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FMUP FACULDADE DE MEDICINA
UNIVERSIDADE DO PORTO

METAL-INDUCED OXIDATIVE STRESS IN HUMAN FIBROBLASTS: A ROLE IN CELLULAR AGEING

LILIANA RAQUEL CASAIS DE MATOS SILVA

TESE DE DOUTORAMENTO APRESENTADA
À FACULDADE DE MEDICINA DA UNIVERSIDADE DO PORTO EM
METABOLISMO: CLÍNICA E EXPERIMENTAÇÃO

PDM*CE*

Programa **D**outoral em **M**etabolismo:
***C**línica e **E**xperimentação*

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Orientação do Professor Doutor Henrique Manuel Nunes de Almeida
Co-orientação da Professora Doutora Alexandra Maria Monteiro Gouveia

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O PAPEL DO STRESSE OXIDATIVO INDUZIDO POR METAIS NO ENVELHECIMENTO CELULAR

LILIANA RAQUEL CASAIS DE MATOS SILVA

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Aos meus pais e irmãos

Ao André

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RESUMO

O envelhecimento tornou-se uma das grandes preocupações das sociedades modernas, devido ao seu elevado impacto social e económico. Por isso, tem sido amplamente investigado com o intuito de organizar estratégias capazes de retardar a deterioração do estado de saúde e o aparecimento de doenças associadas à idade. Neste cenário, é fundamental identificar potenciais intervenientes e desvendar os mecanismos moleculares subjacentes que se encontram implicados no processo de envelhecimento.

Ao nível celular, sabe-se que o processo é caracterizado pela acumulação progressiva de biomoléculas oxidadas que eventualmente levam à disfunção celular. Entre as condições que favorecem esse processo encontram-se alterações na homeostasia de metais, que resultam no seu aumento ou acumulação em células e tecidos, onde promovem a formação de espécies reactivas de oxigénio (ROS), capazes de danificar biomoléculas. Na verdade, foi já descrito que os níveis de cobre aumentam com o envelhecimento e em entidades clínicas associadas à idade, tais como a diabetes tipo II, a aterosclerose e doenças neurodegenerativas, corroborando a contribuição do cobre para o stress oxidativo e o declínio funcional.

Os modelos celulares de senescência *in vitro* são essenciais para o estudo dos eventos moleculares implicados no processo do envelhecimento. Modelos de senescência prematura induzida pelo stresse (SIPS) são frequentemente utilizados, uma vez que apresentam várias vantagens quando comparados com o modelo clássico de senescência replicativa (RS): demoram menos tempo a ser estabelecidos, são mais fáceis de manipular e apresentam uma resposta celular heterogénea semelhante ao que acontece *in vivo*. O indutor de SIPS mais frequentemente utilizado é o peróxido de hidrogénio (H_2O_2), mas outros indutores de stresse oxidativo podem ser utilizados com sucesso.

Atendendo à capacidade do cobre para promover a formação de radicais livres, é aqui descrito pela primeira vez que a incubação de fibroblastos WI-38 com uma concentração sub-citotóxica de sulfato de cobre ($CuSO_4$) é capaz de induzir SIPS. De facto, as células tratadas com o cobre exibem características típicas do fenótipo senescente, que incluem as alterações morfológicas, o aumento da beta-galactosidase associada a senescência (SA βgal), a inibição da proliferação celular e o aumento da expressão de genes e proteínas associadas à senescência.

Acresce que o estabelecimento do novo modelo de $CuSO_4$-SIPS permitiu estudar mecanismos moleculares subjacentes à contribuição do cobre para o processo de envelhecimento. Um destes mecanismos relaciona-se com a perda de proteostasia,

considerada uma das principais características do envelhecimento. Assim, formulou-se a hipótese de que na senescência celular a proteostasia altera-se e são activados mecanismos celulares compensatórios, como a resposta do retículo endoplasmático (ER) a proteínas com conformação anómala (UPR). Os resultados evidenciam que os modelos de RS e $CuSO_4$-SIPS exibem alterações moleculares semelhantes ao nível do ER, em contraste com as observadas no modelo de H_2O_2-SIPS. Além disso, para ambos os modelos SIPS é demonstrado que a activação da UPR do ER é necessária para a indução do fenótipo senescente. No entanto, enquanto a inibição do ciclo celular induzida pelo $CuSO_4$ depende da ativação da UPR mediada pela PERK, isso não se verifica no modelo H_2O_2-SIPS. Assim, no que diz respeito aos mecanismos moleculares envolvidos, o modelo $CuSO_4$-SIPS mostrou ser mais semelhante ao RS do que o modelo H_2O_2-SIPS, o mais frequentemente utilizado.

Com o objetivo de desvendar potenciais mecanismos moleculares anti-senescência, o composto polifenólico antioxidante resveratrol, conhecido por apresentar propriedades anti-envelhecimento, foi utilizado. Da suplementação com resveratrol resultou a atenuação do aparecimento de alterações típicas da senescência induzidas pelo $CuSO_4$ ao nível da morfologia celular, da actividade da SA βgal e da proliferação celular. Além disso, os efeitos benéficos do resveratrol mostraram ser independentes da regulação de genes e proteínas associadas à senescência, mas depender da melhoria da proteostasia celular. Na verdade, o resveratrol atenuou o aumento do teor de proteína total e de BiP induzido pelo cobre, e reduziu o nível de proteínas carboniladas e poli-ubiquitinadas, pela indução de autofagia lisossomal.

No seu conjunto, os resultados apresentados nesta tese apoiam a intervenção activa de cobre no processo de envelhecimento, e propõem um modelo celular que provou ser semelhante aos modelos de RS e SIPS anteriormente reconhecidos. Além disso, verificou-se que a contribuição de cobre para o envelhecimento é acompanhada pela ativação de processos biológicos compensatórios, que visam promover a proteostasia celular. Estamos convencidos de que a identificação de alvos moleculares específicos, que medeiam a senescência celular e são passíveis de ser modulados, terá grande relevância para o desenvolvimento de estratégias destinadas a atenuar os processos de deterioração relacionados com o aumento da idade e melhorar o estado de saúde durante o envelhecimento.

ABSTRACT

Ageing became a major concern of modern societies due to its vast social and financial impact. Therefore, it has been widely investigated in an attempt to find strategies able to delay the age-related health deterioration and the establishment of diseases. In this setting, it is crucial to identify potential contributors and unravel their inherent molecular mechanisms implicated in the ageing process.

At the cellular level, it is characterized by the progressive accumulation of oxidatively damaged biomolecules that ultimately lead to cellular dysfunction. Among the conditions favoring the process may be the alterations on metal homeostasis that result in their increased levels or accumulation within cells and tissues, where they foster the generation of reactive oxygen species (ROS) that target biomolecules. In fact, enhanced copper levels were reported during ageing and in age-associated diseases such as type II diabetes, atherosclerosis and neurodegenerative disorders, further supporting the contribution of copper for oxidative stress and the overall age-related functional deterioration.

In vitro cellular models of senescence are essential to study molecular events during the ageing process. Stress-induced premature senescence (SIPS) models are frequently employed as they present various advantages when compared to the classical replicative senescence (RS) model: they are faster to obtain, easier to manipulate and exhibit a heterogeneous cellular response similar to the *in vivo* condition. The most frequently used SIPS inducer is hydrogen peroxide (H_2O_2), but other oxidative stress inducers may be successfully employed.

Based on copper ability to mediate the formation of free radicals, the current work reported, for the first time, that incubation of WI-38 fibroblasts with a subcytotoxic concentration of copper sulfate ($CuSO_4$) induces SIPS. In fact, $CuSO_4$-SIPS fibroblasts exhibit typical senescent phenotype features, including altered morphology, increased senescence-associated beta-galactosidase (SA βgal), cell proliferation inhibition and several senescence-associated genes and proteins upregulation.

In addition, the establishment of the novel $CuSO_4$-SIPS model allowed to study the molecular mechanisms underlying copper contribution to the ageing process. One such mechanism relates to proteostasis disruption, considered a major hallmark of ageing. Thus, it was hypothesized that proteostasis was altered during cellular senescence and compensatory mechanisms as the endoplasmic reticulum (ER) unfolded protein response (UPR) would be activated. The studies evidenced that both RS and $CuSO_4$-SIPS models

exhibit similar ER molecular alterations, in contrast to the data shown for H_2O_2-SIPS model. Moreover, for both SIPS models it is shown that ER UPR activation is required for the induction of the senescent phenotype. However, while $CuSO_4$-induced cell cycle arrest depends on PERK-mediated UPR activation, this was not verified to H_2O_2-SIPS condition. Therefore, regarding the molecular mechanisms involved, $CuSO_4$-SIPS model proved to be more similar to RS than the most frequently used H_2O_2-SIPS.

Aiming at unraveling potential anti-senescence molecular mechanisms, the polyphenolic antioxidant compound resveratrol, known to exhibit anti-ageing properties, was used. Resveratrol supplementation was shown to attenuate the appearance of $CuSO_4$-induced typical senescence alterations on cell morphology, SA βgal activity and cell proliferation. Moreover, the beneficial effects of resveratrol were shown to be independent of the regulation of some senescence-associated genes and proteins, but to require the improvement of cellular proteostasis. In fact, resveratrol attenuated copper-induced increased levels of total protein content and BiP chaperone and reduced carbonylated and poly-ubiquitinated proteins by lysosomal autophagy induction.

Altogether, the results presented in this thesis support the active intervention of copper in the ageing process, and propose a valuable cellular model that proved to be similar to the previously recognized RS and SIPS models. Moreover, it was found that the contribution of copper to ageing is accompanied by the activation of compensatory biological processes that aim at promoting a balanced cellular proteostasis. We are convinced that the identification of specific molecular targets that mediate cellular senescence, and are ameanable to modulation, will have major relevance for the development of strategies intended to attenuate age-related deterioration processes and enhance human healthspan.

INDEX

I

INTRODUCTION

The progressive ageing of modern societies' population constitutes a major concern due to its vast social and economic implications. Ageing is not a disease but it increases the susceptibility to disease, as it is accompanied by a gradual deterioration in overall functions leading to increased vulnerability and occurrence of entities such as cancer, type II diabetes, neurodegenerative diseases and cardiovascular disease caused by atherosclerosis.

The ageing process has been characterized as consequent to a progressive accumulation of oxidatively damaged biomolecules within cells that ultimately impinges on their function. It is unknown what triggers the process, which would direct us to the profound causes of ageing, but an imbalance in metal homeostasis is a likely condition. Indeed, some metals as copper or iron are contributors to ROS generation that target most biomolecule classes afterwards. It was shown that copper levels increase with ageing in rat brain and serum (Fu *et al.*, 2015), bovine brain (Zatta *et al.*, 2008) and human serum (Harman, 1965, Sohler and Pfeiffer, 1987). Moreover, alterations in copper homeostasis have also been reported in age-associated pathologies, such as atherosclerosis (Apostolova *et al.*, 2003), type II diabetes (Eaton and Qian, 2002) and neurodegenerative disorders, as Huntington (Fox *et al.*, 2007), Parkinson (PD; Rasia *et al.*, 2005) and Alzheimer (AD; James *et al.*, 2012) diseases. In fact, increased level of free serum copper was described for PD (Jimenez-Jimenez *et al.*, 1992) and AD (Brewer *et al.*, 2010), adding further evidence to the role of copper-induced oxidative stress in the etiology of these neurodegenerative diseases. Altogether, the data favour the involvement of this redox active metal in the overall deterioration process that is associated with ageing.

Unravelling the molecular mechanisms underlying the contribution of copper to the ageing process may therefore bring further insights to the identification of therapeutic targets aiming at attenuating age-related deterioration processes.

1. THE HALLMARKS OF AGEING

Since long ago, ageing has been subjected to scientific scrutiny in order to expand the knowledge of its inherent molecular and cellular mechanisms. It is widely accepted that the general cause of ageing is the time-dependent accumulation of cellular damage; however pivotal questions have arisen regarding the physiological sources of such damage, the compensatory responses that are activated and the possibility to delay ageing by exogenous interventions. In a noteworthy review published in *Cell* (López-Ótin *et al.*, 2013),

nine cellular and molecular hallmarks of ageing were identified and generally considered to contribute to the ageing process and to determine its phenotype *(Figure 1)*. Therein, the authors referred the occurrence of genomic instability, telomere attrition, epigenetic alterations and loss of proteostasis as primary hallmarks of ageing, as all these events are clearly negative and may be the initiating causes of cellular damage. Then, deregulated nutrient sensing, mitochondrial dysfunction and cellular senescence were pointed as antagonistic hallmarks because, depending on their intensity they might have opposite effects. At low levels, they are beneficial and consist of compensatory responses to cellular damage, but as the primary hallmarks persist or intensify, their level rises, they become deleterious and generate further damage. At last, stem cell exhaustion and altered intercellular communication, designated integrative hallmarks, arise when cellular damage is not efficiently compensated and eventually affect tissue homeostasis leading to the functional decline associated with ageing. Since the above mentioned hallmarks are interconnected and occur simultaneously during ageing, the exact hierarchic relation between them is yet to be determined.

Independently of their specific contribution to ageing, all these hallmarks consist of stressful conditions that will eventually damage cells and tissues. Therefore, understanding the complex biological processes linking ageing and stress will facilitate future interventions to circumvent age-related functional deterioration and improve human healthspan.

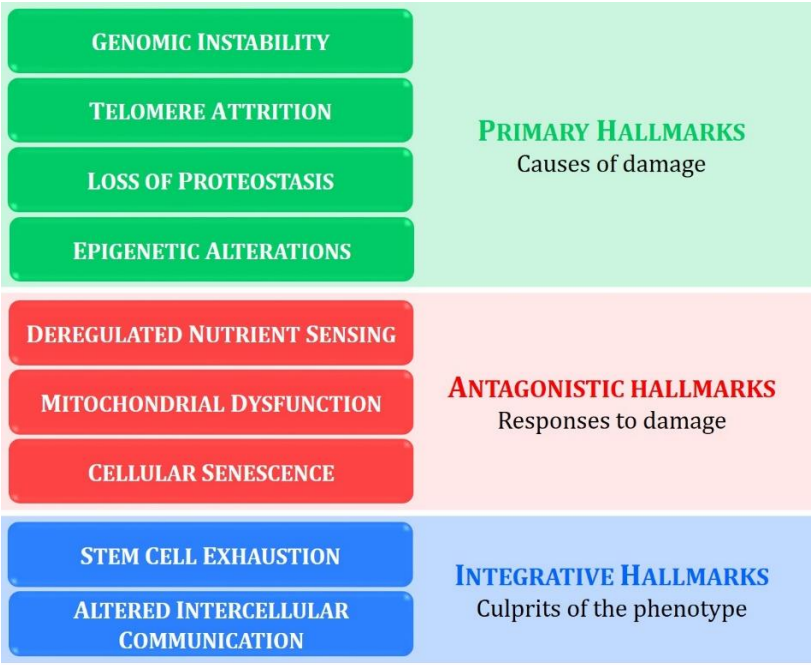

Figure 1: **Hallmarks of Ageing**. The proposed nine hallmarks of ageing are grouped into three categories: the primary hallmarks, which are considered to be the initiating causes of cellular damage; the antagonistic hallmarks that consist in compensatory responses that initially attenuate the damage but, when the damage persists, become deleterious; and the integrative hallmarks that are the major culprits of age-related functional decline. (Adapted from López-Ótin *et al.*, 2013)

2. AGEING AND STRESS

2.1. FREE RADICAL THEORY OF AGEING

Several different theories exist aiming at explaining the biological process of ageing. Among them, the most prominent and experimentally tested is the free radical theory of ageing (FRTA), proposed more than 50 years ago by Denham Harman, who stated that ageing results from the progressive accumulation of cellular damage caused by reactive oxygen species (ROS) generated during normal cellular function (Harman, 1956). Later on, in 1972, Harman renamed the theory as mitochondrial free radical theory of ageing (MFRTA), after he recognized that mitochondria were the main source and target of ROS (Harman, 1972). Ever since, the involvement of mitochondria in the ageing process has been widely investigated and reviewed (Sastre *et al.*, 2003, Gomez-Cabrera *et al.*, 2012, Bratic and Larsson, 2013). Actually, there are numerous age-associated evidences in favour of the MFRTA. For example, several mitochondrial functions decline with ageing, as both the efficiency of electron transport through the respiratory chain and the activity of mitochondrial ROS scavenging enzymes decreases. In contrast, ROS generation increases and the incidence of mitochondrial DNA damage rises (Shigenaga *et al.*, 1994, Castro *et al.*, 2012, Sastre *et al.*, 2002). In further support to the involvement of mitochondrial ROS in ageing, it was shown that the microinjection of aged mitochondria in young fibroblast resulted in the rapid acquisition of aged properties (Corbisier and Remacle, 1990). Interestingly, a higher rate of mitochondrial ROS production is observed in short-lived species, when compared to long-lived ones (Perez-Campo *et al.*, 1998).

Nowadays, it is consensual that the occurrence and accumulation of oxidatively damaged biomolecules are implicated in age-associated functional deterioration of cells and tissues. The main contributors for this process are the increased levels of ROS and the reduced efficiency of the antioxidant protective mechanisms that usually associates with ageing.

2.2. OXIDATIVE STRESS

Oxidative stress was first defined as a condition characterized by an imbalance between the intracellular levels of ROS and the cellular antioxidant capacity, either by an increase in pro-oxidants formation or a decrease in antioxidant defences (Sies, 1985). Meanwhile, the perception that oxidation-reduction (redox) reactions promoted by ROS are fundamental for several biologic signalling pathways led to the redefinition of oxidative stress in order to include the role of redox signalling (Jones, 2006). Nowadays, oxidative stress is described as “an imbalance between oxidants and antioxidants in favour of the oxidants, leading to a disruption of redox signalling and control and/or molecular damage” (Sies and Jones, 2007, Sies, 2015).

ROS designate a group of highly reactive molecules that comprises oxygen-centred free radicals (i.e. species capable of independent existence that have at least one unpaired valence electron) such as hydroxyl radical ($HO^{\cdot}$) and superoxide anion ($O_2^{\cdot-}$), but also includes some oxygen derived non-radicals as hydrogen peroxide (H_2O_2). The reactivity of these species is variable, but $HO^{\cdot}$ is the most reactive as upon its *in vivo* generation, it reacts immediately at the formation site (Halliwell and Cross, 1994). Under aerobic physiological conditions, mitochondrial generation of $O_2^{\cdot-}$ and H_2O_2 constitutes the major intracellular source of ROS (*as reviewed in:* Cadenas and Davies, 2000). $O_2^{\cdot-}$ is formed by the partial reduction of molecular oxygen (O_2) caused by electrons that accidentally leak from the inner mitochondrial membrane respiratory chain (Barja and Herrero, 1998). It was stated that about 2 % of the molecular oxygen utilized by mitochondria would originate $O_2^{\cdot-}$ (Chance *et al.*, 1979) but this value was considered overestimated; it is now believed that the O_2 fraction leading to $O_2^{\cdot-}$ generation is 0,15 % (St-Pierre *et al.*, 2002). Another quantitatively large mitochondrial source of ROS is the outer membrane bound monoamine oxidases (MAOs), whose function is to catalyse the oxidative deamination of biogenic amines using O_2 as an electron acceptor forming H_2O_2 (Edmondson, 2014).

The deleterious effects that can result from ROS action can be prevented by several antioxidant mechanisms. Manganese superoxide dismutase (MnSOD), a mitochondrial matrix enzyme, readily neutralizes $O_2^{\cdot-}$ by converting it into O_2 and H_2O_2 (Weisiger and Fridovich, 1973). In turn, in the mitochondrial intermembrane space, $O_2^{\cdot-}$ level is controlled either by conversion to O_2 upon cytochrome c reduction, or by enzymatic dismutation catalysed by a copper and zinc containing superoxide dismutase (CuZnSOD), which is also present in the cytoplasm of eukaryotic cells (Okado-Matsumoto and Fridovich, 2001). The action of SOD enzymes further increases H_2O_2 levels both in mitochondria and cytoplasm.

To protect biomolecules from consequent oxidative damage, cells contain glutathione peroxidases and catalase enzymes whose function is to keep H_2O_2 levels low (Sies, 2014). However, the existence of high levels of H_2O_2 in the presence of reduced redox active metal ions, such as Fe (II) or Cu(I), may originate the much reactive hydroxyl radical ($HO^{\cdot}$) by Fenton reaction *(Figure 2)* (Kehrer, 2000), that readily reacts at its site of production and attacks whichever biomolecule it encounters to initiate free radical chain reactions (Halliwell and Gutteridge, 2007). In fact, $HO^{\cdot}$ is able to oxidatively damage DNA, lipids and proteins and affect their functions, which eventually impact on cell and tissue integrity (Halliwell and Cross, 1994).

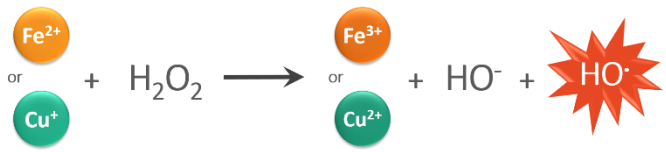

***Figure 2*: Fenton reaction**. In the presence of reduced redox active metals, such as Fe(II) or Cu(I), hydrogen peroxide (H_2O_2) is decomposed and originate the extremely reactive hydroxyl radical ($HO^{\cdot}$) that may oxidize biomolecules, such as proteins, lipids and DNA, and affect their functions.

2.3. ENDOPLASMIC RETICULUM STRESS

As a result of the age-related increase in ROS generation, there is a progressive accumulation of oxidatively damaged proteins within cells and tissues (Levine and Stadtman, 2001). To repair or eliminate such altered proteins and avoid cell dysfunction, cells activate quality control chaperoning and degradation mechanisms. But, then again, the efficiency of these protective cellular responses is decreased with ageing (Hayashi and Goto, 1998, Petropoulos *et al.*, 2000, Soti and Csermely, 2000, Viteri *et al.*, 2004). Altogether, the age-related impairment of protein quality control mechanisms and the accumulation of altered damaged proteins are the major culprits for the occurrence of proteostasis disruption during ageing and frequently correlate with age-associated diseases. Actually, it is well established that protein misfolding, accumulation and aggregation are well known contributors to the pathogenesis of several age-associated disorders, such as Alzheimer and Parkinson neurodegenerative diseases (Kopito and Ron, 2000, Selkoe, 2003), adding further relevance to the study of the molecular mechanisms behind the loss of proteostasis during the ageing process.

Eukaryotic cells possess an important protein quality control mechanism located within the endoplasmic reticulum (ER), which has been shown to play a crucial role in maintaining cellular proteostasis (as reviewed in: Noack *et al.*, 2014). The ER is an organelle composed of a vast network of interconnected membranous cisternae with a continuous intraluminal space that extends throughout the cytoplasm of eukaryotic cells. Its structural organization is highly dynamic as it can assume multiple configurations that allow the performance of different functions in distinct regions within the cell (Voeltz *et al.*, 2002). The ER can be morphologically and functionally divided in two major regions, the smooth (SER) and the rough endoplasmic reticulum (RER). RER is composed of flattened sacks with membrane-bound ribosomes whose main function is the synthesis of proteins to be targeted mainly to the cytoplasmic membrane, the extracellular space, the lysosomes, the ER and the Golgi complex. In turn, SER consists of an interconnected meshwork of tubules, free of ribosomes, which are responsible for the biosynthesis of sterols and phospholipids. Moreover, the ER is also the main site for the storage of intracellular calcium ions and play an essential role in many cellular signalling processes (Berridge, 2002). The relative amount of RER and SER found within different cell types depends on their main functions. For instance, RER is much more abundant in protein secreting cells, while steroid-synthesizing cells exhibit a higher extension of SER.

RER-synthesised proteins constitute a significant fraction of the overall cellular proteome. In order to maintain cellular functions, it is essential to the ER to assure that only correctly folded and functional proteins are able to reach their final destinations. Such quality control depend on the fine regulation of molecular chaperones and folding enzymes that exist within the ER (Hammond and Helenius, 1995). One of the first molecular chaperones encountered by newly ER synthesized polypeptides is the immunoglobulin binding protein (BiP), a member of HSP70 family, whose levels are adjusted according to the cellular needs. In fact, besides being crucial to promote the correct folding of nascent

proteins, BiP is also recruited by misfolded proteins in an attempt to refold, avoid protein aggregation and prevent cellular damage (Haas, 1994). Another chaperone whose activity is fundamental for protein homeostasis is calnexin, an ER transmembrane lectin that controls the quality of newly synthesized glycoproteins (Hammond *et al.*, 1994). A critical step for most ER-synthesized proteins to acquire their native conformation is the formation of disulphide bonds. This process involves thiol-disulphide oxidation, reduction and isomerisation and is catalysed by protein disulphide isomerase (PDI) in the optimal oxidizing environment of the ER, which is maintained by ER oxireductin-1 (Ero1; Ellgaard and Ruddock, 2005).

The occurrence of stress that compromises ER function is likely to impair protein folding and maturation processes and lead to the accumulation of unfolded and misfolded proteins in the lumen of the organelle, a condition termed ER stress. The ER responds to such proteostasis disruption by activating the ER stress response or unfolded protein response (UPR; Schroder and Kaufman, 2005). The UPR is an adaptive coordinated response that can be triggered by three ER transmembrane transducers: inositol-requiring enzyme-1 (IRE1), pancreatic ER kinase -like ER kinase (PERK) and activating transcription factor-6 (ATF6). The activation of these pathways aims to increase ER folding ability, to reduce global protein translation (Harding *et al.*, 1999) and, when necessary, to enhance ER-associated proteasome degradation (ERAD) of misfolded proteins (Friedlander *et al.*, 2000). In baseline conditions, IRE1, PERK and ATF6 activities are inhibited by the binding of BiP to their luminal domains. However, in the presence of ER stress and misfolded protein accumulation, BiP is unbound from the ER transducers, which leads to their activation in order to prevent protein aggregation *(Figure 3)*.

