other pro-survival proteins such as IAPs (XIAP, cIAP1 and cIAP2 in mammals), accumulate in response to proteotoxic stress dependent UPS saturation [149]. The activities of these proteins can be instrumental to maintain cell survival under stress conditions. For example AIRAP, a proteotoxic-stress gene regulated by the master TF HSF1 (Heat-shock factor 1), can regulate cell survival by controlling the levels of cIAP2 [150]. The switch between cell survival/death must imply a control also over the IAPs. An example is the ability of tunicamycin and thapsigargin (two ER stressors) of reducing XIAP levels in a number of mammalian cell lines [114]. XIAP translation can be reduced in a PERK-mediated manner, and ATF4 promotes its degradation. A new scenario that can contribute to reduce the threshold required for caspases activation.

As indirect consequences elicited by proteotoxic stress that can favor cell death, must be mentioned the accumulation of ROS and the alterations of calcium homeostasis. These co-factors can be the deleterious corollaries of the progressive impairments in the clearance capacities, normally operated by the UPS and by autophagy. Accumulations of unfolded proteins and of aggregates impact on ER and mitochondrial functions thus leading to alterations in ROS and calcium levels that in turn engage further signaling events leading to cell death. How these events integrate with the classical apoptotic responses is not clear. In some studies induction of oxidative stress can be

observed in the initial phases of the proteotoxic stress [84,151,152]. Certainly, the augmented levels of ROS and of Calcium can be responsible for the induction of alternative forms of deaths in response to proteotoxic stress observed in different studies [153,154]. In general, the appearance of different forms of cell death in response to proteotoxic stress is a less investigated item [155-157]. Frequently, these necrotic-like responses appear when apoptosis is defective. Interestingly, in a model of toxicity elicited by mutant Huntingtin, a new hypothesis to explain the apoptotic/necrotic switch has been proposed. If the sequestered mutant protein is soluble, cells are characterized by hyperpolarized mitochondrial membrane potential, an increased levels of reactive oxygen species and cell death by apoptosis. Instead, when mutant Huntingtin is present as aggregates, where other cellular proteins can be sequestered, a collapse in mitochondrial potential, cellular quiescence, and deactivated apoptosis occurs. Overall, this response curtails cellular metabolism and leads to a slow death by necrosis [158]. Clearly this model must be verified with general inducers of proteotoxic stress, but it is an interesting hypothesis that deserves further studies. Necrotic proteotoxicity can be hampered by NRF2, possibly through the formation of autophagosomes aimed to decrease the ubiquitinated protein aggregates [159]. Finally, in the necrotic arena a role of NOXA cannot be excluded, since its mitochondrial targeting domain (MTD) can trigger mitochondrial fragmentation and necrosis [160].

Necroptosis is a specific form of cell death activated through the serine/threonine kinases RIPK1, RIPK3 and the pseudokinase MLKL [161]. Compounds that trigger necroptosis can also activate the UPR [162,163]. This observation can suggest some links between proteotoxic stress and necroptosis. However, as similarly discussed below for ferroptosis, it is not simple to discriminate if the UPR engagement is within a pro-survival effort, rather than an effective contribution to the cell death process. Importantly, a study aimed to investigate the involvement of the UPR in the classical necroptosis-induced cell death by TNF-α, discovered that two commonly used PERK inhibitors, GSK2606414 and GSK2656157 are indeed potent RIPK1 inhibitors [164]. Certainly, RIPK1 in its pleiotropic activities can also antagonize proteotoxic stress-induced cell death. Overexpression of RIPK1 enhances induction of autophagy and confers resistance of melanoma cells to ER stress-induced cell death [165]. Finally, in a hypoxia-induced condition of UPR and ER-stress that characterize preeclampsia, the contribution of necroptosis has been excluded. Instead pyroptosis linked to the activation of NLRP3 inflammasome, through the activity of Thioredoxin-interacting protein (TXNIP) has been proposed [166].

Ferroptosis is a specific form of iron-dependent cell death, characterized by the accumulation of lipid peroxides due to the failure of glutathione-dependent antioxidant defenses [167,168]. Few data are available about the implications of ferroptosis in the proteotoxic stress-induced cell death. It is possible that connections exist, as recently discussed [169]. Particularly, if we take into account that different ferroptotic agents can also trigger the UPR [170,171]. The involvement of the UPR, at least in the initial phase can be viewed as pro-survival strategy [172], as discussed above for necroptosis. On the other side, ROS could be the link between ferroptosis and proteotoxic stress. For example, Glutathione peroxidases can regulate ferroptosis through their ability to reduce hydroperoxy groups of complex lipids and to silence lipoxygenases. But they can also take a part during the oxidative protein folding control in the ER, by reacting with protein isomerase as an alternate substrate [173].