IRE1 protein contains an N-terminal portion located in the ER lumen, a single transmembrane segment and a C-terminal cytosolic region with a Ser/Thr kinase and an endoribonuclease (RNase) domain (Tirasophon *et al.*, 1998). Upon BiP unbinding, IRE1 is activated by oligomerization and trans-autophosphorylation of the cytosolic domains. Activated RNase domain of IRE1 is able to remove a 26-nucleotide intron from the X-box binding protein 1 (XBP1) mRNA, generating a more stable XBP1 spliced variant (XBP1s) that, upon translation, acts as a transcription factor that is able to promote the expression of several genes involved in maintaining ER homeostasis (Lee *et al.*, 2003). IRE1 RNase activity is also capable to degrade a pool of mRNAs that generally encode proteins from the secretory pathway through a process designated regulated IRE1-dependent decay of mRNA (RIDD; Hollien and Weissman, 2006), reducing the load of ER client proteins. In turn, the activated kinase domain of IRE1 phosphorylates c-Jun N-terminal kinase (JNK) through the interaction with the adaptor protein tumour necrosis factor (TNF) receptor-associated factor 2 (TRAF2; Urano *et al.*, 2000). Then, activated JNK is able to regulate downstream effectors either towards apoptosis (Szegezdi *et al.*, 2006) or cell survival (Ogata *et al.*, 2006).

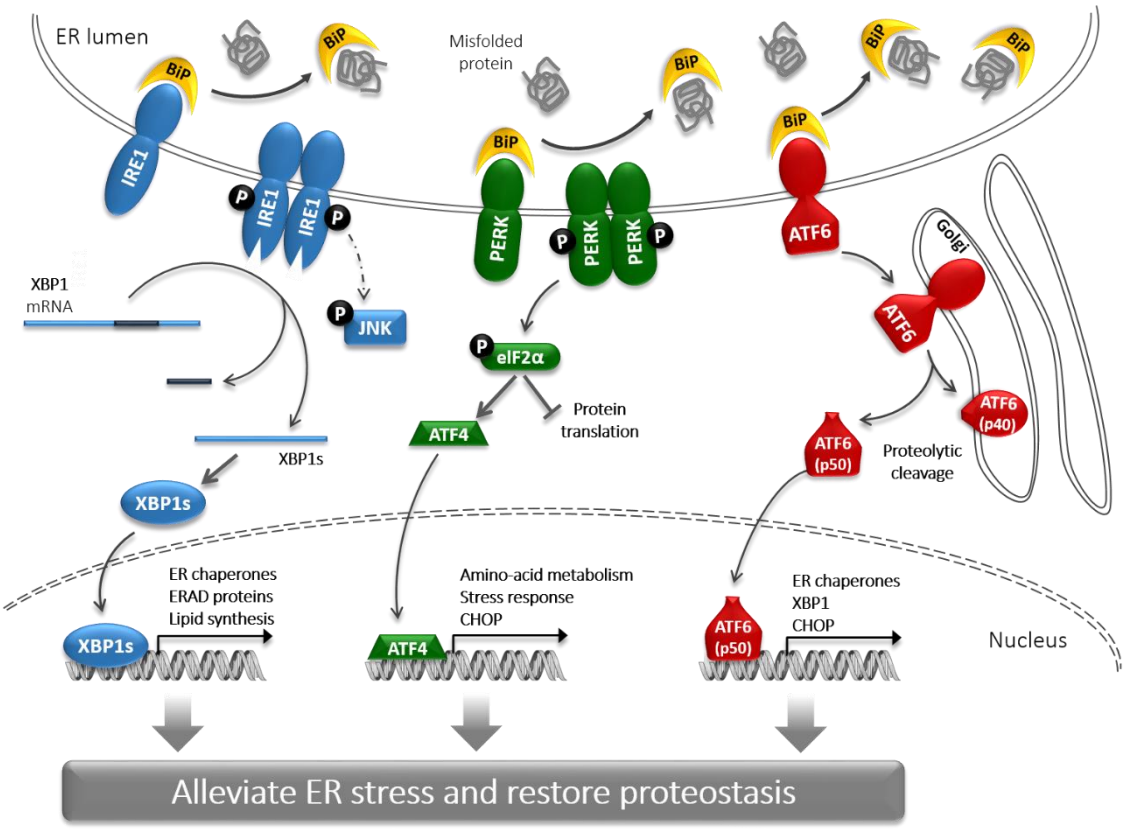

Figure 3: **Endoplasmic reticulum unfolded protein response.** In baseline conditions, BiP chaperone is bound to three ER transmembrane sensors: IRE1, PERK and ATF6. In ER stress conditions, unfolded and misfolded proteins accumulate within the organelle and recruit BiP to aid in their refolding and prevent their aggregation. Upon BiP unbinding, the ER sensors are activated and initiate the unfolded protein response (UPR) that aims at alleviating ER stress and restoring ER homeostasis. IRE1-mediated UPR *(in blue)* is initiated by IRE1 oligomerization and activation by phosphorylation. Once activated, IRE1 kinase domain indirectly leads to JNK phosphorylation, which is then able to regulate downstream effectors either towards apoptosis or cell survival. On the other hand, IRE1 ribonuclease activity promotes the removal of a 26-nucleotide intron from XBP1, originating the spliced variant XBP1s that, upon translation, acts as a transcription factor that promotes the expression of genes involved in maintaining ER homeostasis. Similarly to IRE1, PERK activation also depends on its oligomerization and phosphorylation *(in green)*. Upon activation, PERK phosphorylates eIF2, which causes the inhibition of general protein translation in an attempt to reduce ER protein load. In addition, phospho-eIF2 allows the selective translation of ATF4, which is a transcription factor that regulates the expression of genes involved in amino-acid metabolism and stress response aiming at increasing cell resistance to stress and ameliorating ER stress. ATF4 also promotes the expression of the transcription factor CHOP that at initial stages mediates a pro-survival response by upregulating autophagy-related genes, but as stress is prolonged, regulates pro-apoptotic target genes leading to cell death. Finally, ATF6-mediated UPR *(in red)* is initiated by ATF6 translocation to the Golgi compartment upon BiP dissociation. There, ATF6 is submitted to proteolytic cleavage by cytosolic proteases and releases a 50kDa cytosolic fragment, the ATF6(p50), that becomes an active transcription factor, able to regulate the expression XBP1, CHOP, ER chaperones and folding enzymes in order to restore ER proteostasis.

Similarly to IRE1, PERK protein contains a Ser/Thr kinase domain in its cytosolic C-terminal, and its activation involves dimerization and trans-autophosphorylation upon BiP release from its N-terminal domain in the ER lumen (Bertolotti *et al.*, 2000). Activated PERK phosphorylates the eukaryotic translation initiation factor 2 α (eIF2α), which results in the inhibition of global protein synthesis and consequently in the reduction of protein load into the ER (Harding *et al.*, 2000). Also, phosphorylated eIF2α allows the selective translation of

activating transcription factor-4 (ATF4), which is able to regulate the expression of several genes involved in amino-acid metabolism, stress response and protein secretion, aiming at alleviating ER stress and increase cell resistance to stress (Harding *et al.*, 2003). Besides, ATF4 can also promote the expression of C/EBP-homologous protein (CHOP), a transcription factor that is classically known as being able to upregulate pro-apoptotic genes leading to ER stress-induced apoptotic cell death (Zinszner *et al.*, 1998). However, CHOP was additionally described as having differential effects depending on the duration of stress: at an initial stage it mediated a protective pro-survival response by upregulating autophagy-related genes, but as stress was prolonged, CHOP limited autophagy and promoted apoptosis through the transcriptional control of specific target genes (B'chir *et al.*, 2014).

Finally, ATF6 is an ER transmembrane protein that contains a DNA binding motif in its cytosolic N-terminal portion. In ER stress conditions, BiP dissociates from the C-terminal domain of ATF6 located in the ER lumen and allows ATF6 translocation to the Golgi compartment. There it is submitted to proteolytic cleavage by site 1 and site 2 proteases and releases a 50kDa cytosolic fragment (ATF6(p50)) that becomes an active transcription factor, able to migrate to the nucleus and promote the expression of XBP1, CHOP, ER chaperones and folding enzymes, in an attempt to restore ER proteostasis (Yoshida *et al.*, 2000).

The activation of the UPR usually leads to an increase of cellular tolerance to higher amounts of abnormal ER proteins by folding promotion. However, when proteins fail to properly fold and assembly to achieve their functional conformation, the UPR chaperone system acts in coordination with the ERAD degradative machinery to eliminate misfolded proteins preventing the occurrence of damage (Friedlander *et al.*, 2000). Furthermore, ERAD also plays an important role in the regulation of ER resident proteins levels according to the cellular needs. ERAD is thus a complex process through which either abnormal or no longer needed ER proteins are targeted and ultimately degraded by the cytosolic ubiquitin-proteasome system. This occurs in four main steps, which are: substrate selection, dislocation across the ER membrane to the cytosol, poly-ubiquitination and proteasomal degradation (Olzmann *et al.*, 2013).

During ageing, the efficiency of UPR seems to be compromised, as shown by age-related alterations of ER molecular chaperones and folding enzymes expression (*as reviewed in:* Naidoo, 2009). Several studies provided evidence that BiP (Paz Gavilan *et al.*, 2006, Erickson *et al.*, 2006, Hussain and Ramaiah, 2007, Naidoo *et al.*, 2008), calnexin (Paz Gavilan *et al.*, 2006, Erickson *et al.*, 2006) and PDI (Paz Gavilan *et al.*, 2006) protein levels decrease with ageing in rodent tissues. In addition, both BiP and PDI were found to be oxidized (Rabek *et al.*, 2003) and consequently became less functional (Nuss *et al.*, 2008) in aged mice livers, indicating that ER proteins are vulnerable to the increased oxidative stress that accompanies ageing. Besides the alterations on ER lumen proteins, PERK-mediated UPR pathway was also reported to be affected during ageing, as PERK mRNA was found decreased in the hippocampus of aged rats (Paz Gavilan *et al.*, 2006) and eIF2α

phosphorylation was lower in several tissues from aged rats, when compared to young animals (Hussain and Ramaiah, 2007). The UPR has also been implicated in the pathogenesis of a variety of age-associated diseases, such as type II diabetes, atherosclerosis and neurodegenerative disorders (*as reviewed in:* Brown and Naidoo, 2012).

So, accumulated evidence shows UPR activation involvement in the ageing process, but its actual contribution to the cellular and molecular changes that accompany cellular ageing remain unclear.

3. CELLULAR SENESCENCE

3.1. THE ROLE OF SENESCENT CELLS IN ORGANISMAL AGEING

At the cellular level, the occurrence of ageing is usually termed cellular senescence and is characterized by the appearance of typical morphological and molecular alterations that affect cellular function. The actual existence of senescent cells in living organisms had been extensively debated (Ben-Porath and Weinberg, 2004) mainly due to two major limitations. First, most cells in organismal tissues are post-mitotic, which led researchers to question whether such cells, similarly to proliferating ones, could also become senescent; the second limitation was the lack of markers able to exclusive and unequivocally detect the senescent cells. Therefore, to circumvent the general lack of specificity of such senescence biomarkers, they are often used in combination, which apart from allowing the identification of senescent cells also provide further clues on the mechanisms behind their appearance. The histochemical detection of senescence associated β-galactosidase (SA βgal), a lysosomal enzyme whose activity is increased in senescent cells, was the first and is the most widely used marker of senescence (Dimri *et al.*, 1995, Pendergrass *et al.*, 1999, Paradis *et al.*, 2001, Ding *et al.*, 2001, Geng *et al.*, 2010). However, its use has been questioned as it was also identified in non-senescent cells (Yegorov *et al.*, 1998, Severino *et al.*, 2000, Yang and Hu, 2005). The search for the perfect senescence biomarker is still a matter of intensive research, which, nevertheless, revealed many strong candidates. These include genes involved in the establishment and maintenance of senescence, as p21 (Ding *et al.*, 2001) and p16 (Janzen *et al.*, 2006, Krishnamurthy *et al.*, 2006, Molofsky *et al.*, 2006, Ressler *et al.*, 2006); genotoxic stress markers located at the telomeres, as p53 binding protein 1 (Herbig *et al.*, 2006, Jeyapalan *et al.*, 2007) or phosphorylated histone H2AX (Wang *et al.*, 2009); and distinctive and highly dense nuclear regions, the senescence-associated heterochromatin foci (SAHF; Jeyapalan *et al.*, 2007, Kreiling *et al.*, 2011).

Nevertheless, in the last two decades, the use of the above mentioned senescence markers allowed researchers to demonstrate that the abundance of senescent cells increase in multiple tissues from rodents and primates (including humans) along chronological ageing and in age-associated pathologies, suggesting that these cells might play a role in the typical age-related functional involution (*as reviewed in:* Burton and Krizhanovsky, 2014). In aged mice, senescent cells were detected in diverse tissues and organs, such as brain

(Molofsky *et al.*, 2006, Jurk *et al.*, 2012), pancreas (Krishnamurthy *et al.*, 2006), skeletal muscle (Kreiling *et al.*, 2011), bone marrow (Janzen *et al.*, 2006), lung (Wang *et al.*, 2009, Kreiling *et al.*, 2011), liver (Wang *et al.*, 2009, Kreiling *et al.*, 2011, Hewitt *et al.*, 2012), adipose tissue (Tchkonia *et al.*, 2010), small intestine (Wang *et al.*, 2009, Hewitt *et al.*, 2012), spleen and skin (Wang *et al.*, 2009). In addition, using aged rats, the occurrence of cellular senescence was also described in a specific hippocampus region (Geng *et al.*, 2010) and in kidney (Ding *et al.*, 2001). Only a few studies employed tissues from aged primates. More than fifteen years ago it was shown that the frequency of senescent cells was increased in liver biopsies from aged monkeys (Pendergrass *et al.*, 1999). Moreover, studies using skin and muscle biopsies from aged baboons also reported increased number of senescent cells when compared to young animals tissues (Herbig *et al.*, 2006, Jeyapalan *et al.*, 2007, Kreiling *et al.*, 2011). In humans, despite the inherent difficulties in obtaining human samples, cellular senescence *in vivo* was described in skin (Dimri *et al.*, 1995, Ressler *et al.*, 2006) and liver (Paradis *et al.*, 2001) samples from aged individuals. Furthermore, an increased incidence of senescent cells was reported in tissues of patients with age-associated disorders, such as atherosclerosis (Minamino *et al.*, 2002, Gorgoulis *et al.*, 2005, Erusalimsky, 2009), type II diabetes (Sone and Kagawa, 2005, Markowski *et al.*, 2013), osteoporosis (Benisch *et al.*, 2012), cataracts (Babizhayev *et al.*, 2011) and Alzheimer disease (Malavolta *et al.*, 2013).

The identification of senescent cells in tissues predominantly composed of post-mitotic cells proved that, similarly to proliferating cells, mature post-mitotic cells are also able to develop a senescent phenotype *in situ*, even though the underlying mechanisms may be different. Despite the usually low frequencies reported, the gradual accumulation of senescent cells within tissues appears to reflect the existence of a general cellular response to stress. As a state of permanent inhibition of cell proliferation, senescence was pointed out as an appealing tumour suppressive cellular mechanism, thus able to prevent the development of malignancies (Campisi, 1996).

Paradoxically, it was later shown that senescent cells promoted the growth of premalignant cells by altering cellular neighbourhood (Krtolica *et al.*, 2001, Liu and Hornsby, 2007). In fact, senescent cells are not passive bystanders within tissues, as they remain metabolically active and profoundly affect the surrounding environment through the secretion of factors that collectively constitute the senescent-associated secretory phenotype (SASP; Coppe *et al.*, 2008). The SASP mainly consists of pro-inflammatory cytokines, growth factors and matrix metalloproteinases (Kuilman and Peeper, 2009) that act either in an autocrine or a paracrine fashion. Actually, the secretion of chemokines and growth factor binding proteins by senescent cells was able to further reinforce the senescent phenotype within the secreting cells (Acosta *et al.*, 2008, Kuilman *et al.*, 2008, Wajapeyee *et al.*, 2008). Besides, the SASP can also exert either beneficial or detrimental paracrine actions depending on the context. In younger individuals, a localized and time-limited pro-inflammatory SASP is crucial to alert nearby cells to potential danger and to promote immune clearance of damaged senescent cells, thus avoiding tissue injury and

dysfunction (Xue *et al.*, 2007, Krizhanovsky *et al.*, 2008, Kang *et al.*, 2011). However, during ageing, the effectiveness of the immune system is reduced and, as it becomes less competent to clear senescent cells, their age-related accumulation ensues. Therefore, in older individuals, the presence of a pronounced and persistent SASP causes chronic inflammation, and negatively affects the surrounding environment by disrupting tissue structure and organization (Parrinello *et al.*, 2005), by stimulating tumourigenesis of nearby pre-malignant cells (Krtolica *et al.*, 2001, Coppe *et al.*, 2008) or by inducing senescence in normal neighbour cells (Hubackova *et al.*, 2012, Acosta *et al.*, 2013).

There is a vast amount of data relating the deleterious effects of the SASP from senescent cells *in vivo* to age-associated tissue degeneration and organ dysfunction. However, the first direct evidence for a causal link between the existence of senescent cells and the development of age-related pathologies was demonstrated using a mouse model where the elimination of p16-positive senescent cells prevented or delayed tissue dysfunction and extended healthspan (Baker *et al.*, 2011). Then, using a similar experimental approach, it was demonstrated a positive role of senescent cells and their SASP in tissue repair and regeneration because the removal of p16-positive senescent cells *in vivo* resulted in delayed skin wound healing (Demaria *et al.*, 2014). These distinct roles of cellular senescence *in vivo,* either as a tissue remodelling aiding mechanism or a noxious chronic inflammatory source, encourage further investigation aiming at developing potential therapeutic tools to enhance or block senescence, depending on the context. The best experimental approach to unravel the detailed mechanisms behind human cellular senescence is to use *in vitro* cellular models as they are easy to obtain and manipulate.

3.2. *IN VITRO* CELLULAR MODELS

REPLICATIVE SENESCENCE

In 1965, Leonard Hayflick observed that serially cultivated human diploid fibroblasts (HDFs) exhibited a limited proliferative potential and became irreversibly arrested after a finite number of population doublings (PDs), even in the presence of mitogens (Hayflick, 1965). Such condition was considered a manifestation of ageing at a cellular level, and was designated cellular senescence or replicative senescence (RS). Since then, *in vitro* models of dividing somatic cells, including fibroblasts (Hayflick, 1965), endothelial cells (Mueller *et al.*, 1980), vascular smooth muscle cells (Bierman, 1978), keratinocytes (Rheinwald and Green, 1975) and lymphocytes (Tice *et al.*, 1979), have been preferred systems utilized by researchers to unravel the complex molecular mechanisms and pathways underlying the human ageing process.

Upon the early observations, it was noticed that the number of doublings of a cell population in culture before attaining RS was fairly reproducible, which indicated the existence of a cell division counting mechanism. Telomeres could function as sensors for such mechanism, as they were found to shorten as cells age (Harley *et al.*, 1990) and when

they reached a critical minimum size, a cell cycle inhibition response was triggered leading to cellular senescence. Actually, the ectopic expression of telomerase, a specialized enzyme able to compensate telomeric DNA losses, was found sufficient to revert RS cells into a phenotypically youthful state (Bodnar *et al.*, 1998).

Telomere attrition is unlikely to be solely a cell division counting mechanism as it was shown that a given cell population in culture exhibit a large heterogeneity in telomere length both within individual cells and between cells (Lansdorp *et al.*, 1996, Baird *et al.*, 2003, Zou *et al.*, 2004). One explanation for such heterogeneity is the occurrence of telomere damage caused by oxidative stress (Sozou and Kirkwood, 2001). Being so, the lifespan of an individual cell population is not only dependent on telomere length but is also affected by external stochastic factors upstream of telomere shortening (Passos *et al.*, 2007). Whichever the cause for telomere attrition, this event will activate a DNA damage response that culminates in a G1 phase cell cycle arrest (Huang *et al.*, 1996, Saretzki *et al.*, 1999).

Even though the major hallmarks of RS are the cell cycle arrest and the insensitivity to mitogens, RS cells also exhibit other typical structural and functional changes that are collectively designated as the senescent phenotype. When compared to young proliferating cells, RS fibroblasts experience dramatic morphological alterations that include the increase in cell surface area and volume (Greenberg *et al.*, 1977), the loss of typical small spindle-fusiform shape and the acquisition of large, flat, morphology (Nishio *et al.*, 2001). In addition, senescent cells also undergo several organellar changes. Their lysosomes increase in number and size (Lipetz and Cristofalo, 1972) and accumulate granules of lipofuscin (Brunk and Terman, 2002), the so-called “age-pigment” that is essentially composed of oxidized material (proteins, lipids and, in a lesser amount, carbohydrates) and transition metals (Benavides *et al.*, 2002, Double *et al.*, 2008).

In part, as a consequence of the rise in lysosomal content, RS cells exhibit an increased amount of SA βgal (Dimri *et al.*, 1995). Regarding mitochondria, although they exist in higher number in RS cells (Lee *et al.*, 2002), they are oxidatively affected (Mecocci *et al.*, 1993, Ahmed *et al.*, 2010) and less functional (Boffoli *et al.*, 1994). Moreover, when compared to actively proliferating cells, senescent cells exhibit larger nuclei (Mitsui and Schneider, 1976) where they show highly condensed regions, the SAHF. Such regions seem to relate to transcriptionally suppressed E2F-responsive genes, thus contributing to cell cycle arrest (Narita *et al.*, 2003).

In fact, RS cells present an altered gene expression profile that differ depending on cell type (Shelton *et al.*, 1999). Many of the altered genes are involved in a diversity of processes such as cell cycle regulation, inflammation and immune response, cytoskeleton organization, stress response and metabolism (Zhang *et al.*, 2003, Ly *et al.*, 2000, Yoon *et al.*, 2004). Some genes and their respective proteins are overexpressed and their assessment has been used to evidence cellular senescence; they include apolipoprotein J (ApoJ), fibronectin, osteonectin, transforming growth factor β-1 (TGFβ1) and insulin growth factor binding protein 3 (IGFBP3) (Debacq-Chainiaux *et al.*, 2008). In addition, overexpression of genes encoding the cyclin dependent kinase inhibitors (CDKIs) p21 and p16 is also typical

for RS cells and leads to an irreversible cell cycle arrest in G1 phase (Alcorta *et al.*, 1996, Pignolo *et al.*, 1998). Moreover, the cytoskeleton β-actin and vimentin proteins were also reported to be differentially expressed in RS cells (Trougakos *et al.*, 2006).

In general, RS cells are resistant to apoptosis, although this effect may depend on the cell type and the pro-apoptotic signal. For instance, RS endothelial cells, but not fibroblasts, are susceptible to ceramide-induced apoptosis (Hampel *et al.*, 2004). In addition, RS fibroblasts resist to growth factor deprivation- and oxidative stress-induced cell death but not Fas-mediated apoptosis (Chen *et al.*, 2000a, Tepper *et al.*, 2000). The mechanism behind senescence-associated apoptosis resistance is not clear, but the up-regulation of genes encoding survivin (Al-Khalaf and Aboussekhra, 2013), c-myb (Lee *et al.*, 2010), major vault protein (Ryu *et al.*, 2008) and bcl-2 (Ryu *et al.*, 2007) have already been implicated. In addition, epigenetic mechanisms able to regulate *bcl-2:bax* gene expression in RS fibroblasts, were proposed to contribute to the apoptosis-resistant phenotype (Sanders *et al.*, 2013).

PREMATURE SENESCENCE

Allowing cells to reach their proliferative limit in culture is not the only way to obtain senescent cells, because cellular senescence can be experimentally induced either by oxidative stress or oncogene activation. In fact, it was suggested that non-tumour cells present mechanisms that override the proliferative activity of oncogenes by promoting senescence (Serrano *et al.*, 1997). Since then, several oncogenes were described as capable to trigger oncogene-induced senescence (OIS), including H-Ras^{G12V} (Serrano *et al.*, 1997), BRAF^{V600E} (Michaloglou *et al.*, 2005) and NF-κB (Bernard *et al.*, 2004). The obvious limitations of the *in vitro* studies, consequent to inherent differences between *in vivo* and *in vitro* microenvironments, initially brought some confusion and scepticism on the potential role of OIS *in vivo* (Sherr and Depinho, 2000). However, later, animal model studies or intact human tissues provided substantial evidence in favour of the occurrence of OIS as a physiologic protective mechanism against cancer (Braig *et al.*, 2005, Michaloglou *et al.*, 2005, Lazzerini Denchi *et al.*, 2005).

Alternatively, premature senescence can be induced by oxidative stress, a condition named stress-induced premature senescence (SIPS). Actually, cellular models of SIPS are the most frequently used to study the molecular mechanisms underlying the ageing process. They are established upon exposure of actively dividing cells to subcytotoxic doses of oxidative stress inducers, such as hydrogen peroxide (H_2O_2) (Chen and Ames, 1994), tert-butyl hydroperoxide (tBHP) (Dumont *et al.*, 2000), hyperoxia (Saretzki *et al.*, 1998) or ultra-violet B radiation (UVB) (Debacq-Chainlaux *et al.*, 2005), among others. Similarly to RS, SIPS model exhibits an irreversible cell cycle arrest in the G1 phase (Chen *et al.*, 1998, Von Zglinicki *et al.*, 1995) and the senescent phenotype typical structural and molecular features; therefore, it is an excellent model for the study of cellular senescence.

The most widely utilized SIPS model is obtained by exposing early passage fibroblasts to a subcytotoxic concentration of H_2O_2 for 2h (H_2O_2-SIPS). After a three-day period of recovery, most treated cells cease to proliferate and exhibit a senescent-like morphology. Moreover, they present an increased SA βgal activity and altered gene and protein expression profiles, resembling RS cells (Chen and Ames, 1994, Chen *et al.*, 1998, Chen *et al.*, 2000b). In fact, similarly to RS fibroblasts, hyperoxia- (Saretzki *et al.*, 1998), tBHP- or H_2O_2-SIPS (Dumont *et al.*, 2000) cells exhibit overexpression of several senescence-associated genes, as p21, ApoJ, fibronectin, IGFBP3 and TGFβ1. In addition, using the H_2O_2-SIPS model, it was demonstrated that TGFβ1 overexpression is required to promote the typical senescent morphological changes, the increased activity of SA βgal and the overexpression of fibronectin, ApoJ and osteonectin (Frippiat *et al.*, 2001).

Still, RS and SIPS are not the same and some differences were described. For example, the involvement of telomere attrition in the establishment of SIPS is controversial. While some studies demonstrated that in hyperoxia- or H_2O_2-SIPS cells, telomeres shorten faster than in young fibroblasts (Von Zglinicki *et al.*, 1995, Duan *et al.*, 2005), others failed to show that and suggested that SIPS can be induced without telomere shortening (Chen *et al.*, 2001). Such conflicting evidence may result from experimental variations regarding the type and intensity of the oxidative stress inducer used in the different studies. However, despite these controversial facts, telomere attrition seems to be affected by oxidative stress as cell culture supplementation with antioxidants showed to be efficient in slowing telomere shortening (Furumoto *et al.*, 1998, Von Zglinicki *et al.*, 2000). Moreover, in SIPS cells, telomeres were found to sense the presence of oxidative stress, accumulate damage in their DNA and activate a cellular response that resulted in the inhibition of cell proliferation through p21 overexpression (Saretzki *et al.*, 1999). Furthermore, comparative proteomic studies using SIPS and RS cells showed that each condition has its own protein expression pattern, although they exhibit the same, typical, senescent phenotype (Dierick *et al.*, 2002, Aan *et al.*, 2013). This indicates that different pathways may be involved, but all seem to converge at the senescent phenotype.

One major advantage of using SIPS models is the fact that the induction of senescence can be studied independently from purely adaptive responses; indeed, SIPS induction protocols include a cell recovery period of at least three days after stress, before the analysis of the senescent biomarkers. Another important point is that after exposure to stress, either acute or chronic, there is not an all-or-none response of cells. In fact, a single acute exposure to a stress inducer inhibits proliferation of a fraction of cells whereas the other fraction remains unharmed and able to continue proliferating. Such heterogeneous cellular response makes SIPS an ideal model to study cellular senescence, as a similar response is observed at the organismal level during ageing. Besides, these *in vitro* cellular models of senescence are also a valuable tool in the search for compounds with the potential to modulate ageing progression. Such research might result in the discovery of specific targets that ultimately may be modulated to delay or attenuate age-related functional deterioration and the establishment or progression of age-associated diseases.

3.3. ATTENUATION OF CELLULAR SENESCENCE

So far, caloric restriction (CR) has been described as the best strategy to increase lifespan in a variety of organisms, including yeast, worms, flies, fish, mice and rats (*as reviewed in:* Heilbronn and Ravussin, 2003). Furthermore, in mammals, CR induces metabolic changes known to improve healthspan and reduces the incidence and delays the onset of age-associated disorders (Halagappa *et al.*, 2007, Speakman and Mitchell, 2011). In an attempt to explain the anti-ageing effects of CR, a number of studies repeatedly demonstrated its ability to promote stress resistance and attenuate the appearance and accumulation of oxidative stress induced cellular damage (Matsuo *et al.*, 1993, Sohal *et al.*, 1994, Dubey *et al.*, 1996, Lee *et al.*, 1999, Hamilton *et al.*, 2001).

The silent information regulator 2 (Sir2) protein was identified in yeast as a key mediator in lifespan extension promoted by CR (Lin *et al.*, 2000). In addition, Sir2 homologues were shown to contribute to CR-associated anti-ageing effects in flies (Rogina and Helfand, 2004), worms (Tissenbaum and Guarente, 2001) and mammals (Chen *et al.*, 2005). The mammalian orthologue protein of Sir2 is sirtuin 1 (Sirt1), which belongs to a family of 7 homologous proteins (Sirt1-7) that are NAD^+-dependent deacetylases known to regulate molecular pathways involved in energy metabolism, cell survival and longevity (*as reviewed in:* Michan and Sinclair, 2007). Sirt1 substrates include histones (Imai *et al.*, 2000, Vaquero *et al.*, 2004), tumour suppressor p53 protein (Vaziri *et al.*, 2001) and the family of forkhead box O (FOXO) transcription factors (Brunet *et al.*, 2004).

During ageing, Sirt1 expression was shown to decrease in tissues from rodents (Lafontaine-Lacasse *et al.*, 2010, Jin *et al.*, 2011, Quintas *et al.*, 2012, Gong *et al.*, 2014) and humans (Lin *et al.*, 2011, Lu *et al.*, 2014, Thompson *et al.*, 2014). In addition, the enzyme activity was diminished in the liver, heart, kidney and lung from aged rats when compared to young controls (Braidy *et al.*, 2011). Moreover, Sirt1 expression was reduced in the affected human tissues of age-associated disorders, such as cataracts (Lin *et al.*, 2011), Huntington (Pallas *et al.*, 2008) and Alzheimer (Julien *et al.*, 2009) diseases. At the cellular level, various *in vitro* models of cellular senescence also exhibit reduced expression of Sirt1 (Sasaki *et al.*, 2006, Furukawa *et al.*, 2007, Ho *et al.*, 2009, Song *et al.*, 2014). In fact, it was experimentally demonstrated that the inhibition of Sirt1 was sufficient to induce cellular senescence both in endothelial cells (Ota *et al.*, 2007) and fibroblasts (Volonte *et al.*, 2015), while Sirt1 overexpression was able to attenuate senescence and to extend the replicative lifespan of different cell types in culture (Ota *et al.*, 2007, Huang *et al.*, 2008, Ho *et al.*, 2009, Yamashita *et al.*, 2012). Altogether, these evidences clearly support the beneficial effects of Sirt1 in the ageing process both *in vivo* and *in vitro*.

The identification of molecules that could induce or activate Sirt1, thus mimicking CR anti-ageing effects, emerged as a promising research area within gerontology. So far, a large number of dietary and synthetic compounds were reported to induce Sirt1 activity (*as reviewed in:* Sinclair and Guarente, 2014). Among them, an important part has been ascribed to polyphenols that include resveratrol found in red wine grape skins, some berries and peanuts. Resveratrol was initially described as a phytoalexin, with antimicrobial activity

(Langcake and Pryce, 1976) and attracted more attention after being pointed as responsible for the cardioprotective effects of red wine (Siemann and Creasy, 1992). Nowadays, resveratrol is known to exert protective effects in various pathological entities, such as obesity, diabetes and cardiovascular disease (as reviewed in: Smoliga *et al.*, 2011). Moreover, its ability to modulate ageing has been exhaustively tested. Actually, it was shown to increase maximum lifespan of a variety of organisms, such as *Saccharomyces cerevisiae* (Howitz *et al.*, 2003), *Caenorhabditis elegans* (Wood *et al.*, 2004, Viswanathan *et al.*, 2005), *Drosophila melanogaster* (Wood *et al.*, 2004, Bauer *et al.*, 2004) and the short-lived fish *Nothobranchius furzeri* (Valenzano *et al.*, 2006). However, resveratrol failed to increase longevity in rodents, even though it improved their healthspan by exerting protective actions against age-related deterioration (Baur *et al.*, 2006, Pearson *et al.*, 2008). Similarly, at the cellular level, resveratrol was shown to attenuate senescence in various cell types in culture, through the activation of mechanisms that favour increased stress resistance and activation of DNA repair mechanisms (Giovannelli *et al.*, 2011, Mikula-Pietrasik *et al.*, 2012), induction of telomerase activity (Xia *et al.*, 2008), decreased secretion of senescence-associated pro-inflammatory proteins (Csiszar *et al.*, 2012), inhibition of the mechanistic target of rapamycin, mTOR (Demidenko and Blagosklonny, 2009) and activation of Sirt1-mediated pathways (Kao *et al.*, 2010, Ido *et al.*, 2015). Furthermore, resveratrol has also been considered a promising anti-tumour compound as it was capable to inhibit cell proliferation and induce cellular senescence in several tumour cell lines (Fang *et al.*, 2012, Yang *et al.*, 2013).

Despite the large amount of existing data on the effects of resveratrol, the actual molecular mechanisms that contribute to its anti-ageing effects are unclear. Thus, understanding the molecular dynamics behind the action of this polyphenolic compound may allow the identification of specific molecular targets amenable to therapeutic modulation aiming at delaying age-associated functional deterioration and improve healthspan.

4. OBJECTIVES AND STUDY OUTLINE

In vitro cellular models of senescence have been considered essential tools to unravel the molecular mechanisms responsible for the typical age-associated cell function deterioration (Passos *et al.*, 2009). SIPS models are often used with such purpose, as they exhibit several advantages when compared to the original RS cells: they are faster to obtain, easier to manipulate and exhibit a heterogeneous cellular response similar to what happens in tissues *in vivo*. The oxidative stress inducer that is most frequently employed to establish SIPS is H_2O_2, but other agents may also be successfully used.

Aiming at finding a SIPS model that may truthfully reflect the molecular events behind the physiological ageing process, it was hypothesized that the redox active metal copper would also be capable of inducing SIPS, due to its ability to mediate Fenton reaction and promote oxidative stress. Also, several evidences indicate that copper homeostasis is disrupted during ageing and therefore may be an important contributor to the ageing process. In fact, increased *in vivo* copper level was repeatedly associated with chronological ageing (Harman, 1965, Sohler and Pfeiffer, 1987, Zatta *et al.*, 2008) and age-associated entities, such as Parkinson and Alzheimer diseases (Jimenez-Jimenez *et al.*, 1992, Brewer *et al.*, 2010). In turn, studies using the fungal ageing model *Podospora anserina* demonstrated delayed cellular ageing in conditions of low intracellular copper, either due to an impairment in its cellular uptake (Borghouts *et al.*, 2002) or to the presence of a copper chelator (Borghouts *et al.*, 2001). In addition, RS fibroblasts were shown to have increased intracellular copper levels when compared to young fibroblasts (Boilan *et al.*, 2013). Altogether, these data add further relevance to the development of a copper-induced SIPS model, as it could become a valuable tool to evaluate the potential contribution of copper-induced oxidative stress for age-associated cellular deterioration and to characterize and identify the involved molecular processes.

Therefore, the first goal of the present work was to establish a SIPS model induced by copper, using WI-38 human diploid fibroblasts. Following the general study design employed in other SIPS models, cells were submitted to subcytotoxic concentrations of copper sulfate ($CuSO_4$) for 24h and, upon recovery and adaptation for an additional 72h period, they evidenced characteristic features of the senescent phenotype. They included the typical senescent morphology, cell proliferation inhibition, increased percentage of SA βgal positive cells, and overexpression of several genes and proteins typically associated with senescence, such as p21, ApoJ and TGFβ1. These findings demonstrated for the first time that $CuSO_4$ was actually able to induce premature senescence in cultivated human fibroblasts **(Publication I)**. Next, in the search for underlying mechanisms, it was hypothesized that proteostasis disruption, a typical hallmark of ageing, would take place and activate compensatory mechanisms as the ER UPR. For its assessment the levels of ER resident molecular chaperones and enzymes and the activation of PERK-, IRE1- and ATF6-mediated UPR pathways were evaluated. Both RS and $CuSO_4$-SIPS models exhibited similar results regarding the ER molecular alterations, in contrast to the data obtained for H_2O_2-

SIPS fibroblasts. Thereafter, the contribution of UPR activation for the induction of cellular senescence was assessed using specific PERK and IRE1 inhibitors. The results showed that ER UPR activation was required for the induction of both SIPS models, but, while $CuSO_4$-induced cell cycle arrest was mediated by PERK activation, this was not verified in H_2O_2-SIPS **(Publication II)**. At this point, on account of the molecular mechanisms involved, $CuSO_4$-SIPS proved to be more similar to the RS model, compared to the most frequently used H_2O_2-SIPS.

Finally, a third goal was to test resveratrol ability to modulate the $CuSO_4$-SIPS model and unravel additional molecular mechanisms involved. It was demonstrated that the polyphenolic compound attenuates the appearance of some typical senescence-associated features as SA βgal activity, morphological alterations and cell cycle inhibition. Such anti-senescence effects, proven to be independent of the regulation of classical senescence-associated genes and proteins, were shown to improve cellular proteostasis. In fact, $CuSO_4$-SIPS cells exhibited higher total protein content and increased accumulation of carbonylated and poly-ubiquitinated modified proteins **(Publication III)**, thus lending additional support to proteostasis disruption in this model as previously suggested **(Publication II)**. It is noteworthy that $CuSO_4$-treated cells recovery in the presence of resveratrol resulted in improved cellular proteostasis. Moreover, these beneficial effects were shown to be associated with the activation of lysosomal autophagy **(Publication III)**.

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II

PUBLICATIONS

PUBLICATION I

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Copper ability to induce premature senescence in human fibroblasts

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Abstract Human diploid fibroblasts (HDFs) exposed to subcytotoxic concentrations of oxidative or stressful agents, such as hydrogen peroxide, *tert*-butylhydroperoxide, or ethanol, undergo stress-induced premature senescence (SIPS). This condition is characterized by the appearance of replicative senescence biomarkers such as irreversible growth arrest, increase in senescence-associated β-galactosidase (SA β-gal) activity, altered cell morphology, and overexpression of several senescence-associated genes. Copper is an essential trace element known to accumulate with ageing and to be involved in the pathogenesis of some age-related disorders. Past studies using either yeast or human cellular models of ageing provided evidence in favor of the role of intracellular copper as a longevity modulator. In the present study, copper ability to cause the appearance of senescent features in HDFs was assessed. WI-38 fibroblasts exposed to a subcytotoxic concentration of copper sulfate presented inhibition of cell proliferation, cell enlargement, increased SA β-gal activity, and mRNA overexpression of several senescence-associated genes such as p21, apolipoprotein J (ApoJ), fibronectin, transforming growth factor β-1 (TGF β1), insulin growth factor binding protein 3, and heme oxygenase 1. Western blotting results confirmed enhanced intracellular p21, ApoJ, and TGF β1 in copper-treated cells. Thus, similar to other SIPS-inducing agents, HDF exposure to subcytotoxic concentration of copper results in premature senescence. Further studies will unravel molecular mechanisms and the biological meaning of copper-associated senescence and lead to a better understanding of copper-related disorder establishment and progression.

Keywords Cellular senescence · Copper · Metals · Oxidative stress · Human fibroblasts · Ageing

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Introduction

Cellular senescence was described more than four decades ago when it was demonstrated that normal

cells in culture had a limited ability to proliferate (Hayflick 1965). It was shown that serially cultivated human diploid fibroblasts (HDFs) initially exhibit active cell division, but, after a number of population doublings, dividing cell number decreases. Eventually, they cease dividing, become unresponsive to mitogenic stimuli and enter in a condition termed replicative senescence (RS). In addition, cells in the senescent state exhibit dramatic alterations in structure, mass, and functioning of their subcellular organelles, when compared with proliferating cells. They include an enlarged, flat morphology, increased reactive oxygen species production, lipofuscin accumulation, altered mass and functionality of mitochondria and lysosomes, and enhanced activity of senescence-associated β-galactosidase (SA β-gal; Hwang et al. 2009). Moreover, RS cells present altered expression of several senescence-associated genes (Debacq-Chainiaux et al. 2008).

A senescent phenotype may be achieved earlier, when HDFs in vitro are submitted to subcytotoxic doses of oxidants or other stressful agents such as hydrogen peroxide (Frippiat et al. 2001), *tert*-butylhydroperoxide (t-BHP), ethanol (Dumont et al. 2002), or ultraviolet B (UVB) radiation (Debacq-Chainiaux et al. 2005). Experimentally, when such agents are used, immediate adaptative responses are expected to occur. In order to circumvent them, the senescent features are assessed only 2 or 3 days after exposure to the last stress. Cells in this condition, termed stress-induced premature senescence (SIPS), remain alive for months and display many features of RS, including the typical senescent phenotype, cell cycle arrest, increased activity of SA β-gal, and gene expression profile alteration (Toussaint et al. 2000). Since SIPS cells are able to mimic many of the processes that occur during RS, they are frequently used to study the mechanisms of cellular ageing.

In biological systems, copper is an essential trace element since it acts as a co-factor of different enzymes such as cytochrome c oxidase, Cu/Zn superoxide dismutase, and others (Gupta and Lutsenko 2009). Apart from this essential role, ionic copper in excess becomes toxic and mediates the generation of the highly reactive hydroxyl radical, able to damage different kinds of biomolecules (Valko et al. 2005).

This dual role implies that copper uptake and utilization is under narrow regulation in order to allow for cell needs and prevent harmful effects. In many cells, most of the copper is taken up through evolutionary conserved copper transporters (CTRs) which include efficient orthologs in humans, *hCTR* (Zhou and Gitschier 1997), yeasts, *yCtr1,* and the filamentous fungi *Podospora anserina*, *PaCtr3* (Borghouts et al. 2002). After copper is taken inside liver cells, a Cu-ATPase (ATP7B) transports it across intracellular membranes. These may be vesicles to fuse with the cell membrane and excrete the copper into the bile, or vesicles that may enter the secretory compartment and supply copper as co-factor to the synthesis of the cuproenzymes (Gupta and Lutsenko 2009). Additional copper ions bind metallothionein, but, when in excess, they accumulate in the cytoplasm and cause oxidative damage (Gaetke and Chow 2003). These deleterious effects of copper are not evident early in life but may accumulate as we age. Actually, it is clear that copper is involved in the pathogenesis of age-associated disorders as Alzheimer's and Parkinson's disease (Barnham and Bush 2008; Brewer 2010).

The *P. anserina* ageing model provides interesting information into putative mechanisms on cellular ageing. In contrast to most filamentous fungi, the mycelia of *P. anserina* wild-type strains attain senescence and may die, after some time of active growth. However, a mutation in the *grisea* gene results in strains with life extension and delay in senescence. This gene induces the expression of the PaCTR3 permease but, when mutated, leads to its loss of function and decrease in the uptake of copper into the cell (Borghouts et al. 2002). Interestingly, senescence delay observed in the mutant strains may be lost when they are transformed with a constitutively active construct containing *PaCtr3*-cDNA, and senescence may be postponed when wild-type strains are grown in media added with a copper chelator (Borghouts et al. 2001, 2002). Senescent *P. anserina* wild-type strains exhibit a PaCTR3 downregulation. However, they evidence an enhanced metallothionein 1 expression, confirming an increase in cytosolic copper, thought to derive from mitochondria (Borghouts et al. 2002), and further adding to copper intervention in senescence.

In senescent HDFs, copper-regulated genes such as heat-shock protein-70 (*hspa1a*) and metallothionein 2A (*mt2a*) were found upregulated, indicating that cytosolic copper levels also increase during senescence of

HDFs. Thus, the evidence favoring enhanced intracellular copper as longevity modulator, possibly through the generation of ROS, is strong but is much limited regarding cellular senescence (Scheckhuber et al. 2009).

Departing from the hypothesis that the oxidative effect of copper is able to cause cellular senescence, we aimed to verify whether copper exposure of HDFs was able to cause the morphological and molecular alterations typically associated with it. Here, we provide data showing that copper induces biomarkers of senescence similarly to other SIPS agents.

Methods

Cell culture procedures

WI-38 HDFs were purchased from The European Collection of Cell Cultures and were routinely cultivated in 75 cm^2 culture flasks containing 15 mL of basal medium Eagle (BME) supplemented with 10% fetal bovine serum (FBS) at 37°C in an atmosphere containing 5% CO_2. When confluent, cells were subcultivated as previously described (Hayflick and Moorhead 1961). In slowly growing cultures, the medium was changed every 4 days. To analyze the effect of copper, subconfluent WI-38 cultures at early cumulative population doublings (CPDs≤30) were submitted to 250, 500, 750, or 1,000 μM copper sulfate ($CuSO_4$) in BME containing 10% FBS for 24 h. At the end of the exposure, cells were washed twice with pre-warmed phosphate buffer saline (PBS) and complete medium (BME with 10% FBS) was added. Control cultures at the same early CPDs were incubated with equivalent doses of sodium sulfate (Na_2SO_4). BME condition represents cells treated with complete medium without sodium or copper sulfate.

Copper cytotoxicity

To assess copper cytotoxicity, cell survival was measured by neutral red assay immediately after cell exposure to several concentrations of copper sulfate (250, 500, 750, and 1,000 μM), as described by Repetto et al. (2008), and compared with controls. Briefly, after copper treatment, the medium was removed, and the cells were incubated with neutral red solution (40 μg/mL) in BME for 3 h at 37°C. The cells were subsequently washed with PBS, and the dye was extracted from the viable cells by their lysis with acetic acid (1% *v*/*v*) in 50% (*v*/*v*) ethanol. Optical density was then measured at 540 nm (Abs_{540nm}) using a microplate reader (Infinite®200-TECAN). Control cells, treated with sodium sulfate, represented 100% viability.

Cell proliferation assay

To assess the effect of copper treatment on cell proliferation, the sulforhodamine B (SRB) assay was employed (Vichai and Kirtikara 2006). SRB assay was used for cell density determination, based on the measurement of cellular protein content (arbitrary units). For the assay, 10,000 cells per well were plated onto 96-well plates, submitted to the different treatments, and then fixed at different time-points after stress (1, 2, 3, and 4 days after exposure) with 10% trichloroacetic acid (TCA) during 1 h at 4°C. The TCA-fixed cells were stained for 30 min with 0.057% (*w*/*v*) SRB in 1% acetic acid solution and then were washed four times with 1% acetic acid. Bound dye was solubilized with 10 mM Tris base solution (pH 10), and the absorbance at 510 nm of each well was recorded using a microplate reader (Infinite®200-TECAN). Cell growth was estimated considering that, for each condition, the respective Abs_{510nm} (day 1)=1 arbitrary unit of proliferation index.

Senescence-associated β-galactosidase

At 24 h after copper treatment, cells were trypsinized and seeded in six-well culture plates at a density of 20,000 cells per well. Forty-eight hours after plating, the activity of senescence-associated β-galactosidase (SA β-gal) was determined as described by Dimri et al. (1995). The proportion of SA β-gal-positive cells was determined by counting 400 cells per dish under a microscope, using duplicates. The proportions of cells positive for SA β-gal activity are given as percentages of the total number of cells counted.

Real-time PCR analysis

Total RNA was extracted (RNeasy Plus Mini Kit, Qiagen™) from cells 3 days after treatments, in at

least three independent cultures. Total RNA (2 μg) was converted in cDNA by reverse transcription reaction. Amplification reaction assays contained 1× SYBR Green Mastermix (Bio-Rad™) and primers (STAB VIDA, Lda.) at optimal concentration. The sequences of gene-specific primers are shown in Table 1. A hot start at 94°C for 3 min was followed by 40 cycles at 94°C for 1 min, 60/65°C during 1 min, and 72°C for 1 min using the iCycler iQ5 real-time polymerase chain reaction (PCR; Bio-Rad™) thermal cycler. The specificity of amplification was checked by performing melting curves and electrophoresis of the amplification products.

Western blot analysis

After treatments, WI-38 cells were washed once with ice-cold PBS and scrapped on ice in a lysis buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.1% Triton X-100). Cells were homogenized and sonicated for 5 min. The protein content of these cell extracts was quantified using Bradford assay (Bradford 1976). An equal amount of protein (20 μg/lane) from each cell extract was resolved on SDS-PAGE gels with appropriate polyacrylamide concentrations. Proteins were blotted to a nitrocellulose membrane and, after blocking with 5% non-fat dry milk diluted in Tris buffer saline 0.1 M/0.1% Tween 20 (TBST), were subsequently probed with the specific primary antibodies overnight at 4°C (mouse monoclonal antibody anti-p21, Cell Signaling Technology; rabbit polyclonal antibody anti-fibronectin or anti-TGF β1, Santa Cruz Biotechnology, Inc.; and mouse monoclonal antibody anti-apolipoprotein J, Millipore). After extensive washing with TBST, immunoblots were then incubated with an appropriate peroxidase-conjugated secondary antibody for 1 h at room temperature. After three washes in TBST, immunoblots were detected using the ECL Western Blotting Substrate (Pierce™-Thermo Scientific) and recorded by exposure to an X-ray film. Tubulin was also detected and used as control of protein loading.

Statistical analysis

Student *t* test was used to compare the means between two different conditions. A *p* value lower than 0.05 was considered statistically significant.

Results

Copper sulfate effect on cellular viability

For the determination of the highest dose of copper that could be used without being toxic to WI-38 fibroblasts, several concentrations of copper sulfate were tested. Cells were submitted to 250, 500, 750,

Table 1 Primers used for real-time PCR

Gene	Sequences (5′→3′)	Amplicon size (bp)
Apolipoprotein J–F	GGA TGA AGG ACC AGT GTG ACA AG	114
Apolipoprotein J–R	CAG CGA CCT GGA GGG ATT C	
TGF-β1–F	AGG GCT ACC ATG CCA ACT TCT	102
TGF-β1–R	CCG GGT TAT GCT GGT TGT ACA	
p21–F	CTG GAG ACT CTC AGG GTC GAA	123
p21–R	CCA GGA CTG CAG GCT TCC T	
IGFBP3–F	CAG AGC ACA GAT ACC CAG AAC TTC	111
IGFBP3–R	CAC ATT GAG GAA CTT CAG GTG ATT	
Fibronectin–F	TGT GGT TGC CTT GCA CGA T	109
Fibronectin–R	GCT TGT GGG TGT GAC CTG AGT	
HO1–F	CCA GCA ACA AGG TGC AAG ATT C	148
HO1–R	CAC ATG GCA TAA AGC CCT ACA G	
TBP–F	TCA AAC CCA GAA TTG TTC TCC TTA T	122
TBP–R	CCT GAA TCC CTT TAG AAT AGG GTA GA	

and 1,000 μM $CuSO_4$ for 24 h, and the mean viability from three independent experiments was determined for each concentration, assuming that control cells presented 100% viability. Copper cytotoxicity was determined by neutral red assay performed immediately after exposure. As can be seen in Fig. 1, control cells (incubated with the highest dose of sodium sulfate) did not show significant differences in cell viability when compared with BME cells (101.3%). However, cell viability decreased with increasing concentrations of copper sulfate. Cells exposed to 250 μM copper sulfate presented 96.4% of cell viability when compared with controls. This lowest copper sulfate concentration was considered as a subcytotoxic dose, on account that cell exposure to 500, 750, and 1,000 μM resulted in a substantial decrease in cellular viability to 41.0%, 21.8%, and 17.8%, respectively, when compared with controls. The three highest doses of copper sulfate were considered cytotoxic since they yielded cell viabilities lower than 50%. Thus, we decided to emphasize on 250 μM $CuSO_4$ for all the experiments throughout this study. However, in order to evaluate if a higher copper concentration was able to provoke more pronounced senescent effects on cells, the concentration of 500 μM $CuSO_4$ was also tested.