A final important point concerns the heterogenous response of cell population to proteotoxic stress. It is well known that although exposed to the same intensity of proteotoxic stress some cells die while others survive. Clearly the availability of a pool of chaperons is a critical condition. Particularly for ER-stress, the switch from proteostasis to proteotoxicity the ER-resident chaperone BIP is a key factor [174]. HSF1 is the master regulator of chaperones expression in response to proteotoxic stress. Under stress conditions HSF1 is phosphorylated, trimerizes, binds regulative elements in chaperones genes driving their transcription [175]. Recently, a model has been proposed where membrane-less organelles foci of HSF1 regulate the cell decision in terms of survival/death. In the presence of prolonged stress, the biophysical properties of HSF1 foci can undergo to a change.

Small, fluid condensates enlarge into indissoluble gel-like arrangements, where HSF1 is immobilize. Consequently chaperones genes expression decrease leading to cell death by apoptosis [176].

5. Proteotoxic stress in cancer cells

The protein synthesis process is intrinsically prone to errors. It has been estimated that in mammalian cells more than 30% of newly synthesized proteins are degraded by the proteasome within minutes from their translation [177]. These quickly degraded proteins are called Defective Ribosomal Proteins (DRiPs) or Rapidly Degraded Polypeptides (RDPs). If not removed, DRiPs can increase proteasome loading and the consequent induction of proteotoxic stress [178]. Cancer cells generally enhance protein synthesis and therefore DRiPs accumulate more rapidly than in normal cells [179]. For example, cancer cells frequently over-activate mTORC1 pathway. This pathway is required to promote elevated levels of protein synthesis. A condition that obliges cancer cells to pay a tribute to the proteasome to avoid the accumulation of misfolded proteins. This dependence from the proteasome has been exploited to kill cancer cells via small compounds blocking the UPS [36, 180-182]. The fundamental role of the proteostasis in cancer cells is further underlined by the formation of immunoproteasomes, as a secondary mechanism to manage the increased proteotoxic stress arose in cells mutated for RAS, PTEN, TSC1, or mTORC1 [180,183]. Environmental conditions, which are commonly exacerbated in tumors, such as hypoxia, oxidative stress, and nutrient deprivation are additional inducers of protein misfolding and of proteotoxic stress [84,94,178].

A still poorly explored aspect of the proteotoxic stress is its connection with cellular metabolism [184,185]. It seems that the switch towards an oxidative metabolism rather than glycolysis renders cancer cells resistant to the UPS inhibitor bortezomib. The regulation of the mitochondrial state could represent an additional mechanism of adaptation to proteotoxic stress that could be addressed in therapeutic perspective [186].

In addition to the enhanced proteins synthesis and environmental conditions, genetic alterations accumulated in cancer are other sources of proteotoxic stress. Aneuploidy, copy number variations and point mutations are common genetic alterations in cancers that can induce proteotoxic stress [187-191]. Aneuploidy is also associated with many types of stresses in cancer cells, which include both metabolic and oxidative stresses [192]. In aneuploid cells, protein complexes stoichiometry imbalances are important causes of protein aggregation and of proteotoxic stress induction. The uncoordinated expression of a single subunit of protein complexes, because encoded on excess chromosomes, leads to its aggregate state. The excess subunits are either degraded or aggregate, with protein aggregation nearly as effective as protein degradation for lowering the levels of excess proteins [193]. In aneuploid cells also the induction of HSF1 is in some way compromised. This deficit is transduced in an impaired expression of HSP90, accumulation of misfolded proteins and the appearance of proteotoxic stress [194]. Similarly, overexpression of genes, as well as the accumulation of mutations in coding regions can alter the normal proteostasis [195]. These mutations would produce protein variants that more are prone to misfolding, degradation, and aggregation [191].

Cancer cells convive with proteotoxic stress by up-regulating all the possible mechanisms able to maintain proteostasis. [196-204]. As a consequence, cancer cells are more dependent on the presence of HSP and from the UPS for their growth and survival [205,206]. Among the HSPs, the HSP90s and the HSP70s are critical for escaping from anti-proliferative signals, resisting to cell death and evading senescence. Additionally, these chaperones are involved in many distinct tracts of the cancer cells including drug resistance, angiogenesis and metastasis [207,208]. Clearly, impacting on these adaptive mechanisms has important consequence on the survival of cancer cells [209,210]. This dependence has attracted several interests for the developing of therapeutic approaches aimed to switch-off these adaptations and thus unleash all the dramatic consequences of the unresolved proteotoxic stress [210-218]. In some circumstances adaptations to proteotoxic stress can favor the resistance to other therapeutic regiments, as observed for HSF1 and the resistance to the receptor

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tyrosine kinase (RTK) inhibitor lapatinib in breast cancer [219]. Interestingly the master regulators of ER-stress and of the UPR (ATF3/4/5/6 and CHOP) are highly expressed in a fraction of bladder kidney and prostate cancers, indicative of a high levels of proteotoxic stress (Fig. 3A). These sub-groups of tumors exhibit an aggressive behavior characterized by a reduction of the overall survival (Fig. 3B).