Effect of copper on cell morphology and senescence-associated β-galactosidase activity

The most evident morphological changes occurring in cellular senescence of fibroblasts are the increase in cell surface area/volume and the alteration of their morphology from small spindle–fusiform to large flat spread (Greenberg et al. 1977; Bayreuther et al. 1988). On the present investigation, cells exposed to 250 or 500 μM copper sulfate presented altered morphological features (Fig. 2a), such as enlarged cell surface as well as stellate outline with thin extensions resembling the typical senescent-like cell morphology. In agreement with the results obtained for cellular viability, cell incubation with 500 μM copper sulfate resulted in a much lower cell density when compared with the other conditions (BME, control, and 250 μM copper), as can be seen in Fig. 2a.

The increased activity of SA β-gal was shown to be a reliable marker of senescence in non-confluent fibroblasts (Dimri et al. 1995) and is commonly used to evidence that. To verify the effects of copper sulfate

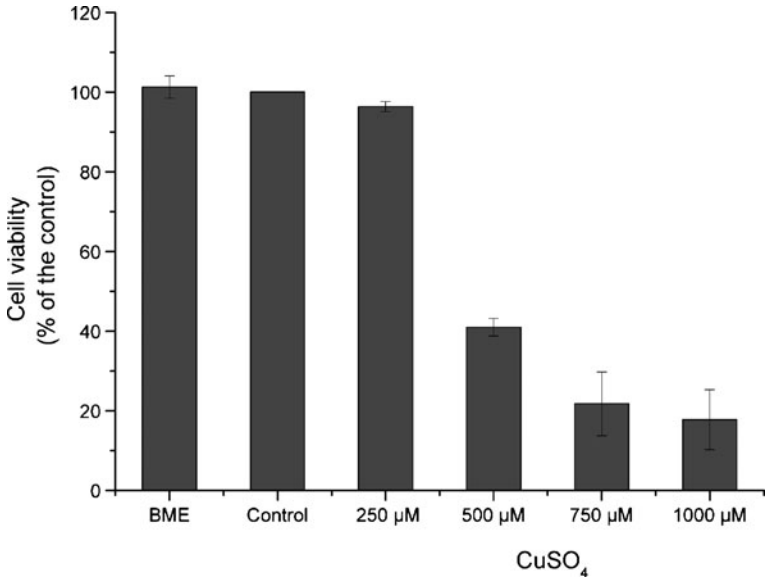


Fig. 1 Cell viability after exposure to copper sulfate at different concentrations for 24 h. Cell viability decreases with increasing doses of copper sulfate. Control cells, submitted to 1,000 μM sodium sulfate for 24 h, represent 100% viability and did not present significant alteration in viability when compared with cells treated with complete medium (BME). Cell viability was copper sulfate dose-dependent from 250 to 1,000 μM. However, in contrast to 250 μM, a subcytotoxic dose that led to 94.6% of cell viability, cell exposure to the higher doses of copper sulfate, 500, 750, and 1,000 μM, resulted in a decrease in cell viability to 41.0%, 21.8%, and 17.8%, respectively. Data are expressed as mean±SEM from three independent experiments

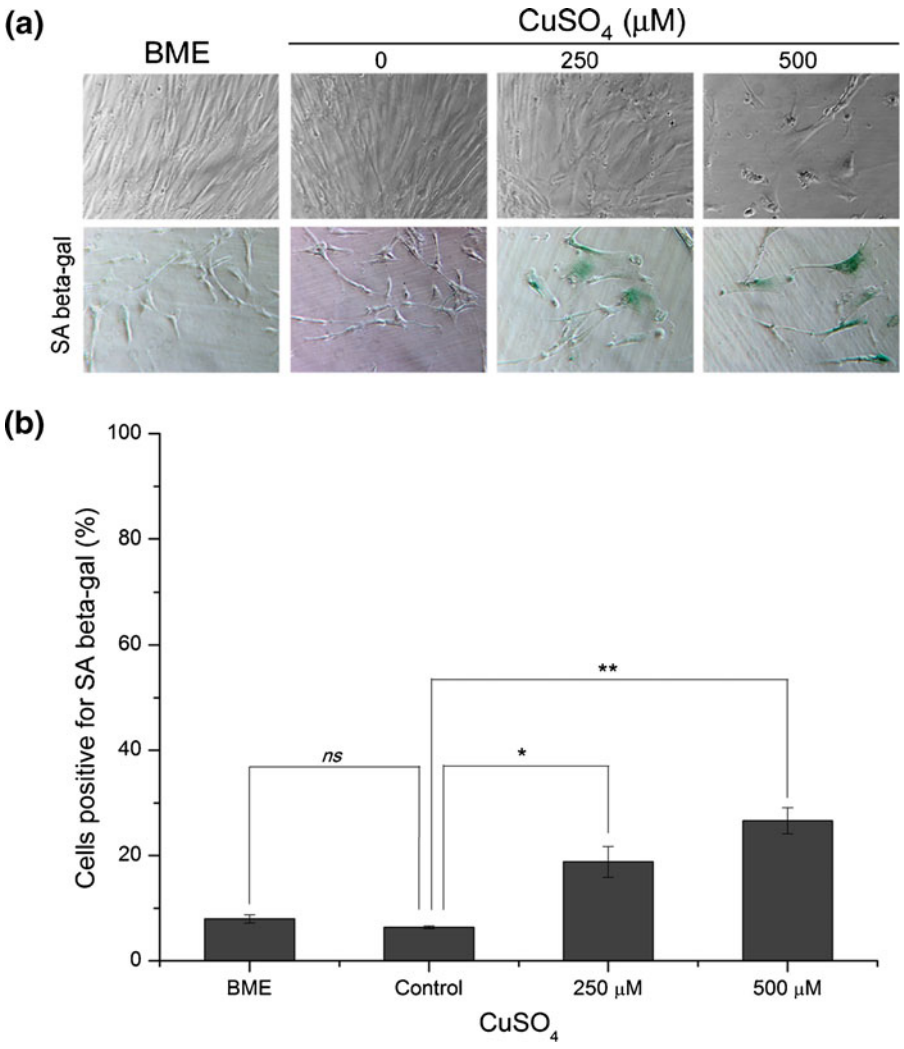


Fig. 2 Cell morphology and senescence-associated β-galactosidase activity detection on fibroblasts exposed to 250 or 500 μM of copper sulfate. **a** Wi-38 HDFs exposed to 250 or 500 μM $CuSO_4$ presented enlarged cellular volume and altered shape, resembling the typical senescent phenotype that normally appears on replicatively senescent fibroblasts. **b** SA β-gal-positive cells increased to 19% and 27% in cells exposed to 250 and 500 μM copper sulfate, respectively, when compared with control cells (6%). Data are expressed as mean±SEM from three independent experiments. *$p<0.05$; **$p<0.01$ and *ns* non-significant, when compared with control

on SA β-gal activity, 500 μM sodium sulfate (control) and BME (complete medium) were compared, but no significant differences were detected between both groups ($p=0.23$). However, as shown in Fig. 2b, the percentage of cells positive for the SA β-gal increased significantly to 19% ($p<0.05$) and 27% ($p<0.01$) in cells exposed to 250 and 500 μM copper sulfate, respectively, when compared with the 6% of the control. These phenotypic changes are usually taken as indicating cellular senescence (Hwang et al. 2009).

Effect of copper sulfate on cell proliferation

Either replicatively or stress-induced, senescent cells cease proliferating and exhibit cell cycle arrest in G1. To assess the effect of copper in cell proliferation, the total mass of cell proteins was determined in arbitrary units, at different time-points (1, 2, 3, and 4 days) after stress. For each condition, it was assumed that, at day 1, the proliferation index was 1. Fig. 3 shows cell proliferation curves for the different conditions during 4 days. Both BME and control cells proliferated approximately at the same rate, showing an increase in protein content from 1-(day 1) to 3.16- and 2.91-fold (day 4), respectively. Cells treated with 250 or 500 μM copper sulfate presented smaller increases in protein content from 1 (day 1) to 1.81 and 1.40 on day 4, respectively. This lesser increase in protein content observed for cells treated with 250 μM represent a decrease in cell proliferation of about 58% while, for cells exposed to 500 μM, the proliferation was reduced by 79%, when compared with the proliferation rate of control cells.

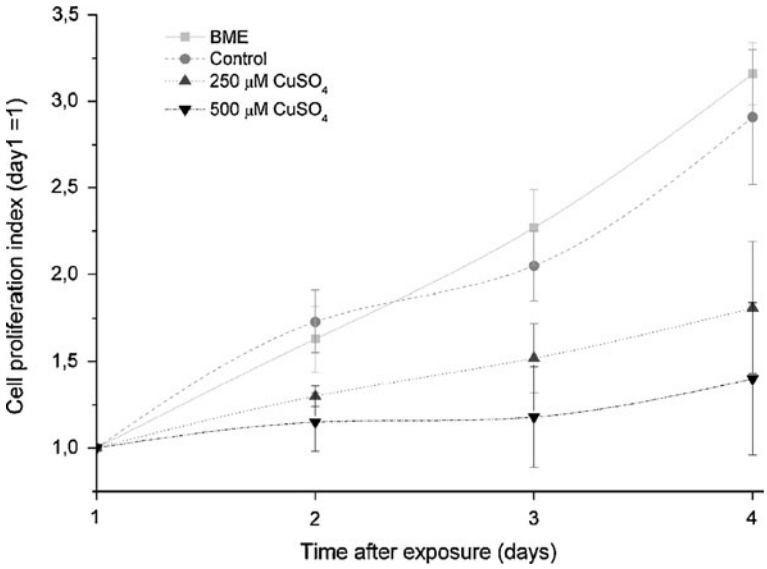


Fig. 3 Cell proliferation curves of human diploid fibroblasts exposed to 250 or 500 μM of copper sulfate. Both BME and control cells presented approximately the same proliferation rate during the 4 days after stress. At the fourth day after treatment, cells exposed to 250 or 500 μM $CuSO_4$ showed a decrease in cell proliferation of about 58% and 79%, respectively, when compared with control cells. Data are expressed as mean±SEM from three independent experiments. *$p<0.05$ when compared with the same time-point of the control

Gene expression in fibroblasts exposed to copper sulfate

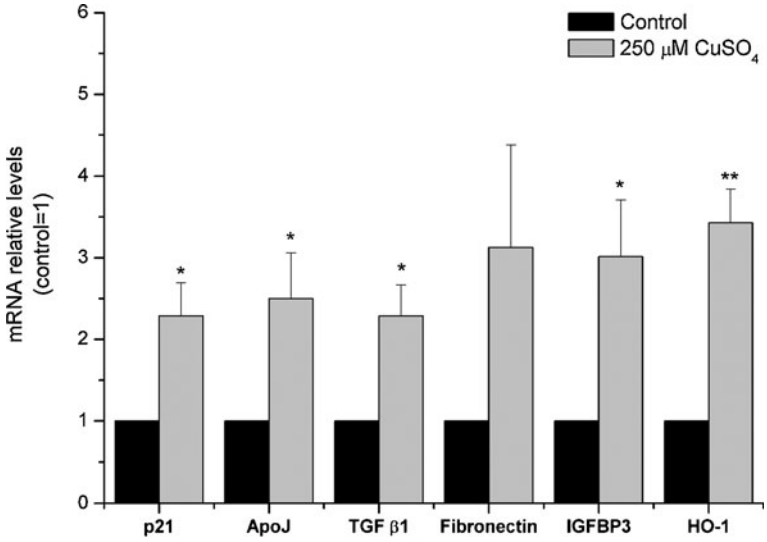
Senescent cells display several typical features which include an alteration in the expression level of several senescence-associated genes (Dumont et al. 2000; Debacq-Chainiaux et al. 2008). The genes encoding cyclin-dependent kinase inhibitor 1A (p21), apolipoprotein J (ApoJ), transforming growth factor beta 1 (TGF β1), fibronectin, insulin growth factor binding protein 3 (IGFBP3), and heme oxygenase-1 (HO-1) are known to be overexpressed both in replicatively, and in prematurely stress-induced, senescent cells. Transcript levels of these genes were quantified by real-time PCR in human fibroblasts 72 h after exposure to copper sulfate, in at least three independent experiments. Results obtained from real-time PCR are depicted in Fig. 4. Cells submitted to 250 μM $CuSO_4$ presented statistically significant increase in mRNA levels of p21, ApoJ, TGF β1, fibronectin, IGFBP3, and HO-1 (respectively 2.3-, 2.5-, 2.3-, 3.1-, 3.0-, and 3.4-fold) when compared with control cells. Cells exposed to 500 μM copper sulfate did not present consistent variations regarding mRNA expression of the selected genes (online resource 1). Such results were interpreted as a consequence of the toxic effects obtained for this concentration.

Western blot analysis in fibroblasts exposed to copper sulfate

The protein levels of some of the senescence-associated genes (fibronectin, p21, TGF β1, and ApoJ) were assessed by Western blotting. Fig. 5a shows representative blots of the variations obtained in the content of each protein, and, in Fig. 5b, the relative densitometric means from three independent experiments are plotted. Intracellular levels of fibronectin did not change in WI-38 fibroblasts exposed to 250 μM $CuSO_4$ when compared with control cells. However, the same copper-treated cells presented increased protein levels of p21, TGF β1, and ApoJ (3.7-, 2.0-, and 1.4-fold, respectively) when compared with controls. There were several attempts in order to evaluate the protein levels of IGFBP3. However, the antibody utilized with that purpose was not specific, and, for that reason, we could not conclude about the effect of copper on IGFBP3 protein levels. Once again, incubation of fibroblasts with 500 μM copper sulfate resulted in inconsistent protein variations (online resource 2), most probably due to the cytotoxic effects of such copper concentration.

Discussion and conclusions

Some agents causing oxidation may be toxic to cells and impose a dramatic limit to their viability. However, their use in subcytotoxic amount induces a change in cell structure and gene expression pattern that directs cells to the condition of senescence. The present study provides evidence that copper has those properties and acts in a way similar to other oxidative agents such as hydrogen peroxide and t-BHP that



Gene	mRNA relative levels (Control=1) 250 μM $CuSO_4$ Mean ± SEM (*p*)
p21	2.3 ± 0.4 (*0.013*)
Apolipoprotein J	2.5 ± 0.6 (*0.037*)
TGFβ1	2.3 ± 0.4 (*0.015*)
Fibronectin	3.1 ± 1.3 (*0.166*)
IGFBP3	3.0 ± 0.7 (*0.044*)
HO-1	3.4 ± 0.4 (*0.004*)

Fig. 4 Evaluation of mRNA relative levels of several senescence-associated genes by real-time PCR. Cells exposed to 250 μM $CuSO_4$ presented a statistically significant overexpression of p21, ApoJ, TGF β1, IGFBP3, and HO-1 (respectively 2.3-, 2.5-, 2.3-, 3.0-, and 3.4-fold increase compared with control cells). mRNA levels of fibronectin were also increased in cells treated with copper, although this variation was not statistically significant when compared with control cells. Data are presented as mean± SEM from at least three independent experiments. *$p<0.05$; **$p<0.01$ when compared with control

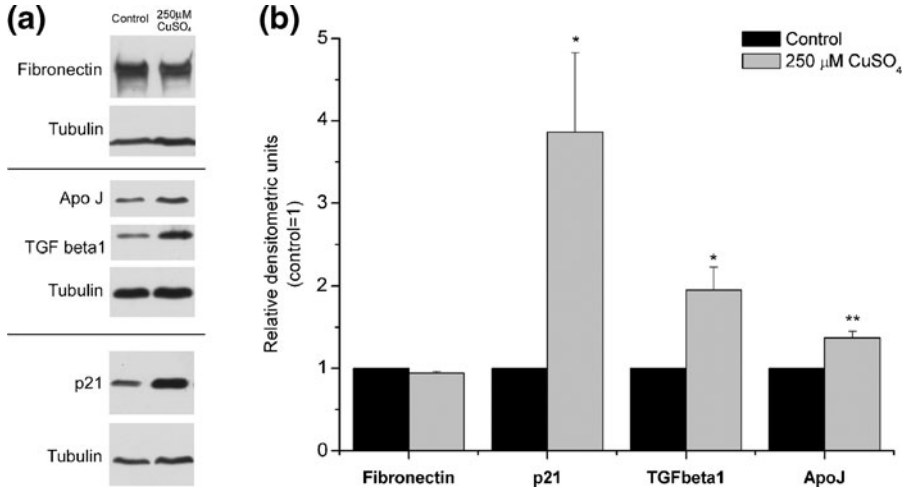


Fig. 5 Western blot analysis of fibronectin, p21, ApoJ, and TGF β1 protein levels. **a** Representative blots for the detection of the different proteins. Tubulin was used as loading control. **b** The resulting bands were quantified using densitometric analysis of the different signals. Fibronectin intracellular content remained unaltered after exposure to 250 μM $CuSO_4$, comparing with controls. Protein levels of p21, TGF β1, and Apo J were 3.9-, 2-, and 1.4-fold increased, after exposure to 250 μM $CuSO_4$, respectively, when compared with control levels. Data are expressed as mean±SEM from at least three independent experiments. *$p<0.05$; **$p<0.01$ when compared with control

cause SIPS. In fact, when WI-38 fibroblasts were submitted to copper sulfate for a limited time, they evidenced elevated viability at 250 μM that was strongly reduced at 500 μM and more. The cytotoxic effects of 500 μM copper sulfate were further evidenced in the study of mRNA and protein expression. For this reason, 250 μM was considered subcytotoxic and employed thereafter to verify copper ability to cause SIPS.

Sound data obtained along decades led to the concept that the progressive decline in the proliferative potential followed by growth arrest is the defining feature of the in vitro replicative senescence of fibroblasts (Goldstein 1990; Smith and Pereira Smith 1996) and SIPS (Toussaint et al. 2000). In the present investigation, cell proliferation was found substantially reduced during the 4 days after treatment with 250 μM of $CuSO_4$, when compared with the control cells. These findings are in agreement with previous SIPS studies. In fact, F65 fibroblasts exposed for 2 h to 200 μM H_2O_2 exhibited a sluggish or absent mitogenic response to several growth stimuli (Chen and Ames 1994). In addition, WI-38 fibroblasts cultivated under mild hyperoxia (von Zglinicki et al. 1995) or submitted to successive stresses under t-BHP treatment (Dumont et al. 2000) showed a drastic decrease in their proliferative capacity, too.

Cell morphology is an important criterion to evaluate the senescence of human fibroblasts in vitro, which usually enlarge, flatten, and loosen spindle morphology and exhibit reduced density. These features were observed in WI-38 fibroblasts 3 days after treatment with copper sulfate, similar to what is seen in other SIPS cellular models (Chen and Ames 1994; Wang et al. 2004). The same stands for SA β-gal activity which was shown to be a reliable marker of senescence in non-confluent cultures of fibroblasts (Dimri et al. 1995) and is used regularly with that purpose. In the present study, WI-38 fibroblast cultures submitted to 250 μM copper sulfate for 24 h showed a higher proportion of cells positive for SA β-gal. This finding parallels other SIPS-inducing conditions such as exposure of cells to successive stresses with t-BHP or ethanol (Debacq-Chainiaux et al. 2008), subcytotoxic levels of hydrogen peroxide (Zdanov et al. 2006), or UVB radiation (Debacq-Chainiaux et al. 2005).

There are several genes whose mRNA levels increase both in replicative and premature senescent HDFs indicating a change in gene expression. Debacq-Chainiaux and colleagues evaluated three different HDF models of senescence and showed that the mRNA levels of senescence-associated genes such as fibronectin, p21, ApoJ, IGFBP3, and TGF β1 were similarly increased in the three cellular models (Debacq-Chainiaux et al. 2008). Those findings prompted us to study their relative transcript levels by real-time PCR, in WI-38 HDFs, 72 h after exposure to copper sulfate.

As already mentioned, the hallmark of cellular senescence is the irreversible arrest of cell division. Senescent cells have been shown to arrest in the G1 phase of cell cycle (Chen et al. 1998) and do not resume proliferation when challenged by physiological mitogens. In mammals, the main regulators of cell cycle progression through G1 phase are heterodimers composed of a cyclin-dependent kinase (CDK4 or CDK6 in this phase) and one member of the D class of cyclins. However, the progression is stalled when the CDK inhibitor p21 exhibits sustained overexpression as occurs in replicative senescence (Stein et al. 1990) and in premature senescence induced by H_2O_2 (Chen et al. 1998), t-BHP (Dumont et al. 2000), or UVB (Debacq-Chainiaux et al. 2005). The present study, in accordance with the reduced proliferation, provides evidence that p21 transcript levels and protein content are also increased in $CuSO_4$-exposed WI-38 cells.

Fibronectin is an essential extracellular matrix component involved in cell adhesion, cytoskeletal organization, mediation of external mitogenic signals, and wound repair. Fibronectin gene has been found to be overexpressed in senescent pig skin fibroblasts (Martin et al. 1990), in fibroblasts derived from patients with Werner ageing syndrome (LeckaCzernik et al. 1996), and also in several ageing models of human fibroblasts, such as replicative senescence, premature senescence induced by t-BHP or ethanol (Debacq-Chainiaux et al. 2008), and premature senescence induced by UVB radiation (Debacq-Chainiaux et al. 2005). Increased expression of fibronectin was found to positively correlate with cell surface increase observed during senescence of HDFs, suggesting that it may underlie or contribute to replicative senescence morphological changes (Kumazaki et al. 1993). Here, copper-treated WI-38 human fibroblasts presented about threefold increased fibronectin mRNA expression when compared with control cells, although its

intracellular protein content, assessed by Western blotting, did not increase after exposure to 250 μM copper sulfate. One possible explanation to this discrepancy is that fibronectin secretion rate might increase with increasing concentrations of copper sulfate, resulting in a faster secretion of fibronectin to the extracellular space, where it usually localizes. This specific point could be elucidated by the quantification of secreted fibronectin in the culture media where cells were grown, but regrettably, it was not evaluated.

Apolipoprotein J, also known as clusterin, is an 80-kDa glycoprotein consisting of two disulfide-linked subunits, alpha and beta, and is constitutively synthesized and secreted by many cell types. ApoJ is mostly recognized as an extracellular chaperone which interacts with many different proteins and inhibits their stress-induced precipitation (Poon et al. 2002) and is involved in numerous physiological processes (Trougakos and Gonos 2006). ApoJ gene is also very sensitive to cellular stressful conditions, especially oxidative stress. ApoJ overexpression was observed in replicatively senescent human fibroblasts and also in prematurely senescent fibroblasts induced by t-BHP, ethanol (Debacq-Chainiaux et al. 2008), or UVB (Debacq-Chainiaux et al. 2005) and is thus considered a faithful biomarker of cellular senescence (Trougakos and Gonos 2006). Its expression was enhanced in the current study too. Due to the varied functional involvement of ApoJ, there is uncertainty regarding its specific intracellular effects. As a wide-range chaperone, a likely explanation is to endow cells with protective survival tools when protein structure may become deranged due to stresses. Yet, another role is to direct damaged proteins for destruction, employing a different mechanism for the same survival purpose. In fact, it was recently reported that ApoJ expression was enhanced in cells grown in excess of copper. Under such conditions, there was evidence for the enhancement of interaction ApoJ/ATP7B copper transporter and its degradation, particularly when mutated (Materia et al. 2011).

TGF β1 is a pleiotropic cytokine involved in many cell functions like cell growth, differentiation, and biosynthesis of extracellular connective tissue. TGF β1 was shown to be overexpressed in replicative, t-BHP-, and ethanol-induced senescence of WI-38 HDFs (Pascal et al. 2005). More specifically, it has already been demonstrated that TGF β1 overexpression is required for the appearance of several biomarkers of cellular senescence, such as the induction of senescent morphogenesis, increased mRNA level of senescence-associated genes, such as ApoJ and fibronectin, and increased senescence-associated β-galactosidase activity, after exposure of HDFs to subcytotoxic stress with H_2O_2 (Frippiat et al. 2001; Frippiat et al. 2002) or UVB (Debacq-Chainiaux et al. 2005). Similar to other stress-induced premature senescence cellular models, WI-38 HDFs exposed to 250 μM copper sulfate was followed by increased mRNA and protein levels of TGF β1.

TGF β1 is known to induce HO-1 expression in pulmonary epithelial cells (Ning et al. 2002), human renal proximal tubule cells (Hill-Kapturczak et al. 2000), and also in WI-38 HDFs (Pascal et al. 2007). HO-1 expression is transcriptionally activated by agents that generate reactive oxygen species (Soares and Bach 2009), and it has been already shown to be overexpressed in prematurely senescent WI-38 HDFs after exposure to t-BHP and ethanol (Pascal et al. 2007). The present study reports a similar effect in WI-38 fibroblasts with 250 μM copper sulfate.

In addition, TGF β1 is also known to control synthesis and secretion of IGFBP3 by HDFs (Martin and Baxter 1991). IGFBP3 expression is associated with inhibition of cell proliferation and cellular senescence, and, again, its overexpression in RS and SIPS fibroblasts induced by t-BHP, ethanol (Debacq-Chainiaux et al. 2008), or UVB (Debacq-Chainiaux et al. 2005) was described. It has been suggested that growth arrest in premature senescence partly depends on TGF β1 via the overexpression of IGFBP3 (Debacq-Chainiaux et al. 2008). In accordance, fibroblasts exposed to copper sulfate presented increased transcript levels of IGFBP3 in a fashion similar to TGF β1.

The results presented herein show that subcytotoxic concentration of copper sulfate is able to induce senescence features on WI-38 human fibroblasts. Similar to the effects of other well-known SIPS agents, they are observed past adaptative conditions related to immediate gene response and are varied: they include changes in proliferation, in SA β-gal activity, and in gene expression. The coherent pattern of their change gives credit to the inclusion of copper as a SIPS-inducing agent.

It is hoped that further studies addressing the mechanism and biological meaning of copper-associated cell senescence will improve the under-

standing of the establishment and progression of brain and liver disorders as Alzheimer's and Wilson's diseases. In the first, copper not only interacts with amyloid precursor protein through a binding domain but also promotes amyloid β peptide crosslinks and aggregation through metal-mediated oxidative stress (Barnham and Bush 2008). In Wilson's disease, a deficient intracellular transport of copper results from a structural abnormality of the ATP7B translocator, whose level is regulated by the senescence biomarker ApoJ/clusterin (Materia et al. 2011).

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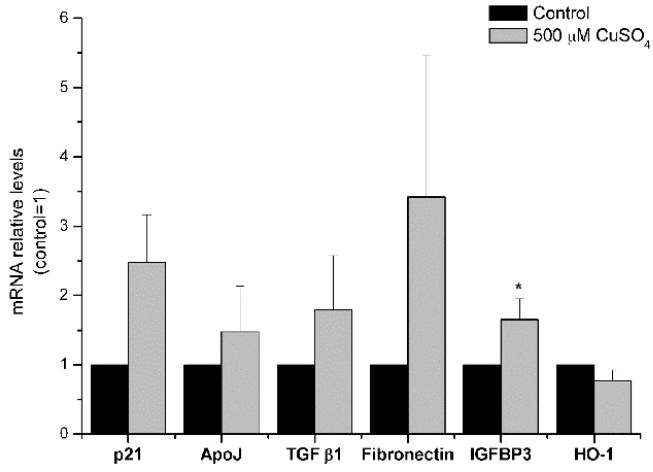
Electronic supplementary material

Online resource 1:



Gene	mRNA relative levels (Control=1) 500 μM $CuSO_4$ Mean ± SEM (*p*)
p21	2.5 ± 0.7 (*0.064*)
Apolipoprotein J	1.5 ± 0.7 (*0.496*)
TGFβ1	1.8 ± 0.8 (*0.349*)
Fibronectin	3.4 ± 2.0 (*0.301*)
IGFBP3	1.7 ± 0.3 (*0.047*)
HO-1	0.8 ± 0.2 (*0.211*)

Effect of 500 μM copper sulfate on mRNA transcript levels of several senescence-associated genes. The incubation of WI-38 fibroblasts with the lower cytotoxic copper concentration (500 μM) resulted in non-significant variations on p21, ApoJ, TGF β1, fibronectin, and HO-1 gene expression, when compared with controls. In addition, IGFBP3 mRNA levels were 1.7-fold increased in 500 μM $CuSO_4$-treated cells, comparing with control cells. When compared with the consistent upregulation of the several senescence-associated genes observed for cells exposed to 250 μM copper, gene expression variations obtained with 500 μM copper sulfate were senseless, suggesting that the transcriptional machinery of these cells were seriously compromised due to the toxic effects of the dose. Data are presented as mean ± SEM from at least three independent experiments. *$p < 0.05$ when compared with control.

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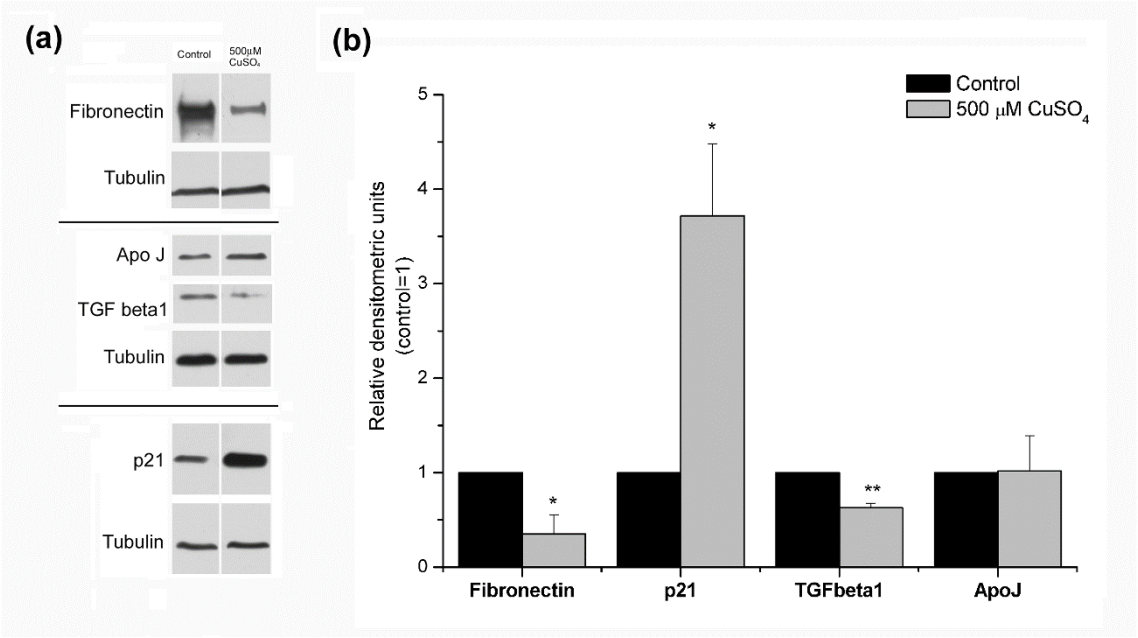

Western blot analysis of fibronectin, p21, ApoJ, and TGF β1 protein levels in cells submitted to 500 µM copper sulfate. **(a)** Representative blots for the detection of the different proteins. Tubulin was used as loading control. **(b)** The resulting bands were quantified using densitometric analysis of the different signals. Protein levels of p21 were found significantly increased (3.7-fold) in WI-38 fibroblasts treated with 500 µM copper sulfate for 24h, when compared with controls. Both fibronectin and TGF β1 intracellular protein levels were decreased, and ApoJ protein content did not alter after exposure to the cytotoxic dose of copper, when compared with control cells. These inconsistent protein variations may originate from cellular metabolic alterations reflecting the cytotoxic effects of copper concentration used. Data are expressed as mean ± SEM from at least three independent experiments. *$p < 0.05$; **$p < 0.01$ when compared with control

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Original Article

ER Stress Response in Human Cellular Models of Senescence

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Abstract

The aging process is characterized by progressive accumulation of damaged biomolecules in the endoplasmic reticulum, as result of increased oxidative stress accompanying cellular senescence. In agreement, we hypothesized that WI-38 human cellular models of replicative senescence and stress-induced premature senescence (SIPS) induced by hydrogen peroxide (H_2O_2-SIPS) or copper sulfate ($CuSO_4$-SIPS) would present endoplasmic reticulum chaperoning mechanisms impairment and unfolded protein response activation. Results show that in replicative senescence and $CuSO_4$-SIPS, immunoglobulin binding protein, calnexin, protein disulfide isomerase, and ER oxireductin-1 levels adjust to restore proteostasis and inositol-requiring enzyme-1 (IRE1)-, activating transcription factor 6 (ATF6)-, and pancreatic ER kinase (PERK)-mediated unfolded protein response are activated. However, H_2O_2-SIPS does not exhibit IRE1 and ATF6 pathways activation but a PERK-mediated upregulation of CCAAT/enhancer-binding protein homologous protein, showing that $CuSO_4$-SIPS mimics better the endoplasmic reticulum molecular events of replicative senescence than H_2O_2-SIPS. Moreover, unfolded protein response activation is required for both SIPS models induction, because PERK and IRE1 inhibitors decreased senescence-associated beta-galactosidase appearance. In $CuSO_4$-SIPS, the decrease in senescence levels is associated with PERK-driven, but IRE1 independent, cell cycle arrest while in H_2O_2-SIPS cell proliferation is PERK independent. These results add a step further on the molecular mechanisms that regulate senescence induction; moreover, they validate $CuSO_4$-SIPS model as a useful tool to study cellular stress responses during aging, hoping to postpone age-related health decline.

Key Words: ER stress—Replicative senescence—Copper—Human fibroblasts—SIPS

The increase of elderly population raised interest in the mechanisms underlying the progressive functional decline and homeostasis imbalance that associate with aging. At the molecular level, it is characterized by the continued accumulation of damaged biomolecules, as proteins, which impinge on cell and tissue function. Actually, a role was ascribed to unfolded/misfolded proteins in the normal aging process and the establishment of age-related entities as Alzheimer and Parkinson diseases (1,2). Oxidative stress seems to be a major contributor to such age-related accumulation of abnormal proteins (3,4).

The endoplasmic reticulum (ER) is a target for endogenously generated reactive oxygen species along aging, as evidenced by increased

specific ER resident proteins oxidation (5) and consequent organelle activity impairment (6). Nevertheless, to prevent protein misfolding, accumulation and aggregation, ER possesses a protein quality control chaperoning system that includes key chaperones and enzymes, such as immunoglobulin binding protein (BiP), calnexin, protein disulfide isomerase (PDI), and ER oxireductin-1 (Ero1). A fine regulation of their expression must exist to avoid protein aggregation and cellular damage, but when proteostasis disruption appears, an adaptive, coordinated ER stress response or unfolded protein response (UPR) is activated (7). At the cellular level, this complex response is mediated by three ER transmembrane sensors: pancreatic ER kinase - like ER kinase (PERK), activating transcription factor-6 (ATF6), and inositol-requiring enzyme-1 (IRE1). These UPR pathways limit ER abnormal protein load and alleviate ER stress by attenuating protein translation (8), upregulating ER chaperones (9), and degrading misfolded proteins in the proteasome by an ER-associated degradation process (10). If these protective cellular responses do not restore normal ER functioning, apoptosis is activated (7,11).

The efficiency of this protein quality control system seems to be compromised during aging, as several studies demonstrated an age-related decrease on the expression levels of some ER molecular chaperones and folding enzymes (12,13). However, most of them used different tissues from aged rodents (14–16), whereas ER stress response in in vitro nonmodified human cellular models of senescence was never addressed.

Cellular senescence was firstly described by Hayflick and Moorhead, who demonstrated that serially cultivated human diploid fibroblasts (HDFs) ceased dividing after a number of population doublings (PDs) and became unresponsive to mitogenic stimuli, thus entering in a condition termed replicative senescence (RS) (17). In addition, cells in RS exhibit dramatic changes in morphology, gene expression and organelle structure, mass, and function when compared with proliferating cells (for a review see Ref. (18)).

Besides RS, a senescent phenotype may be prematurely achieved when HDFs are submitted to subcytotoxic doses of oxidative stress inducers such as hydrogen peroxide (19), ultraviolet-B radiation (20), or copper sulfate (21). Cells in this stress-induced premature senescence (SIPS) condition display features of RS, including the typical senescent morphology and gene expression profile (22). Such induced models are frequently used to study molecular mechanisms of cellular senescence.

Taking into account the increased oxidative stress accompanying RS and SIPS human cellular models of aging, it was hypothesized that such conditions would favor ER stress establishment, disturbance of ER chaperoning mechanisms, and UPR pathways activation. In fact, in this study, the activation of protective IRE1-, ATF6-, and PERK-mediated ER stress responses is shown in RS and SIPS human cellular models. Moreover, our data show that PERK and IRE1 activation are necessary for the induction of senescence in $CuSO_4$- and H_2O_2-SIPS models.

Methods

Cell Culture

WI-38 HDFs, purchased from the European Collection of Cell Cultures, were cultivated in complete medium composed of basal medium Eagle (BME) supplemented with 10% fetal bovine serum, at 37°C in a 5% CO_2 atmosphere. The WI-38 HDFs are considered to be young below 30 PDs and enter senescence at 45 PDs or above. Cells unable to make a PD within 3 weeks were in RS. For the induction of SIPS with hydrogen peroxide (H_2O_2-SIPS) or copper sulfate ($CuSO_4$-SIPS), young WI-38 HDFs were exposed to subcytotoxic concentrations of H_2O_2 (50, 75, or 100 µM) or $CuSO_4$ (250 or 350 µM) for 2 or 24 hours, respectively. Then, cells were washed with phosphate buffered saline (PBS) and replaced with fresh complete medium. After a 72-hour resting period, cells were processed for protein or gene expression assessment techniques. Control conditions for each cellular model were: young HDFs for RS, BME for H_2O_2-SIPS, and sodium sulfate for $CuSO_4$-SIPS. For the inhibition of PERK- or IRE1-mediated ER stress response, 120 nM of PERK-inhibitor GSK2606414 or 10 µM of IRE1-inhibitor 4µ8c (Cat No. 516535 and 412512, respectively, Calbiochem) were added to the medium 1 hour prior to SIPS induction and were maintained throughout the experiments. Control cells were incubated with the inhibitors respective vehicle.

Cell Viability Assay

Cell survival was evaluated using neutral red assay immediately after exposure to $CuSO_4$ or H_2O_2, either in the presence or absence of 4µ8c or GSK2606414 inhibitors, and compared to controls. In brief, after treatments, the medium was removed and cells were incubated with neutral red in BME (40 µg/mL) for 3 hours at 37°C. They were subsequently washed with PBS, and the dye was extracted from viable cells with 1% acetic acid in 50% ethanol. Optical density was measured at 540 nm using a microplate reader (Infinite 200—TECAN). Control cells represented 100% viability.

Senescence-Associated β-Galactosidase Detection

Cells were seeded in six-well culture plates, 20,000 cells/well; after 48 hours, the senescence biomarker senescence-associated β-galactosidase (SA beta-gal) was detected as described (23). The proportion, in percentage, of SA beta-gal positive cells in each condition was determined by microscopically counting 400 total cells/well from at least three independent experiments.

Cell Proliferation

To assess the different conditions effect on cell proliferation, sulforhodamine B (SRB) assay was used for cell density determination, based on cellular protein content (24). Briefly, 10,000 cells/well were plated onto 96-well plates, submitted to the treatments, and fixed at different time-points after stress (0, 1, 2, 3, and 4 days) with 10% trichloroacetic acid (TCA), 1 hour at 4°C. The TCA-fixed cells were stained for 30 minutes with 0.057% (w/v) SRB in 1% acetic acid solution and then washed four times with 1% acetic acid. Bound dye was solubilized with 10 mM Tris base solution and the absorbance at 510 nm of each well was recorded using a microplate reader (Infinite 200—TECAN).

Western Blot

WI-38 cells were washed with PBS and scrapped on ice in a lysis buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.1% Triton X-100) supplemented with protease inhibitors cocktail (Sigma-Aldrich). Upon Bradford assay, 20 µg of protein from each cell extract were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were blotted into a nitrocellulose membrane; after blocking with 5% nonfat dry milk diluted in Tris-buffered saline supplemented with 0.1% tween 20 (TBST), were probed with specific primary antibodies (anti-BiP #3177, anti-calnexin #2679, anti-Ero1 #3264, anti-PDI #3501, anti-IRE1 #3294, anti-PERK #5683, anti-phospho-eIF2α #3398—Cell Signaling Technology; anti-P-JNK sc-6254, anti-ATF6

sc-14250—Santa Cruz Biotechnology, Inc.; and anti-tubulin T5168—SigmaAldrich) overnight at predetermined optimal dilutions. After washing with TBST, immunoblots were incubated with appropriate peroxidase-conjugated secondary antibodies for 1 hour, detected using ECL western blotting substrate (Pierce—Thermo Scientific), and visualized with ChemiDocTM XRS (BioRad Laboratories). Results were quantified by densitometry using the Image Lab software.

Real-Time PCR

Total RNA extracted (PureLink RNA Mini Kit, Ambion) from cells derived from at least three independent cultures was converted into cDNA by reverse transcription reaction. Amplification reaction assays contained SYBR Green Mastermix (Maxima SYBR Green/ROX qPCR Master Mix, Thermo Scientific) and primers (STAB VIDA, Lda.) at optimal concentration. The primer sequences were: BiP, 5-GTT CTT GCCGTTCAAGGTGG-3 and 5-TGGTACAGTAACAACTGCATG-3; CCAAT/enhancer-binding protein homologous protein (CHOP), 5-CAGAAC CAGCA GAGGTCACA-3 and 5-AGCTGTGCCACTTTCCTTTC-3; p21, 5-CTGGA GACT CTCAGGGTCGAA-3 and 5-CCAGGACTGCA GGCTTCCT-3; and TATA box binding protein (TBP), 5-TCAAACCC AGAATTGTTCTCCTTAT-3, and 5-CCT GAATCCCTTTAGAATA GGGTAGA-3. The protocol used for the quantitative real-time polymerase chain reaction (qPCR) was: 95°C (10 minutes), 40 cycles of 95°C (15 seconds), and 60°C (1 minute). For the specific amplification of the spliced variant of human X box-binding protein 1 (XBP1s), the primer sequences used were: 5-GGTCTGC TGAGTCCGCAGCAGG-3' and 5-GGGCTTGGTATATATGTGG-3; and the qPCR protocol was: 95°C (10 minutes), 40 cycles of 95°C (15 seconds), 55°C (30 seconds), and 72°C (30 seconds). The qPCR was performed in the StepOnePlus thermal cycler (Applied Biosystems). The TBP was the selected housekeeping gene when calculating relative transcript levels of the target genes.

Statistical Analysis

Student's *t* test was used to compare the means between two different conditions. A *p* value lower than .05 was considered statistically significant.

Results

Cell Viability, Proliferation and SA β-Gal Detection in $CuSO_4$- and H_2O_2-SIPS Cells

The $CuSO_4$-SIPS cellular model was recently established by exposing WI-38 fibroblasts to 250 μM $CuSO_4$ for 24 hours (21). As 500 μM $CuSO_4$ was found to greatly affect cell viability, here, we evaluated the cytotoxicity of an intermediate concentration (350 μM) to include a higher sublethal dose of copper in all experiments. As presented in Figure 1A, fibroblasts treated with 250 or 350 μM $CuSO_4$ exhibited 75% and 61% viability, respectively. Moreover, cells treated with 50, 75, or 100 μM H_2O_2, the most commonly used SIPS inducer, had 97%, 94%, or 87% of cell survival when compared with controls.

Both SIPS and RS conditions had a significant increase in the percentage of senescent cells as assessed by the positivity for SA beta-gal activity (Figure 1B). Control cells presented 4% of SA beta-gal positive cells, whereas cells submitted to 250 and 350 μM $CuSO_4$ presented 36% and 39%, respectively. In addition, fibroblasts treated with 50, 75, and 100 μM H_2O_2 exhibited 20%, 27%, and 34% of stained cells, respectively. Finally, in RS, 80% of the total number of cells were positive for SA beta-gal.

Cell proliferation inhibition is another typical and frequently evaluated marker of the senescence phenotype. Similarly to previous observation (21), 3 days after removing the stressor, cells exposed to 250 or 350 μM $CuSO_4$ presented 49% and 74% of cell proliferation inhibition, respectively, when compared with controls (Figure 1C). In addition, cell proliferation was 40% and 56% inhibited in fibroblasts treated with 75 or 100 μM H_2O_2, respectively, whereas 50 μM H_2O_2 treated cells did not present significant inhibition (3%) compared with controls (Figure 1D). In turn, RS cells inhibition was 90% of young actively proliferating cells, as expected (Figure 1C and 1D).

Expression of Key ER Chaperones and Enzymes Is Altered in RS and SIPS Fibroblasts

BiP, an hsp70 family member (25), is one of the first molecular chaperones encountered by newly ER synthesized polypeptides. Its expression in all three cellular senescence models was evaluated by qPCR (Figure 2A). Fibroblasts exposed to 250 or 350 μM $CuSO_4$ presented a 2.9- and 5.7-fold increase in BiP transcript level, respectively, compared with controls, and RS cells exhibited a 24-fold BiP mRNA increase comparatively to young cells. In contrast to copper-treated cells, H_2O_2-SIPS cells showed BiP mRNA decrease after exposure to 50, 75, or 100 μM H_2O_2 (0.7-, 0.6- and 0.6-fold variations, respectively) when compared with controls. To confirm these gene expression variations, BiP protein levels were assessed by western blot in the different senescence models (Figure 2B and 2C). Fibroblasts exposed to 250 or 350 μM $CuSO_4$ or in RS exhibited 2.5-, 2.2- and 5.9-fold increase in BiP content; in turn, H_2O_2-treated cells did not present significant changes in BiP protein levels when compared with controls.

Another analyzed chaperone was calnexin, an ER transmembrane lectin, responsible for the quality control of newly synthesized glycoproteins. Calnexin protein levels decreased in all cellular models (Figure 2B and 2C). Fibroblast exposure to 250 or 350 μM $CuSO_4$ or to 50, 75, or 100 μM H_2O_2 resulted in calnexin level reduction of 28% or 23% and 38%, 29%, or 32%, respectively, when compared with controls. Similarly, for the RS cellular model, calnexin levels were also lowered by about 33%.

Disulfide bond formation is an additional critical step in the folding of most newly synthesized proteins that translocate through ER membrane. PDI, a thioredoxin superfamily of proteins member, catalyzes the process through thiol-disulfide oxidation, reduction, and isomerization (26). Moreover, it is promoted with the help of Ero1 that maintains a suitable oxidizing environment and is thus, together with PDI, a major intervener in protein disulfide bond formation. The PDI and Ero1 protein levels were evaluated in the three senescence models (Figure 2B and 2C). Fibroblasts exposed to 250 or 350 μM $CuSO_4$ showed PDI levels reduction to 78% or 55% of the control cells' levels, respectively. In addition, cells treated with 50, 75 or 100 μM H_2O_2 presented a 27%, 34%, and 30% PDI levels decrease, respectively, when compared with controls. In RS model, PDI protein reduced 55% compared with controls. Regarding Ero1 protein, levels were enhanced in all three models comparatively to controls (Figure 2C). $CuSO_4$-SIPS fibroblasts (both 250 and 350 μM treated) presented about 2-fold increase in Ero1 content, whereas in cells treated with 50, 75, or 100 μM H_2O_2 the raise was of 2-, 3.2-, and 3.7-fold, respectively. Furthermore, in RS cells, Ero1 protein levels were 5.6-fold enhanced comparatively to young cells.

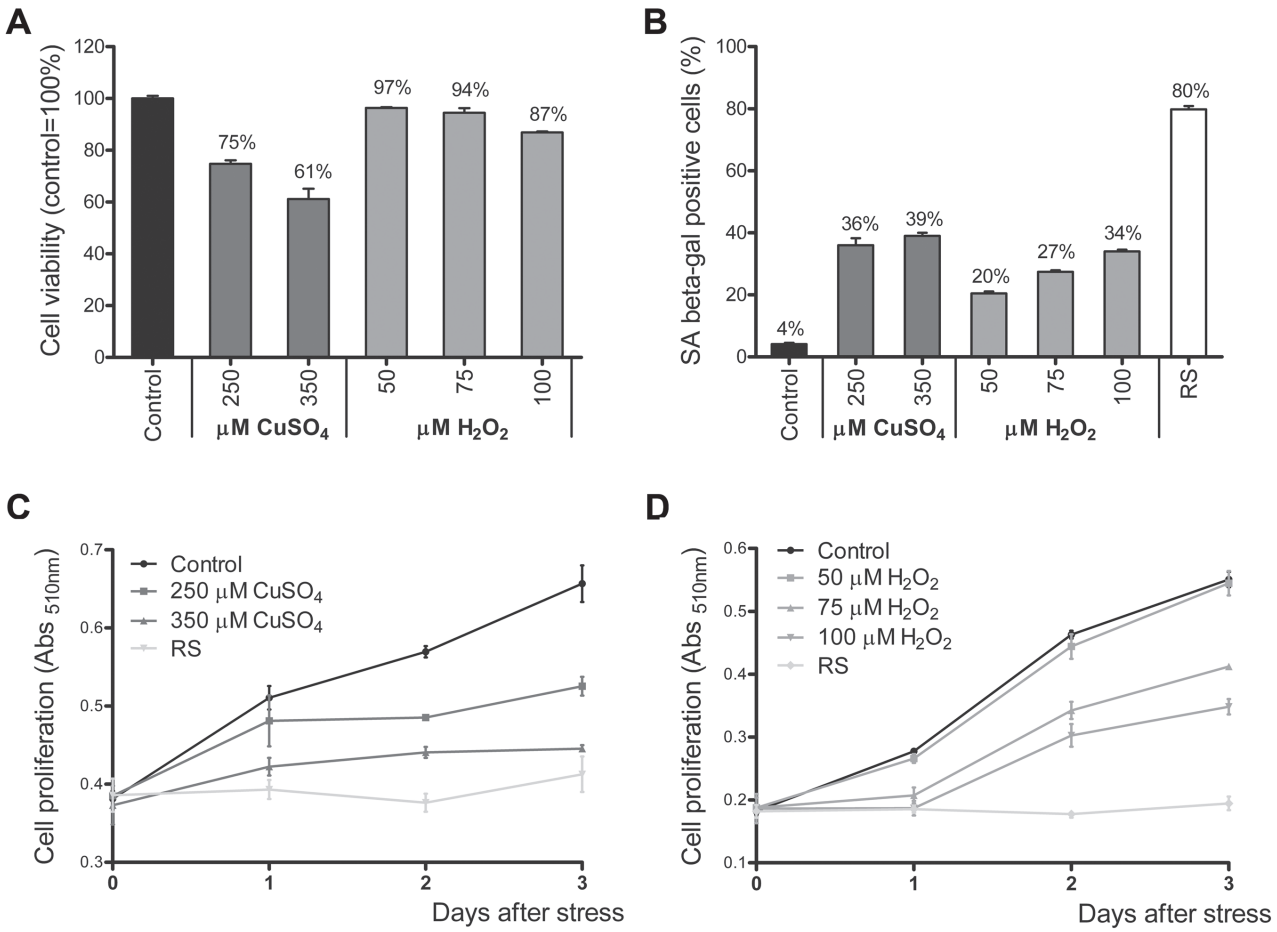

Figure 1. Subcytotoxic concentrations of $CuSO_4$ and H_2O_2 induce senescence in WI-38 human diploid fibroblasts. (**A**) Cell viability was measured using neutral red assay immediately after exposure to the different concentrations of $CuSO_4$ or H_2O_2, assuming that the control condition exhibited 100% of viable cells. (**B**)The percentage of cells positive for senescence associated beta-galactosidase activity (SA beta-gal) is plotted for cells in H_2O_2-stress-induced premature senescence (SIPS), $CuSO_4$-SIPS, or replicative senescence (RS). (**C** and **D**) Cell proliferation in $CuSO_4$-SIPS, H_2O_2-SIPS, and RS fibroblasts was assessed using sulforhodamine B assay. Data are expressed as mean ± *SEM* from at least three independent experiments.

ER Stress Response Transduction in RS and SIPS Human Cellular Models

ER stress is transmitted to the cytoplasm through IRE1, PERK, and ATF6 transmembrane ER sensor proteins that remain inactive while bound to BiP through their luminal domain. However, when unfolded/misfolded proteins accumulate in ER lumen, BiP is recruited to chaperone them, which results in sensors unbinding and consequent activation of their transduction pathways (27).