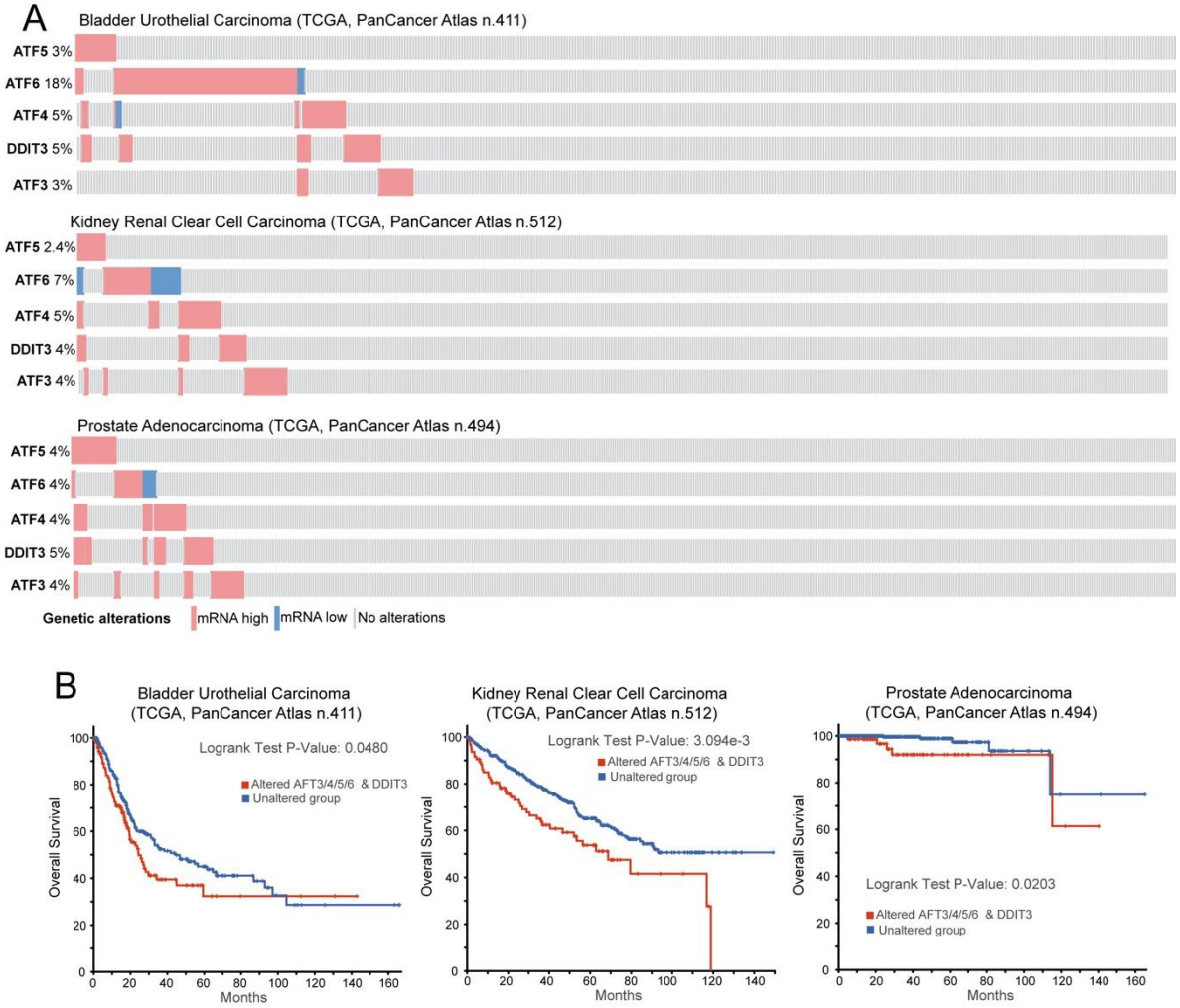

Figure 3. ATFs factors in cancer. A) Oncoprint of mRNA expression variations for the indicated TFs. Data were obtained from the TCGA database and include RNAseq data from patients as indicated. The heatmap shows the alterations in the expression levels and were generated through cBioPortal (http://www.cbioportal.org). mRNA expression z-scores were relative to diploid samples (RNA Seq V2 RSEM). B) Kaplan-Meier survival analysis related to the alterations in the mRNA levels of the ATFs network. All cases were analyzed and clustered into two groups according to ATF3/4/5/6 and DDIT3/CHOP alterations in the expression levels as illustrated in (A). Data were generated through cBioPortal (http://www.cbioportal.org)

6. Conclusions

Proteostasis is a fundamental task for every cell. The evolution has sculptured elaborated interconnect mechanisms to maintain proteostasis. Some of these mechanisms are highly conserved through the evolution and with the appearance of the eukaryotic cells each subcellular compartment has evolved a dedicated set of strategies [220,221]. Proteostasis alterations and induction of proteotoxic stress are responsible for several pathological conditions, particularly in neurodegenerative diseases, including Huntington's, Parkinson's, Amyotrophic Lateral Sclerosis, and Alzheimer's Diseases [222]. On the other side small compounds able to trigger proteotoxic stress or of targeting the machineries resolving the proteotoxic stress, are actively screened as anti-cancer

agents [93]. Undoubtedly, the central role played by the proteotoxic stress in the cell life/death decision guarantees that, by studying its regulation and by developing new compounds aimed to improve or to impair its appearance, benefits for the human health will be generated.

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