IRE1 Signaling: XBP1 Splicing and Jun N-Terminal Kinase Activation

IRE1 has an ER luminal N-terminal domain, a single-pass transmembrane segment and a C-terminal cytosolic region containing both a Ser/Thr protein kinase and a endoribonuclease (RNase) domain (28). In ER stress conditions, BiP is released from IRE1 N-terminal domain allowing oligomerization and phosphorylation of its cytosolic domain, which activates IRE1 signaling pathway (Figure 3A). Once activated, C-terminal cytosolic domain of IRE1 acquires RNase activity and cleaves 26 nucleotides from XBP1 mRNA generating an mRNA spliced variant (XBP1s). In addition, IRE1 also plays a role in signaling because its activated kinase domain interacts with TNF receptor-associated factor 2 leading to the phosphorylation/activation of the c-Jun N-terminal kinase (JNK) (29). Activated JNK can then determine downstream events that may contribute to cell survival or to apoptosis (11,30).

To evaluate whether WI-38 fibroblasts senescence activates the IRE1 branch of ER stress response, its levels were assessed in the three cellular senescence models (Figure 3B and 3C). The IRE1 protein was increased by 2- and 2.5-fold in cells exposed to 250 and 350 µM $CuSO_4$, respectively, when compared with controls. In addition, RS cells presented a 6.7-fold augmentation in IRE1 relatively to young fibroblasts. In addition, both RS and $CuSO_4$-SIPS imparted IRE1 functional changes that activated its kinase and C-terminal RNase domains. Phosphorylation levels of JNK (P-JNK) were raised in copper-treated cells (1.5-fold) and in RS model (3-fold) when compared with their controls. Furthermore, assessment of XBP1s transcript levels by qPCR revealed that both models presented a near 2.5-fold increase in XBP1s mRNA content (Figure 3D). In contrast, H_2O_2-treated cells did not evidence significant variations in IRE1 and P-JNK levels (Figure 3B and 3C), but showed a dose-dependent decrease on XBP1s transcripts (Figure 3D), when compared with controls.

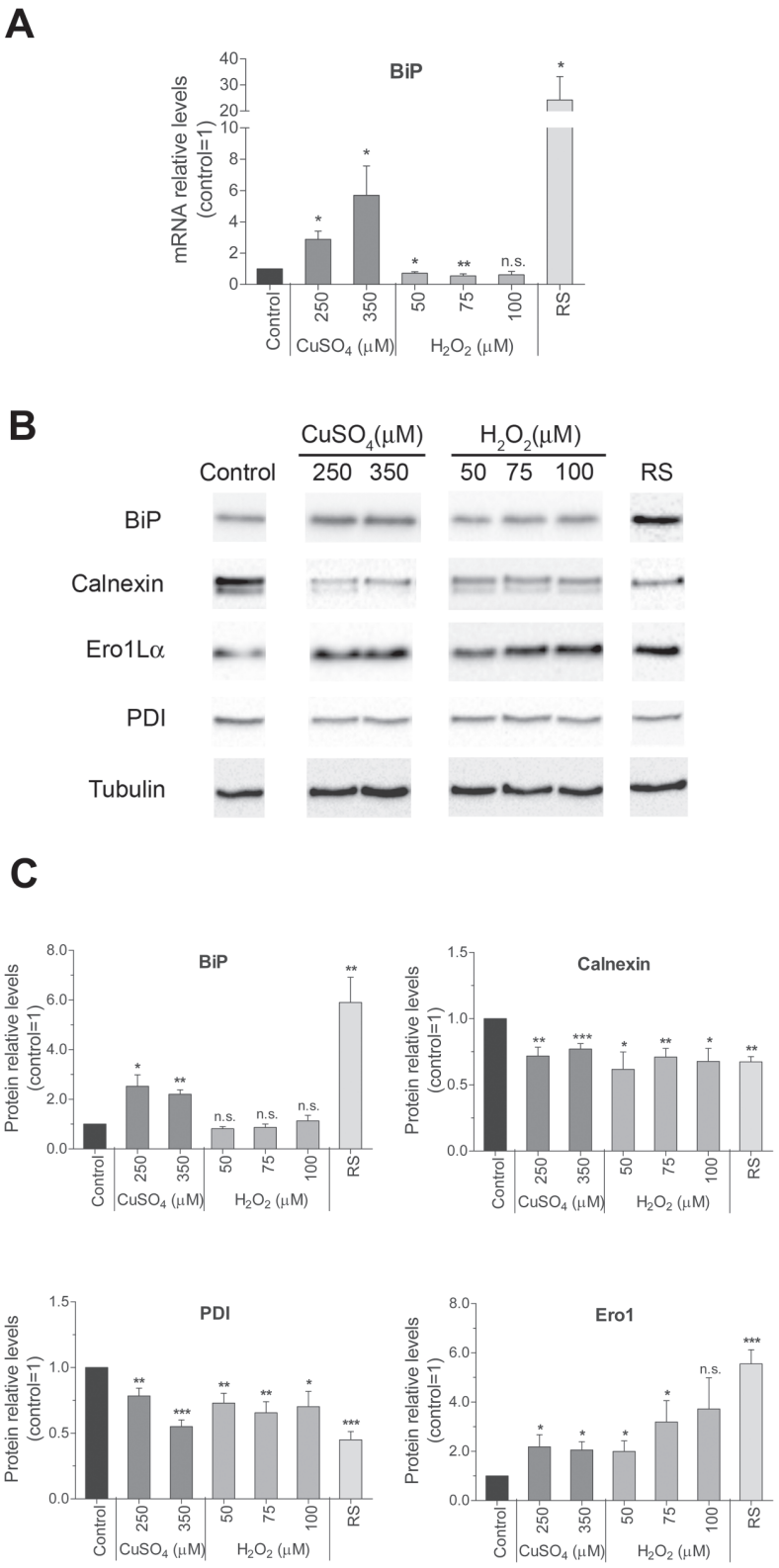

Figure 2. Expression of key endoplasmic reticulum chaperones and enzymes is altered in both replicative senescence (RS) and stress-induced premature senescence (SIPS) fibroblasts. (**A**) Relative transcript levels of immunoglobulin binding protein (BiP) were quantified by qPCR after normalization to TATA box binding protein expression for the three cellular models of senescence. (**B**) BiP, calnexin, ER oxireductin-1, and protein disulfide isomerase proteins were detected by Western blot and (**C**) their relative protein levels were quantified based on densitometry relative to tubulin in the cellular extracts from RS, $CuSO_4$-, and H_2O_2-SIPS cellular models. Data are expressed as mean ± *SEM* from at least three independent experiments. **p* < .05; ***p* < .01; ****p* < .001; and n.s.= nonsignificant when compared with control.

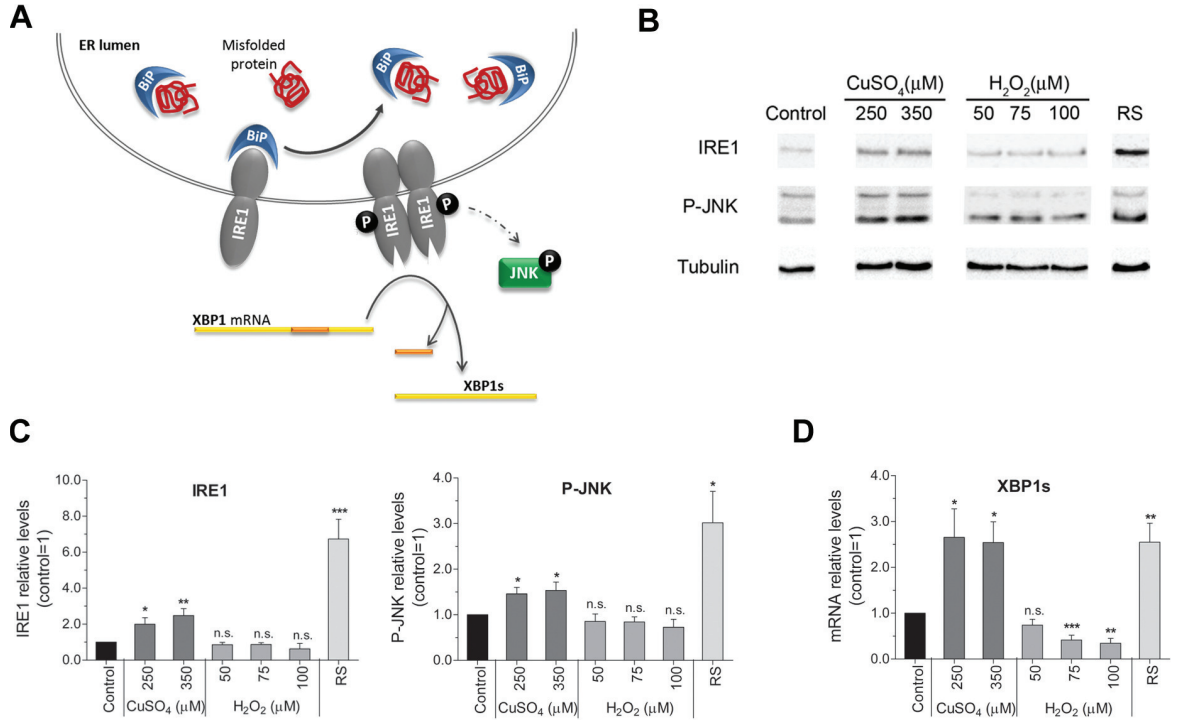

Figure 3. Inositol-requiring enzyme-1 (IRE1)–X box-binding protein 1 (XBP1s) axis of endoplasmic reticulum (ER) stress response is activated in RS and copper-induced senescence. (**A**) Schematic representation of IRE1 pathway activation: the accumulation of misfolded proteins in the ER leads to the release of immunoglobulin binding protein from IRE1 luminal domain resulting in IRE1 oligomerization and phosphorylation. Activated IRE1 kinase domain ultimately induces Jun N-terminal kinase (JNK) phosphorylation and its endoribonuclease domain promotes XBP1 mRNA splicing, originating XBP1s. (**B**) IRE1 and phosphorylated JNK1/2 (P-JNK) were detected by Western blot and (**C**) quantified by densitometry relative to tubulin in RS, $CuSO_4$- and H_2O_2-stress-induced premature senescence cells. (**D**) XBP1s relative transcript levels were assessed by qPCR and normalized to TATA box binding protein expression for the three cellular models of senescence. Data are expressed as mean ± *SEM* from at least three independent experiments. *$p < .05$; **$p < .01$; ***$p < .001$; and n.s. = nonsignificant when compared with control.

PERK Pathway: Phosphorylation of Eukaryotic Translation Initiation Factor 2α and CHOP Induction

PERK is an ER transmembrane protein with a cytoplasmic C-terminal domain with Ser/Thr kinase activity and a luminal N-terminal BiP binding domain, similar to that of IRE1 (31). As misfolded/unfolded proteins increase in ER lumen, BiP dissociates from PERK resulting in its oligomerization, autophosphorylation, and kinase domain activation (Figure 4A). Activated PERK phosphorylates eukaryotic translation initiation factor 2α (eIF2α), which leads to inhibition of protein translation and ER protein-folding load reduction (9). In addition, eIF2α phosphorylation results in the selective translation of activating transcription factor-4 (ATF4) (32), which promotes the expression of CHOP, a transcription factor that can either mediate ER stress-induced apoptosis (33) or an autophagy mediated cytoprotective response (34).

In this study, PERK and phopshorylated-eIF2α (p-eIF2α) protein levels were assessed by western blot (Figure 4B and 4C), and CHOP transcription levels were evaluated by qPCR (Figure 4D) in H_2O_2-SIPS, $CuSO_4$-SIPS, and RS WI-38 human fibroblasts. When compared with controls, PERK protein consistently increased in all three models. Cells exposed to 250 or 350 μM $CuSO_4$ exhibited 2.1- or 2.5-fold increase, respectively; similarly, cells treated with 50, 75, or 100 μM H_2O_2 presented 1.8-, 2.8-, or 2.7-fold augmentation, respectively; and RS cells showed 4.9-fold rise in PERK abundance when comparing with controls. Accordingly, p-eIF2α levels were also increased in the three cellular models studied herein, corroborating PERK activation during cellular senescence: $CuSO_4$-SIPS cells showed a 3.9- or 5.2-fold increase when exposed to 250 or 350 μM $CuSO_4$, respectively; 75 and 100 μM H_2O_2-treated cells presented 2.3- and 3.0-fold higher levels of p-eIF2α, respectively; and RS fibroblasts exhibited a 3.5-fold activation of p-eIF2α when compared with controls. In contrast, CHOP mRNA levels were slightly diminished in $CuSO_4$-SIPS, RS, and 50 μM H_2O_2-treated fibroblasts, corresponding to a decrease of about 25%, whereas cells exposed to 75 or 100 μM H_2O_2 exhibited a statistically significant 1.3-fold increased CHOP expression when compared with controls.

ATF6 Activation by Proteolytic Cleavage

ATF6 is a 90 kDa ER transmembrane protein with a DNA binding motif in its cytosolic portion. In ER stress conditions, BiP dissociates from ATF6 and allows its transport to the Golgi compartment, where it is activated by proteolytic cleavage (Figure 5A). The 50 kDa cleaved form – ATF6(p50), now a transcription factor, binds to a ER stress response element and regulates the expression of genes encoding ER chaperones and folding enzymes (3) and also XBP1 and CHOP (9).

To evaluate ATF6 activation, ATF6(p50) was semi-quantified in extracts from the three cellular models (Figure 5B). Cells exposed to 250 or 350 μM $CuSO_4$ presented a significant 1.7- or 2.2- fold increase in ATF6(p50) levels; RS cells, although in a statistically nonsignificant fashion ($p = .09$), exhibited higher levels (4.5-fold) of activated ATF6 when compared with controls. Cells treated with

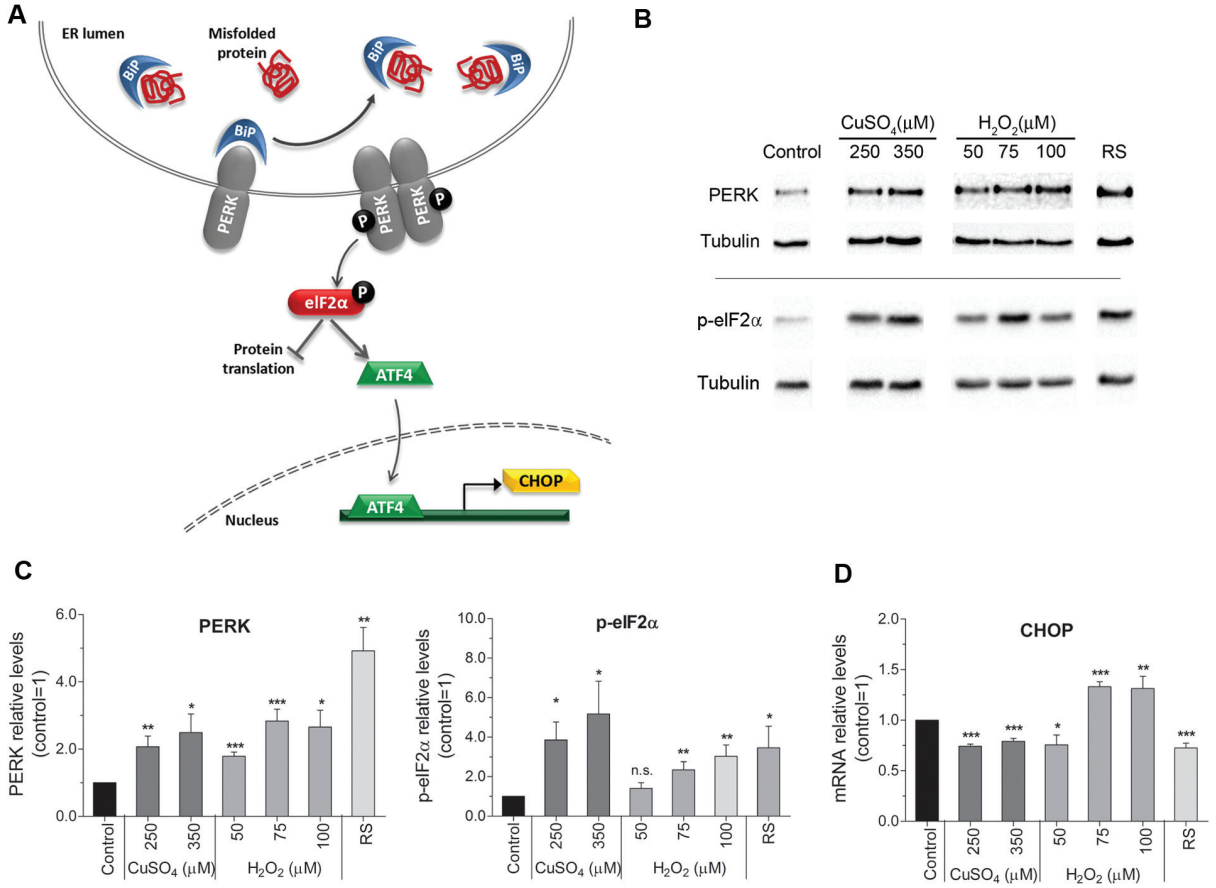

Figure 4. Pancreatic ER kinase (PERK) pathway is activated in H_2O_2- and $CuSO_4$-stress-induced premature senescence (SIPS) cellular models. (**A**) Schematic representation of the activation of PERK pathway: in endoplasmic reticulum (ER) stress conditions, immunoglobulin binding protein is recruited to assist protein folding and releases the ER luminal domain of PERK leading to its oligomerization and phosphorylation. Activated PERK then phosphorylates eIF2α (p-eIF2α) inhibiting general protein translation and activating the translation of specific mRNAs such as the transcription factor ATF4, which in turn promotes the transcription of CHOP. (**B**) PERK and p-eIF2α proteins were detected by Western blot and (**C**) quantified, based on densitometry relative to tubulin in cellular extracts from RS, $CuSO_4$-, and H_2O_2-SIPS models. (**D**) Relative levels of CHOP mRNA were quantified by qPCR after normalization to TATA box binding protein expression in the three cellular models of senescence. Data are expressed as mean ± *SEM* from at least three independent experiments. $^*p < .05$; $^{**}p < .01$; $^{***}p < .001$; and n.s. = nonsignificant when compared with control.

H_2O_2 showed a trend to higher (~1.5-fold) levels of ATF6(p50) when compared with controls, but only the lowest dose tested (50 μM) yielded a statistically significant variation ($p = .04$).

ER Stress Response Inhibition Attenuates the Appearance of SIPS

The importance of ER stress response activation during cellular senescence was evaluated by inhibiting IRE1 or PERK activities in WI-38 fibroblasts prior to the induction of SIPS. The IRE1 inhibitor 4μ8c and PERK inhibitor GSK2606414 were already tested in several cell lines and were shown to selectively inhibit IRE1-dependent XBP1 splicing and eIF2α phosphorylation, respectively (35,36). Herein, the efficacy of each one of these inhibitors was verified in cells treated with dithiothreitol (DTT), a potent ER stress inducer. It was demonstrated that the marked increased in XBP1s mRNA and p-eIF2α protein levels induced by DTT was greatly prevented by the presence of the IRE1 and PERK inhibitors, respectively (see Supplementary Figure 1). Because $CuSO_4$-SIPS cells exhibit both IRE1- and PERK-mediated ER stress response activation (Figures 3 and 4), whereas H_2O_2-SIPS cells just present PERK pathway induction (Figure 4), IRE1 inhibition with 4μ8c was performed prior to SIPS induction with copper sulfate, whereas PERK was inhibited with GSK2606414 before SIPS induction with copper sulfate or hydrogen peroxide. As presented in Figure 6A, GSK2606414 or 4μ8c did not affect cell viability. Regarding cell proliferation, PERK inhibition with GSK2606414 prior to H_2O_2-SIPS induction had no effect when compared with H_2O_2-treated cells without the inhibitor (Figure 6B). However, pretreatment of $CuSO_4$-treated cells with GSK2606414 resulted in a statistically significant increase in cell proliferation as compared to $CuSO_4$-treated cells without the inhibitor (Figure 6C). A divergent result was obtained using 4μ8c: the inhibition of IRE1-mediated ER stress response prior to $CuSO_4$-SIPS induction did not affect copper ability to inhibit cell proliferation (Figure 6D). It is noteworthy to mention that inhibition of IRE1-pathway per se results in a marked inhibition of cell proliferation in WI-38 fibroblasts (control vs. control 4μ8c). This anti-proliferative effect of 4μ8c was already described in KP4 tumor cell line (36), and IRE1 has actually been identified as a cell cycle regulator, as it controls cyclin A1 expression promoting cell proliferation through XBP1 (37).

To evaluate the effect of ER stress response inhibition in the appearance of typical senescence biomarkers, cells were pretreated

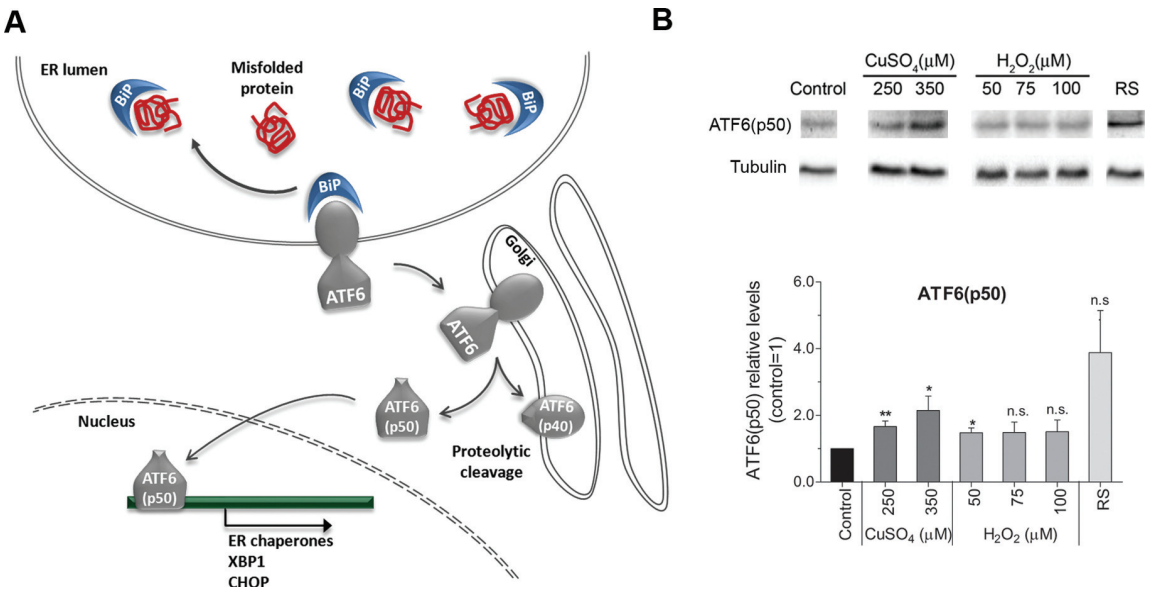

Figure 5. Activating transcription factor 6 (ATF6) is activated by proteolytic cleavage in RS and $CuSO_4$-stress-induced premature senescence. (**A**) Schematic representation ATF6 branch of endoplasmic reticulum (ER) stress response: as immunoglobulin binding protein (BiP) binds to accumulated misfolded proteins in the ER, it releases ATF6 that is then transported to Golgi apparatus. There, the cytosolic domain of ATF6 is cleaved to a 50 kDa form—ATF6(p50)—which translocates to the nucleus where it can activate the transcription of X box-binding protein 1, CCAAT/enhancer-binding protein homologous protein, and ER chaperones, such as BiP. (**B**) ATF6(p50) was detected by Western blot and its relative protein levels were obtained by densitometric quantification relative to tubulin. Data are expressed as mean ± *SEM* from at least three independent experiments. *$p < .05$; **$p < .01$; and n.s. = nonsignificant when compared with control.

with GSK2606414 or 4μ8c before being exposed to hydrogen peroxide or copper sulfate and the percentage of SA beta-gal positive cells and p21 mRNA relative levels were quantified. The PERK inhibition prior to H_2O_2-SIPS induction resulted in a decreased number of cells positive for SA beta-gal from 33.7% to 20.1% and in an attenuation of p21 mRNA levels, from 16.4- to 2.1-fold (Figure 7A and 7C). In addition, PERK- and IRE1-inhibiton prior to cell exposure to 350 μM $CuSO_4$ resulted in a diminished percentage of positive cells for SA beta-gal from 38.6% in $CuSO_4$-treated cells to 17.1% and 17.2% in the presence of GSK2606414 and 4μ8c, respectively (Figure 7B). Moreover, fibroblasts treated with 350 μM $CuSO_4$ exhibited a 4.1-fold increase in p21 mRNA relative levels when compared with control cells (Figure 7D) and this increase was also significantly prevented by the presence of GSK2606414 (2.0-fold). Regarding IRE1-pathway inhibition, cells treated with 4μ8c prior to $CuSO_4$-SIPS induction did not show any significant variation on p21 transcript levels (3.5-fold) when compared with $CuSO_4$-treated cells.

Discussion

This study evaluates the levels of ER chaperoning system interveners and the activation of ER stress response during cellular senescence. The occurrence of ER stress was reported in human senescence induced by tumor-associated mutations (38) or chemotherapeutic agents in neoplastic cells (39), but never in nonmodified senescent cells in vitro. Using RS, H_2O_2- and $CuSO_4$-SIPS human cellular models, it is shown that BiP, calnexin, PDI, and Ero1 levels are adjusted to deal with senescence-associated imbalance in prosteostasis. Moreover, RS triggers a cellular protective ER stress response mediated by IRE1, ATF6, and PERK. This behavior is mimicked by $CuSO_4$-SIPS but not by H_2O_2-SIPS, which does not exhibit the activation of IRE1 and ATF6 axis of ER stress response and, instead, reveals PERK-mediated upregulation of CHOP.

The BiP chaperone expression is usually induced when misfolded polypeptides accumulate in the ER (40). Aging is likely to result in the impairment of this mechanism as BiP expression was lower in the liver (6), brain cortex (41), and other tissues (16) of aged rodents. In contrast, in this study, the human cellular models of RS and $CuSO_4$-SIPS, but not H_2O_2-SIPS, had enhanced BiP mRNA and protein levels when compared with controls. One likely explanation for this discrepancy is the effective percentage of senescent cells present in each case. In fact, aged baboons (42,43), monkeys (44), and humans (23) skin biopsies had senescent cells ranging from 0.1% to 15% of the total number. This implies that most BiP in old tissues results from nonsenescent cells which precludes a direct comparison of tissue homogenates with the senescent cell enriched in vitro cellular models. We are convinced that during cellular senescence, either RS or $CuSO_4$-SIPS, moderate oxidative damage on proteins promotes their misfolding/unfolding and aggregation. In such a proteostasis disruption condition, ER stress response is activated, leading to enhanced BiP transcription in an attempt to assist protein folding and restore proteostasis.

Calnexin and PDI were reduced in aged rodent hippocampus (15) and liver (6,14). In addition, calnexin levels in RS and H_2O_2-SIPS fibroblasts exhibited significant decrease when compared with young cells (45). The current investigation confirmed calnexin downregulation in the three models and further evidenced a similar PDI protein change. The Ero1 protein levels variation along aging, largely unknown so far, evidenced here a senescent-associated increase, probably as a result of an oxidative stress induced effect on transcriptional regulation, as reported in yeast (46).

Besides these chaperones and enzymes, aging modulates ER stress response components such as the PERK, IRE1, and ATF6 transmembrane sensors. The efficiency of these cell protective pathways declines during aging, disturbs protein quality control, and can lead to cell death (13–16). Age-related variations in PERK signaling

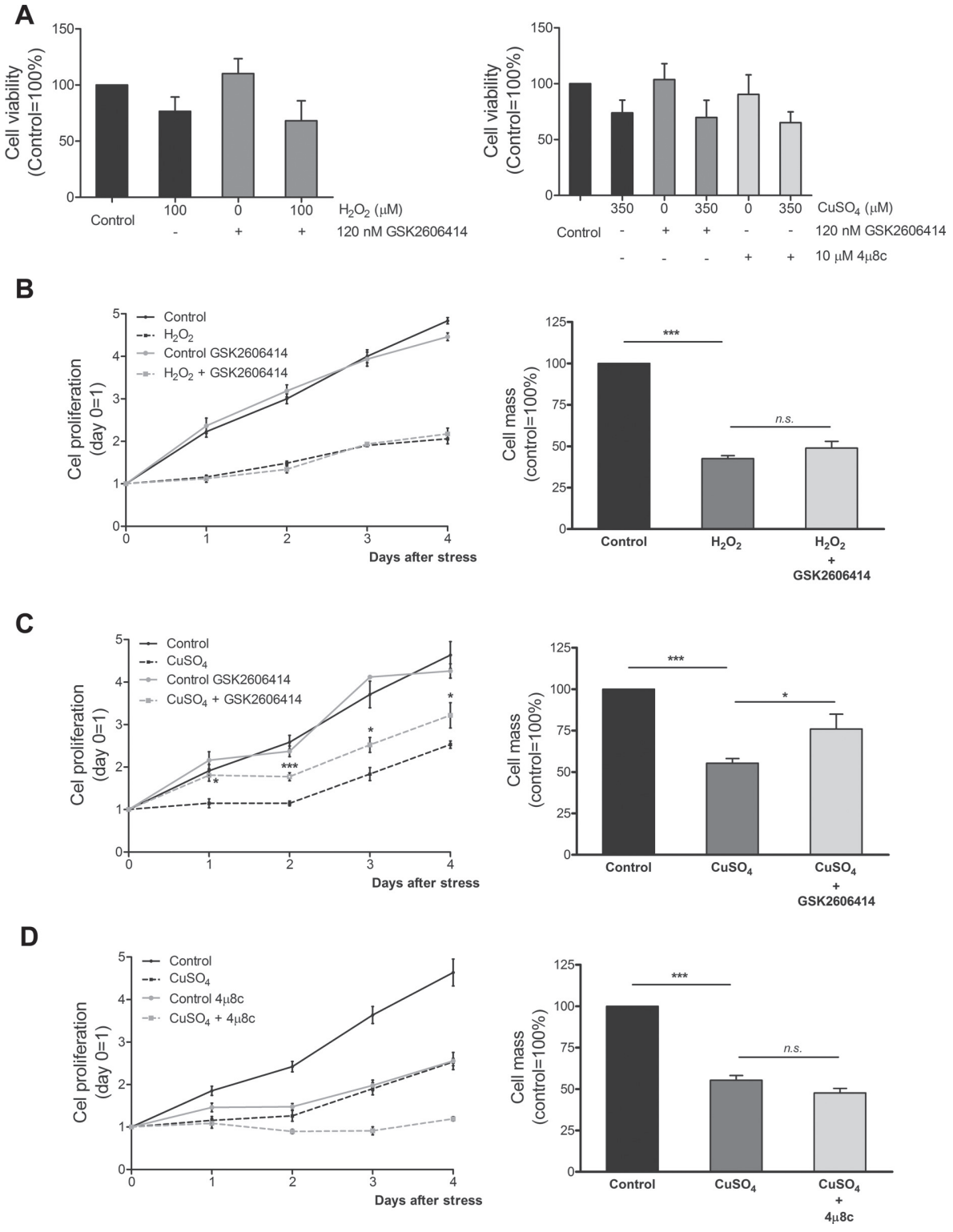

Figure 6. Pancreatic ER kinase (PERK) and inositol-requiring enzyme-1 (IRE1) inhibition prior to stress-induced premature senescence (SIPS) induction does not affect cell viability but present differential effects on cell proliferation. (**A**) Cell viability was assessed using neutral red assay immediately after stress in H_2O_2- and $CuSO_4$-SIPS cells in the presence or absence of PERK (GSK2606414) or IRE1 (4μ8c) inhibitors, assuming that the control condition exhibited 100% of viable cells. Cell proliferation was evaluated using in sulforhodamine B assay at 0, 1, 2, 3, and 4 days after stress in (**B**) H_2O_2-SIPS or (**C** and **D**) $CuSO_4$-SIPS cells pre-treated or not with 120 nM GSK2606414 or 10 μM 4μ8c, as indicated in the respective plots. The percentage of cell mass was calculated at the fourth day after stress for H_2O_2-SIPS and $CuSO_4$-SIPS cells in the presence or absence of the inhibitors, considering that the respective control cells represented 100% of cell mass. Data are expressed as mean ± *SEM* from at least three independent experiments. *$p < .05$; **$p < .01$; ***$p < .001$; and n.s. = nonsignificant when compared with control.

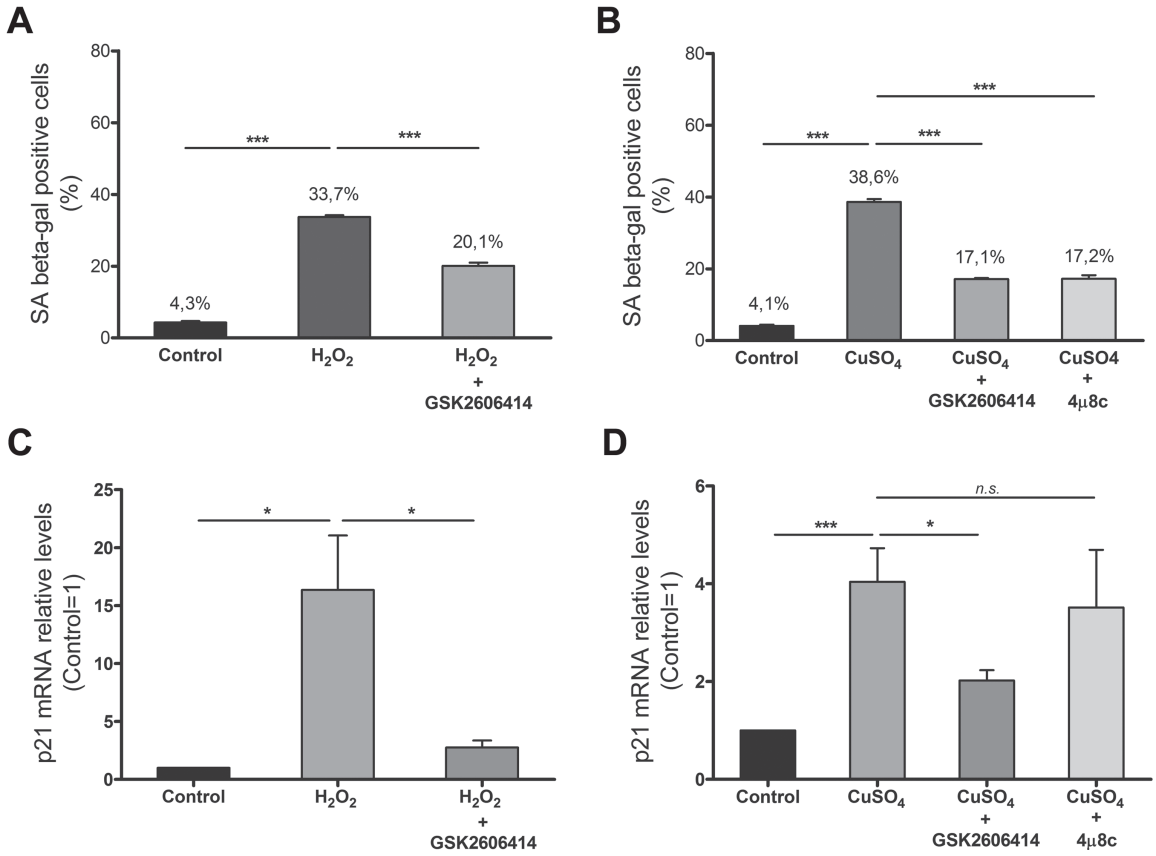

Figure 7. Impact of unfolded protein response inhibition prior to stress-induced premature senescence (SIPS) induction in the appearance of the senescent biomarkers SA beta-gal and p21 mRNA levels. The percentage of cells positive for SA beta-gal is plotted for cells in (**A**) H_2O_2-SIPS and (**B**) $CuSO_4$-SIPS in the presence or absence of pancreatic ER kinase (PERK) (120 nM GSK2606414) or inositol-requiring enzyme-1 (IRE1) (10 μM 4μ8c) inhibitors. Relative levels of p21 mRNA were quantified by qPCR after normalization to TATA box binding protein expression in (**C**) H_2O_2-SIPS or (**D**) $CuSO_4$-SIPS cells treated or not with GSK2606414 or 4μ8c, as indicated. Data are expressed as mean ± *SEM* from at least three independent experiments. *$p < .05$; ***$p < .001$; and n.s. = nonsignificant, when compared with control.

were previously reported, but the results are sparse and conflicting. For instance, in aged rats hippocampus, PERK mRNA was downregulated (15), whereas PERK protein levels were increased in other tissues (16). In addition, it remained to be determined IRE1 or ATF6 pathways involvement in aging as IRE1 inactivation resulted in *Caenorhabditis elegans* decreased life span (2,47) and no change in *Saccharomyces cerevisiae* (48). This study provides compelling evidence that RS and $CuSO_4$-SIPS conditions activated IRE1 pathway, as shown by enhanced expression of IRE1 protein itself and its downstream effectors XBP1s mRNA and phosphorylated JNK; similarly, a clear rise in ATF6(p50) levels is consequent to ATF6 axis activation upon proteolytic cleavage; and PERK pathway activation was also verified by increased total PERK and p-eIF2α levels, although without increased CHOP transcription. In contrast, H_2O_2-SIPS fibroblasts do not reveal IRE1 or ATF6 signaling activation, but instead they exhibit PERK activation with increased p-eIF2α and CHOP overexpression.

These results in RS and $CuSO_4$-SIPS models favor the view that senescence is accompanied by moderate ER stress which activates protective responses, mediated by IRE1, ATF6, and PERK. In this case, however, PERK-mediated UPR response activation is not upregulating CHOP as it occurs in its most classical view. Instead, although uncertain, a differentially regulated PERK-dependent response leading to CHOP downregulation may be activated in these conditions. In fact, it was recently identified a PERK-responsive microRNA (miR-211) that inhibits CHOP expression, allowing cell homeostasis re-establishment (49). However, further investigation is needed to clarify this point.

Although the pattern of chaperone and ER stress sensors activation is considerably coherent in RS and $CuSO_4$-SIPS, it is not so in H_2O_2-SIPS model. The latter is the most frequently used in vitro model to study molecular events of cellular senescence because it largely mimics genotypic and phenotypic features of RS. Surprisingly, in this study, H_2O_2-treated cells failed to activate two of the ER stress response pathways that are triggered in RS. In fact, neither ATF6 nor IRE1 pathways were activated in H_2O_2-SIPS and, actually, XBP1s transcript level decreased in fibroblasts treated with H_2O_2 highest doses. As shown earlier in mesangial cells (50), such oxidant environment is likely to affect XBP1s mRNA, which must be stabilized to promote an efficient IRE1-mediated ER stress response (51). In contrast to ATF6 and IRE1, PERK-CHOP pathway is activated in 75 or 100 μM H_2O_2-induced SIPS fibroblasts. The CHOP, apart from its involvement in ER stress-induced apoptosis (33), was recently recognized as essential for the transcriptional activation of autophagy genes (34), preventing cell death (30). In H_2O_2-SIPS, a condition where the cells are known to acquire resistance to cell death, the slightly overexpressed CHOP might be regulating cytoprotective mechanisms as autophagy instead of inducing apoptosis.

The ER acts as a sensor of the physiological state of the cell and is able to trigger an ER stress response that will decide cell fate. It is shown here that the inhibition of ER stress response (either PERK- or IRE1-mediated) before the induction of H_2O_2- or $CuSO_4$-SIPS leads to the attenuation of the appearance of the senescence biomarker SA beta-gal. Actually, a similar effect had been already described in an oncogene-induced premature senescence model (38). The decrease of $CuSO_4$-induced senescence by GSK2606414 is reinforced by the increase in cell proliferation and decrease in p21 mRNA levels. Actually, PERK activation was already associated with cell cycle blockage, as it was shown that high levels of eIF2α phosphorylation are able to induce G1 cell cycle arrest in mouse fibroblasts submitted to hypoxia (52). Interestingly, PERK inhibition prior to H_2O_2-SIPS did not affect cell proliferation while p21 is still decreased. These results indicate that PERK-mediated ER stress response is implicated in copper ability to induce cell cycle arrest in $CuSO_4$-SIPS model, whereas cell proliferation inhibition by H_2O_2 is independent of PERK-pathway activation. This differential behavior observed between $CuSO_4$- and H_2O_2-SIPS cells, already discussed earlier, may arise from the different intensities of stress provoked by each SIPS inducer and the alternative pathways they might be activating in the cell. The IRE1 inhibition prior to cell exposure to $CuSO_4$ did not alter the effect of copper sulfate on cell proliferation or p21 mRNA levels. Together, the data shown here indicate that the cell cycle arrest occurring in $CuSO_4$-SIPS cells is mediated by PERK activation but does not require IRE1 activity.

This study shows that $CuSO_4$-SIPS model mimics better the ER molecular events of RS than H_2O_2-SIPS and adds further evidence on the molecular mechanisms that regulate the induction of senescence. Copper has recently been involved in the establishment of senescence, because its intracellular levels were increased in RS fibroblasts when compared with young cells (53). Such involvement can be now justified by copper ability to induce the activation of ER stress response, which we demonstrate here to be required for the induction of senescence. Actually, copper homeostasis disruption has been involved in age-related diseases, as Alzheimer's and Parkinson's (for a review, see Ref. (54)), which turns copper-induced senescence model a valuable tool in their study. In addition, its usefulness may extend to identify new mechanisms and potential targets for pharmaceutical interventions, aiming to ameliorate cellular stress responses during aging and postpone age-related health deterioration.

Supplementary Material

Supplementary material can be found at: http://biomedgerontology.oxfordjournals.org

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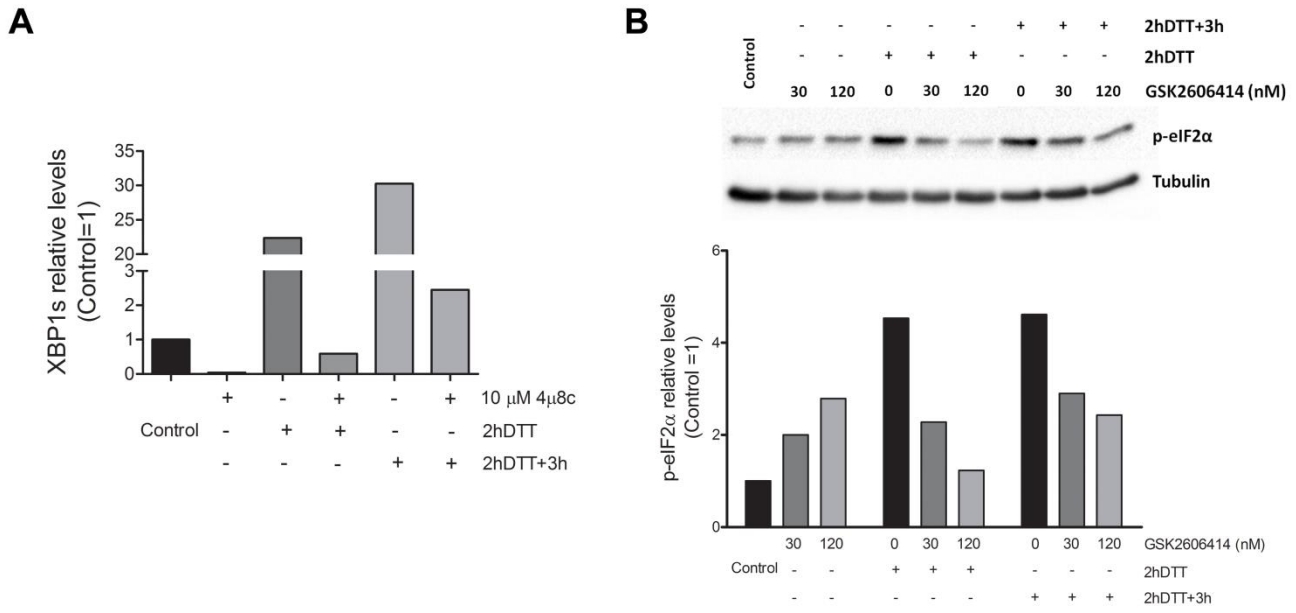
Supplementary material



Supplementary Figure 1: IRE1 and PERK inhibitors were able to effectively decrease XBP1s and p-eIF2α levels in the presence of DTT. (A) XBP1s mRNA levels were evaluated by qPCR immediately after cell exposure to 2 mM DTT for 2h (2hDTT) and after allowing cell recovery from 2hDTT for 3h (2hDTT+3h), in the presence or absence of 10 μM 4μ8c. The presence of 4μ8c was able to reduce XBP1 splicing from 22- and 30-fold to 0.6- and 2.5-fold in 2hDTT and 2hDTT+3h cells, respectively, when compared to control. (B) p-eIF2α protein levels, semi-quantified by western blot, exhibited an increase of about 4.5-fold both in 2hDTT and 2hDTT+3h cells, that was inhibited by 30 or 120nM GSK2606414 to 2.3- and 1.2-fold respectively in 2hDTT cells, and to 2.9- and 2.4-fold in cells that were allowed to recover for 3h after 2hDTT treatment, when compared to control.

PUBLICATION III

"Resveratrol attenuates copper-induced senescence by improving cellular proteostasis"

(submitted)

Matos *et al., (submitted)*

Resveratrol attenuates copper-induced senescence by improving cellular proteostasis

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Copper sulfate-induced premature senescence ($CuSO_4$-SIPS) was found to mimetize molecular mechanisms of replicative senescence in a consistent way, particularly at the level of endoplasmic reticulum proteostasis. In fact, disruption of protein homeostasis has been recognized as leading to age-related cell and tissue dysfunction, and human disorders susceptibility. Resveratrol is a polyphenolic antioxidant compound known to exhibit anti-aging properties under *in vitro* and *in vivo* particular conditions. In this setting, we aimed at evaluating resveratrol ability to attenuate the induction of cellular senescence and to unravel the related, elusive, molecular mechanisms. Using $CuSO_4$-SIPS WI-38 fibroblasts, it is demonstrated that resveratrol attenuates typical senescence alterations on cell morphology, senescence-associated beta-galactosidase activity and cell proliferation. The mechanisms implicated in this anti-senescence effect do not appear to involve the regulation of senescence-associated genes and proteins, but are instead reliant on the improvement of cellular proteostasis. In fact, resveratrol supplementation restores copper-induced increased total intracellular protein content, attenuates BiP chaperone level, and reduces carbonylated and poly-ubiquitinated proteins by lysosomal autophagy induction. Our data provide compelling evidence for the beneficial effects of resveratrol by mitigating $CuSO_4$-SIPS stressful consequences in human fibroblasts by the modulation of protein quality control systems. These findings highlight the importance of a balanced cellular proteostasis and add further knowledge on molecular mechanisms that mediate resveratrol anti-senescence effects. Moreover, they contribute to identify specific molecular targets whose modulation is able to prevent age-associated cell dysfunction and improve human healthspan.

Key words: aging, copper, SIPS, resveratrol, human fibroblasts, proteostasis

Normal somatic dividing cells have been proved to be valuable *in vitro* models to study cellular senescence and unravel molecular mechanisms and pathways implicated in the human aging process. The well known model of replicative senescence (RS) is achieved when human diploid fibroblasts (HDFs) spontaneously stop dividing after an initial active period of population doublings, and become unresponsive to mitogenic stimuli (Hayflick & Moorhead 1961). Besides the irreversible cell cycle arrest, RS fibroblasts exhibit other, typical, morphological and molecular features, such as increased cellular volume, higher senescence associated beta-galactosidase (SA beta-gal) activity and increased expression of senescence-associated genes and proteins (Dimri *et al.* 1995; Debacq-Chainiaux *et al.* 2008). A similar senescent phenotype, termed stress-induced premature senescence (SIPS), can be attained by the exposure of HDFs to subcytotoxic doses of oxidative stress inducers such as hydrogen peroxide (H_2O_2-SIPS) (Chen & Ames 1994), tert-butyl hydroperoxide, ultra-violet B radiation (Debacq-Chainiaux *et al.* 2008) or copper sulfate ($CuSO_4$-SIPS) (Matos *et al.* 2012). Recently, the latter was shown to mimic better the RS model compared to the most frequently used H_2O_2-SIPS model (Matos *et al.* 2014).

Resveratrol is a natural polyphenolic compound that was shown to increase maximum lifespan of several organisms, such as *Saccharomyces cerevisiae* (Howitz *et al.* 2003), *Caenorhabditis elegans* (Viswanathan et al. 2005), *Drosophila melanogaster* (Wood *et al.* 2004) and the short-lived fish *Nothobranchius furzeri* (Valenzano *et al.* 2006). Yet, resveratrol failed to extend longevity in rodent mammals, even though it improved their healthspan, thus evidencing a protective role against age-related deterioration (Pearson *et al.* 2008).

At the cellular level, resveratrol has also been shown to attenuate senescence features in either RS (Giovannelli *et al.* 2011) or H_2O_2-SIPS (Demidenko & Blagosklonny 2009; Ido *et al.* 2015) cellular models. These anti-aging effects have long been associated to resveratrol ability to activate sirtuin1 deacetylase, Sirt1 (for a review see Bhullar & Hubbard 2015). Actually, it was demonstrated that Sirt1 overexpression attenuates senescence and extends replicative lifespan of several cultured cell types (Ota *et al.* 2007; Huang *et al.* 2008; Yamashita *et al.* 2012), while its inhibition results in increased cellular senescence (Ota *et al.* 2007). Also, Sirt1 was shown to be downregulated with aging (Lu *et al.* 2014) and in cellular senescence models (Sasaki *et al.* 2006; Song *et al.* 2014) further favoring its preventive role of senescence features. Besides resveratrol ability to modulate signal transduction pathways through the activation of Sirt1 (Park *et al.* 2012; Ido *et al.* 2015), several other biological events were assigned as responsible for its positive effects, including its ability to increase stress

resistance (Giovannelli *et al.* 2011), to induce telomerase activity (Xia *et al.* 2008), to decrease the secretion of senescence-associated pro-inflammatory proteins (Csiszar *et al.* 2012) and to inhibit the mechanistic target of rapamycin, mTOR (Demidenko & Blagosklonny 2009). Resveratrol was also found to modulate protein quality control cellular responses, as it was shown to regulate the expression of the heat shock molecular chaperones (Putics *et al.* 2008) and to promote cellular protein degradation mechanisms, namely ubiquitin-proteasome system (UPS) (Sato *et al.* 2013; Regitz *et al.* 2015) and lysosomal autophagy (Duan *et al.* 2013; Suzuki & Bartlett 2014). Moreover, resveratrol was able to increase *C. elegans* lifespan through the upregulation of *abu11* (activated in blocked unfolded protein response-11), which encodes a protein involved in the endoplasmic reticulum (ER) unfolded protein response (UPR) that protects the organism from damage by improperly folded proteins (Viswanathan *et al.* 2005).

In the present study we aimed to evaluate the ability of resveratrol to attenuate the establishment of cellular senescence upon $CuSO_4$ induction, unravelling the molecular mechanisms that might be involved. It was found that resveratrol supplementation was able to reduce the appearance of some senescence-associated features by the improvement of cellular proteostasis probably by protecting proteins from oxidative damage and preventing their accumulation by the induction of protein degradation mechanisms.

RESULTS

Sirtuin 1 expression is diminished in $CuSO_4$-SIPS

It was already demonstrated that Sirt1 expression decreases with increasing population doublings (Sasaki *et al.* 2006) and also in H_2O_2-SIPS cellular models (Song *et al.* 2014). Here, Sirt1 transcript and protein levels were evaluated by qPCR and western blot, respectively, in $CuSO_4$-induced senescent WI-38 fibroblasts. Similarly to other RS and SIPS models, $CuSO_4$-SIPS fibroblasts also presented decreased expression of both gene (Fig. 1A) and protein (Fig. 1B) Sirt1. Namely, mRNA and protein relative level presented a 27% and 23% reduction, respectively, in copper-treated cells when compared to controls ($p=0.04$ and $p=0.008$, respectively). The effect of resveratrol (5 or 10 µM), a Sirt1 activator, was evaluated 72h after the 24h-incubation of cells with $CuSO_4$, which is the usual recovery time that cells need to adapt and develop the senescent phenotype (Matos *et al.* 2012). The addition of 10 µM resveratrol attenuated the copper-induced decrease in Sirt1 protein levels ($p=0.047$) to values similar to the young control cells. Incubation of non $CuSO_4$ submitted fibroblasts with 5 and 10 µM resveratrol for 72h increased Sirt1 protein level by 1.6- and 1.3-fold ($p=0.008$ and $p=0.01$), respectively, when compared to young control cells (Fig. 1B).

Resveratrol attenuates the appearance of some typical senescence-associated alterations

Senescent cells usually present typical morphological alterations, increased level of SA beta-gal and irreversible inhibition of cell proliferation. Therefore, these three features were evaluated in order to assess the effect of resveratrol in $CuSO_4$-SIPS fibroblasts. Briefly, cell proliferation was assessed by counting the viable cells at 0, 24, 48 and 72h after copper removal. Then, at the last time-point (72h), cell morphology was observed and the percentage of SA beta-gal positive cells was quantified for each condition. As shown in Fig. 2A, in the absence of resveratrol, $CuSO_4$-SIPS fibroblasts presented the typical senescent morphology, as they were no longer small and fusiform and became enlarged and flattened. However, copper-treated cells recovering in the presence of resveratrol exhibited less pronounced senescent-like alterations, as they appeared thinner and more elongated, when compared to cells in the absence of resveratrol. This was particularly evident for the highest concentration of resveratrol used (10 µM). It is noteworthy to mention that even cells not submitted to copper exhibited a slightly different aspect

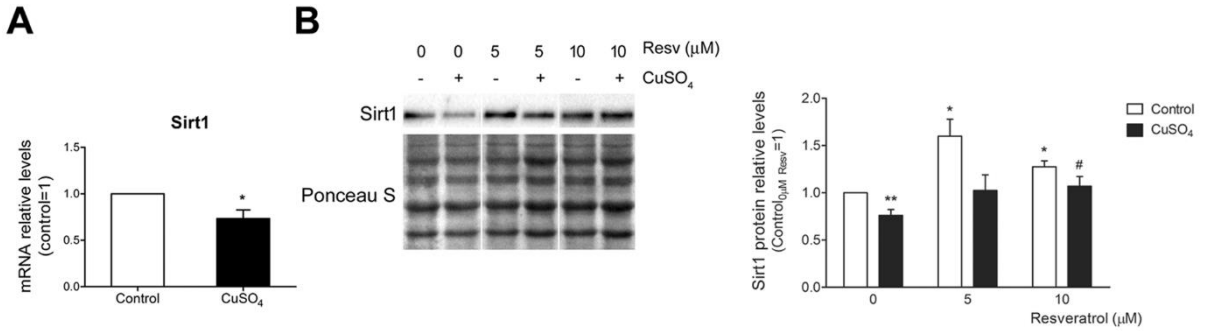

Figure 1: Reduced sirtuin 1 expression in $CuSO_4$-SIPS fibroblasts is restored by the addition of resveratrol. (A-B) WI-38 fibroblasts were incubated with 350 µM $CuSO_4$ (or Na_2SO_4, for controls) for 24h. Then, media were changed and cells were allowed to recover for an additional 72h period in the presence of 5 or 10 µM resveratrol (or 0,1% DMSO, for controls). After this recovery period, fibroblasts were processed for different assays. **(A)** Sirtuin 1 (Sirt1) transcript levels were assessed by qPCR and plotted assuming that mRNA level of controls equals 1. TBP was the selected housekeeping gene. **(B)** Sirt1 relative protein content was determined by western blot, using Ponceau S staining to normalize protein loading. Depicted blots are representative and densitometric quantification is plotted assuming that control cells in the absence of resveratrol presents a relative protein level of 1. Data represent mean ± SEM of at least three independent experiments. * $p<0.05$; and ** $p<0.01$, when compared to control cells in the absence of resveratrol; # $p<0.05$, relatively to $CuSO_4$-treated cells without resveratrol.

in the presence of resveratrol, as they seemed smaller and their cell limits were more clear-cut.

Similar to previously reported results (Matos *et al.* 2014), $CuSO_4$-SIPS cellular model contained 34% of cells positive for SA beta-gal (Fig. 2B), whereas the controls had only 5% of senescent cells. However, the addition of 5 and 10 µM resveratrol to copper-treated cells resulted in a statistically significant reduction in the number of SA beta-gal positive cells (to 16 and 14%, respectively). The ability of copper sulfate to inhibit cell proliferation had previously been described (Matos *et al.* 2014) and herein is again demonstrated (Fig. 2C), as 3 days after stress, copper-treated cells presented a reduction of 88% in their proliferation when compared to controls. Media supplementation with 5 and 10 µM resveratrol during the recovery period resulted in the attenuation of cell proliferation inhibition by 20 and 34%, respectively. In addition, in the absence of copper, the selected concentrations of resveratrol were not able to affect significantly cell proliferation when compared to control cells. Altogether, these data show that resveratrol can actually attenuate the induction of senescence by copper sulfate in WI-38 fibroblasts.

Resveratrol does not alter copper-induced upregulation of senescence-associated genes and proteins

There are several genes and proteins, such as the cyclin-dependent kinase inhibitor 1A (p21), apolipoprotein J (ApoJ) and transforming growth factor beta 1 (TGFβ1), whose overexpression is typical of the senescent phenotype observed in RS and SIPS cellular models. Herein, we evaluated the ability of resveratrol to adjust the levels of p21, ApoJ and TGFβ1 upon copper treatment, in order to justify its effect in the attenuation of copper-induced senescence. Therefore,

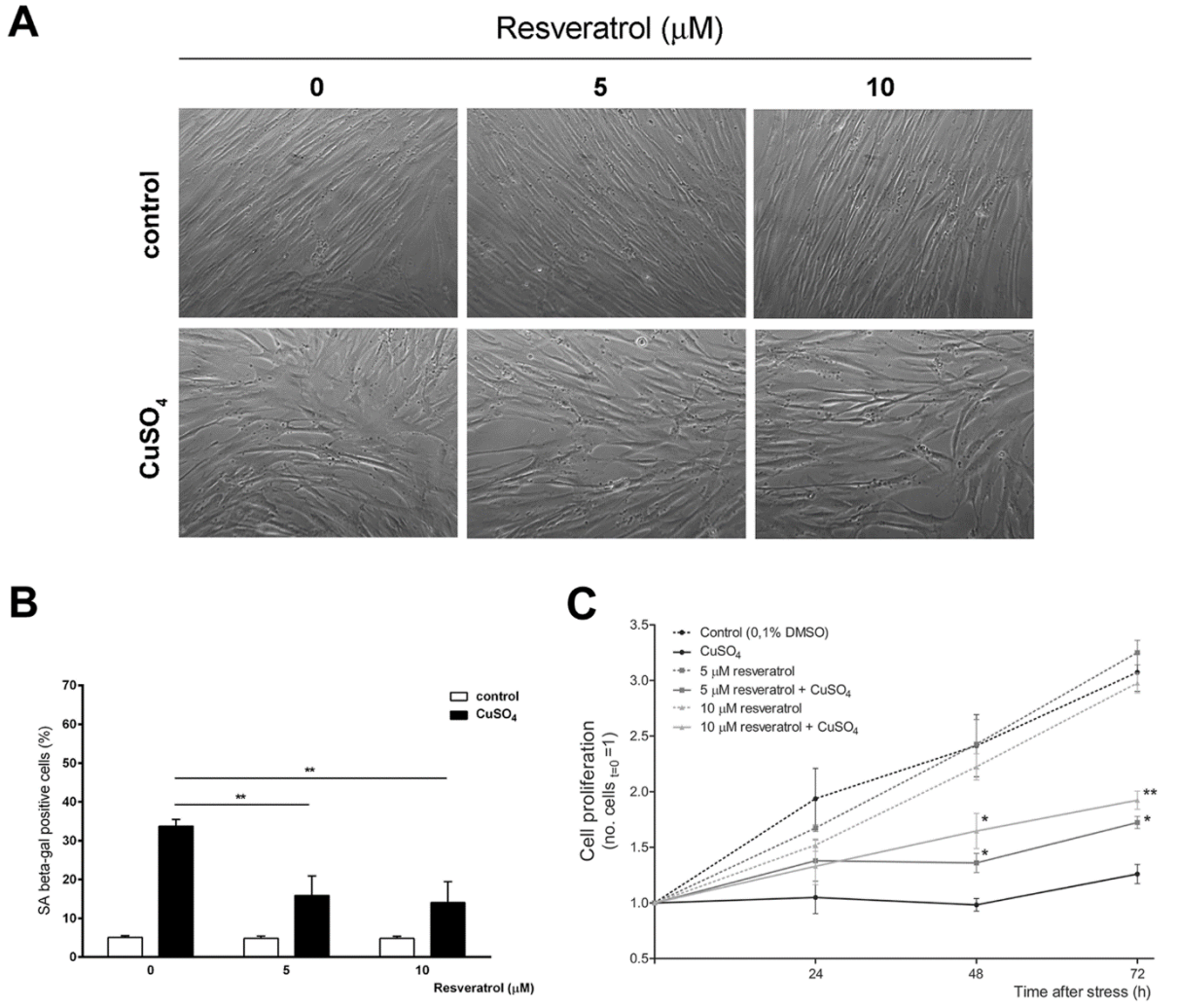

Figure 2: Resveratrol attenuates the appearance of typical senescence-associated features induced by $CuSO_4$. (A) Cell morphology was evaluated 72h after the removal of 350µM $CuSO_4$ (or Na_2SO_4, for controls) in fibroblasts that were allowed to recover in the presence or absence of 5 or 10 µM resveratrol. Representative images from the indicated conditions are depicted. **(B)** Senescence-associated beta-galactosidase (SA beta-gal) activity was detected 72h after $CuSO_4$ removal and the percentage of positive cells was calculated for each condition after counting a minimum of 400 cells/well. **(C)** Cell proliferation was assessed by counting the viable cells in a Neubauer chamber at different time-points after $CuSO_4$ treatment (0, 24, 48, and 72h). To facilitate direct comparison between the indicated conditions along time, the number of viable cells at day 0 was assumed as 1 for all treatments. Data represent mean ± SEM of at least three independent experiments.* $p<0.05$; and ** $p<0.01$, when compared to $CuSO_4$-treated cells without resveratrol at the respective time-point.

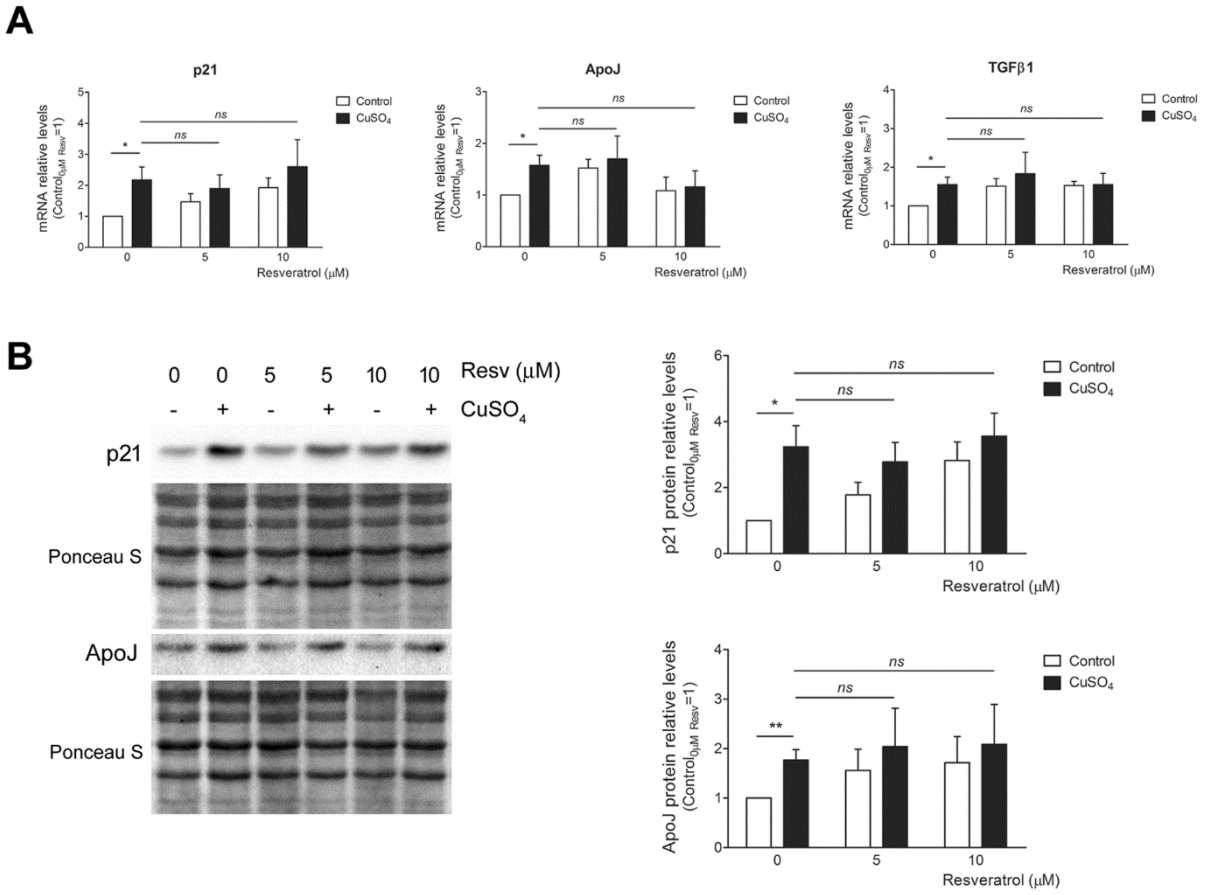

Figure 3: Resveratrol supplementation does not affect copper-induced expression of senescence-associated molecules. (A) Transcript relative levels of cyclin-dependent kinase inhibitor 1A (p21), apolipoprotein J (ApoJ) and transforming growth factor beta 1 (TGFβ1) were assessed by qPCR in 350 µM $CuSO_4$-treated fibroblasts that were allowed to recover in the presence of the indicated doses of resveratrol. **(B)** Representative blots obtained for the determination of p21 and ApoJ protein level by western blot are depicted; the resulting densitometric analysis, normalized for control cells in the absence of resveratrol, is plotted for each analysed protein. Ponceau S staining was used to control protein loading. Data represent mean ± SEM of at least three independent experiments.* $p<0.05$; ** $p<0.01$; and ^{ns} non-significant, for the comparisons between the indicated groups.

the mRNA transcript relative levels of these genes were quantified by qPCR (Fig. 3A). In accordance to previous publication (Matos *et al.* 2012), p21, ApoJ and TGFβ1 mRNA levels were found upregulated by 2.2-, 1.6- and 1.6-fold, respectively, in $CuSO_4$-SIPS fibroblasts when compared to control cells. However, the addition of resveratrol (either 5 or 10 µM) immediately after copper sulfate removal did not have any statistically significant effect on the transcript levels of these genes. To validate these results and exclude the occurrence of post-translational regulation, the relative protein levels pf p21 and ApoJ were evaluated by western blot (Fig. 3B). At the protein level, p21 and ApoJ presented a 3.2- and 1.8-fold increase in copper-treated cells, when compared to controls, thus confirming the previously noticed trend. In addition, similarly to transcript levels results, resveratrol supplementation did not affect copper-induced augmentation of these proteins. Overall, the effect of resveratrol in the attenuation of copper-induced senescence does not involve the regulation of p21, ApoJ and TGFβ1 senescence-associated genes.

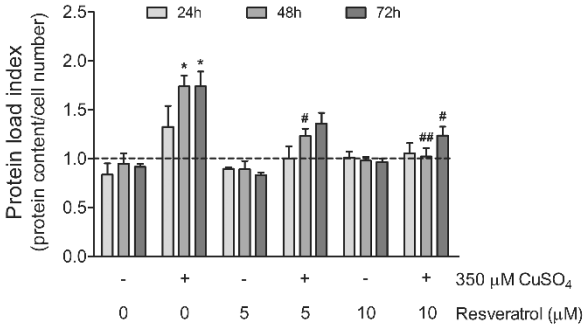

Figure 4: $CuSO_4$-induced proteostasis imbalance is attenuated by resveratrol. Protein load index (PLI), used as a measure of cellular protein accumulation, was calculated as the ratio between total protein content and cell number for each condition, at different time-points after $CuSO_4$ treatment (0, 24, 48 and 72h). PLI values were normalized for the initial time-point (0h) and the relative values are plotted for the indicated conditions. Data represent mean ± SEM of at least three independent experiments. * $p<0.05$, when compared to control cells in the absence of resveratrol; $^{\#}p<0.05$; and $^{\#\#}p<0.01$, relatively to $CuSO_4$-treated cells without resveratrol, at the respective time-points.

$CuSO_4$-induced proteostasis imbalance is attenuated by resveratrol

The occurrence of proteostasis imbalance is a major hallmark of aging (López-Ótin *et al.* 2013). To measure cellular protein accumulation for each experimental condition, the ratio between total protein content and cell number, here defined as the protein load index (PLI), was calculated at 0, 24, 48 and 72h after $CuSO_4$ removal (sodium sulfate for controls). Assuming that immediately after stress removal PLI equals 1, it was shown that it significantly increased 1.7-fold at 48 and 72h time-points in $CuSO_4$-SIPS cells when compared to the respective control conditions (Fig. 4). $CuSO_4$-treated fibroblasts, that were allowed to recover in the presence of 5 µM resveratrol, exhibited a statistically significant 0.5-fold decrease in PLI at 48h, when compared with cells without added resveratrol. Moreover, the addition of 10 µM resveratrol after copper removal totally reverted PLI to the level of controls in the absence of copper at 48h, and presented a statistically significant 0.5-fold decrease at 72h, when compared to copper-treated cells in the absence of resveratrol at the same time-point.

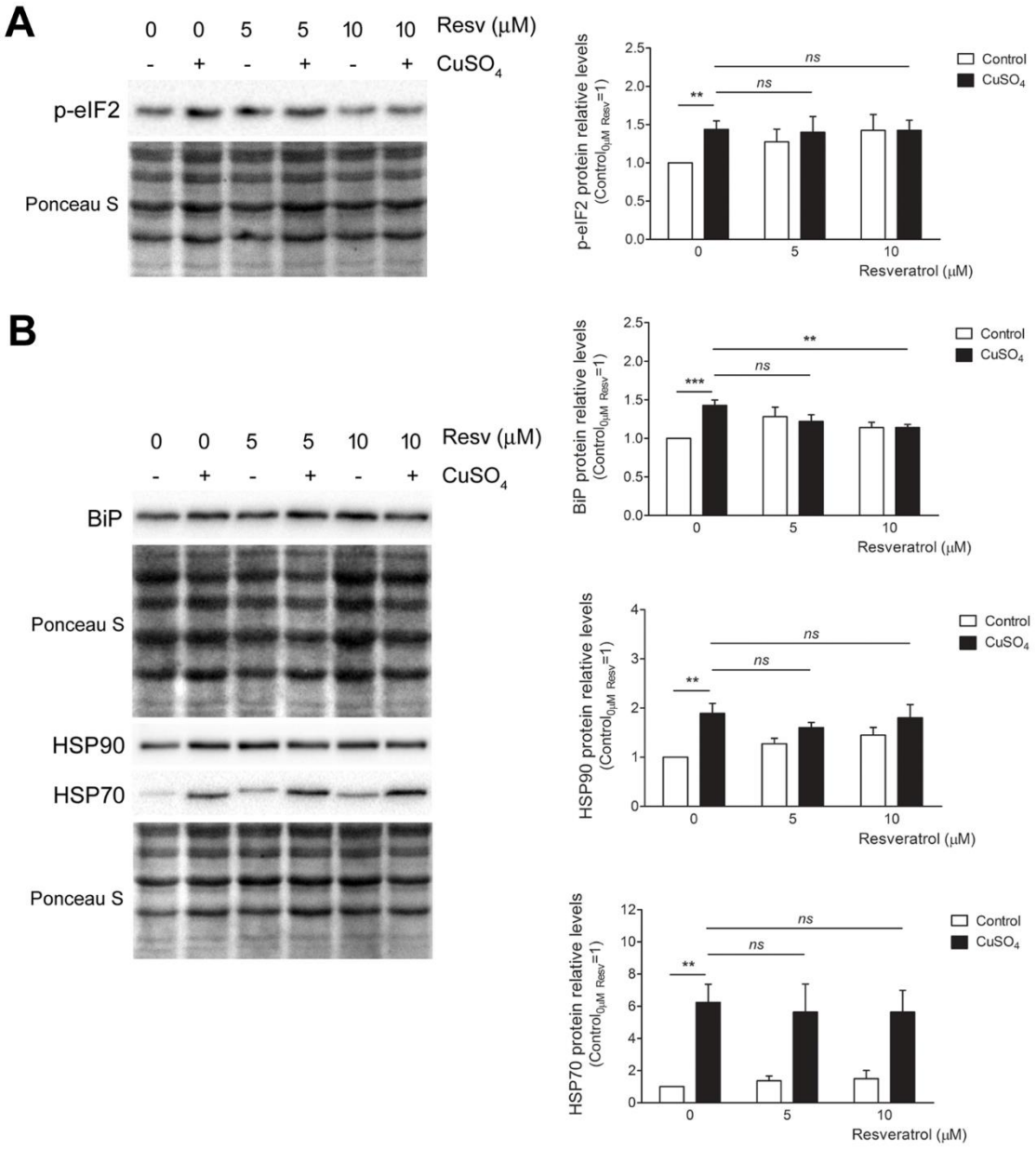

Figure 5: Resveratrol attenuates copper-induced BiP upregulation, but has no effect on eIF2 phosphorylation or HSP90 and HSP70 expression. (A) Phosphorylated eukaryotic translation initiation factor 2 (p-eIF2) and **(B)** immunoglobulin binding protein (BiP), heat shock protein (HSP) 90 and HSP70 protein relative levels were determined by western blot at 72h after the removal of 350µM $CuSO_4$ (or Na_2SO_4, for controls) in fibroblasts that were allowed to recover in the presence or absence of resveratrol (5 or 10µM). Representative blots are depicted and densitometric quantification is plotted assuming that protein levels of each analysed protein in control cells without resveratrol equals 1. Ponceau S staining was used to normalize protein loading. Data represent mean ± SEM of at least three independent experiments. ** $p<0.01$; *** $p<0.001$; and ^{ns} non-significant, for the comparisons between the indicated groups.

To compensate the altered proteostasis, $CuSO_4$-SIPS cells present higher levels of phosphorylated eukaryotic translation initiation factor 2 (p-eIF2) (Matos *et al.* 2014), which inhibits general protein translation and allows cells to restore homeostasis. A possible explanation for the diminished PLI obtained for copper cells recovering in the presence of resveratrol, could be an increased inhibition of overall protein synthesis caused by higher p-eIF2. When p-eIF2 was quantified by western blot (Fig. 5A), as expected it was found increased in $CuSO_4$-treated cells, compared to controls. However, resveratrol supplementation upon copper removal did not result in any additional alteration in p-eIF2 protein level. Next, cell chaperoning ability was evaluated by the quantification of the molecular chaperones immunoglobulin binding protein (BiP), heat shock protein (HSP) 90 and HSP70 by western blot (Fig. 5B). In fact, the intracellular protein levels of BiP, HSP90 and HSP70 were found 1.4-, 1.9- and 6.3-fold increased in $CuSO_4$-SIPS fibroblasts, when compared to control cells. The presence of resveratrol after copper removal had no effect on HSP90 and HSP70 protein levels comparing to the levels of copper-treated cells without resveratrol. However, BiP protein levels were diminished (to 1.1-fold) in copper-treated cells that were allowed to recover in the presence of 10 µM resveratrol, relatively to the condition without resveratrol, reflecting a lower need to buffer defective or damaged proteins.

Resveratrol attenuates $CuSO_4$-induced accumulation of modified proteins by the induction of lysosomal autophagy

The altered proteostasis observed in $CuSO_4$-SIPS fibroblasts could be a consequence of a progressive accumulation of oxidatively modified proteins. Protein carbonylation is a type of irreversible protein oxidation that is frequently used as an indicator of increased permanent levels of oxidative stress. Actually, cellular senescence models (Baraibar & Friguet 2013) and cells treated with oxidative stress inducers (Bollineni *et al.* 2014) were both shown to exhibit increased levels of carbonylated proteins. Herein, carbonyl protein content was evaluated to infer about cellular oxidative status in the different experimental conditions. $CuSO_4$-SIPS cells presented a statistically significant 13% increase ($p=0.0017$) in the relative levels of carbonylated proteins, when compared to control cells (Fig. 6A). The addition of 10 µM resveratrol during cell recovery (but not 5 µM) was able to attenuate such increase in protein oxidation by 34%, a variation that was close to reach statistical significance ($p=0.054$). These data suggest that resveratrol may be able to prevent or attenuate the accumulation of copper-induced oxidized proteins. This may be achieved either by its well described antioxidant properties that might prevent protein damage or by its ability to modulate protein degradation processes. UPS activity is known to be reduced during aging. The accumulation of poly-ubiquitinated (poly-Ub) proteins is usually associated with decreased UPS efficiency and, in fact, here it was observed a 22% increase in the levels of poly-Ub proteins in $CuSO_4$-SIPS fibroblasts (Fig. 6B). In addition, resveratrol supplementation (only at 10 µM) immediately after copper sulfate removal showed to be effective on restoring poly-Ub protein levels to the control cells ones, in a statistically significant manner ($p=0.026$).

Depending on the conformation of the poly-ubiquitin chain that they possess, poly-Ub proteins may be degraded either in the proteasome or by lysosomal macroautophagy (Kirkin *et al.* 2009), mentioned as autophagy from here on in order to simplify. Autophagy plays a crucial role in the recycling of dysfunctional organelles and damaged protein aggregates and it was shown to be induced by resveratrol in order to prevent oxidative stress cellular damage (Duan *et al.* 2013; Suzuki & Bartlett 2014). In the present study, the induction of autophagy was evaluated by the conversion of LC3-I to LC3-II, an essential step for autophagosome formation, by calculating the ratio of the LC3-II/LC3-I protein levels using western blot (Fig. 6C). $CuSO_4$-SIPS cells presented a statistically significant 1.4-fold increase in LC3-II/LC3-I ratio, when compared to young control fibroblasts. Furthermore, cell treatment with 10 µM resveratrol after copper removal, further increased this ratio (to 1.8-fold, $p=0.017$), when compared to copper-treated cells that were allowed to recover in the absence of resveratrol.

DISCUSSION

The $CuSO_4$-SIPS cellular model has proven to have major value for studying molecular events that are responsible for the aging process (Matos *et al.* 2012; Boilan *et al.* 2013; Matos *et al.* 2014). Furthermore, it brought additional evidence supporting copper contribution to the age-related functional deterioration and to the progression of age-related disorders. The present study shows that $CuSO_4$-induced cell senescence results in reduced Sirt1 expression. As Sirt1 is activated by the polyphenolic compound resveratrol, the mechanism and possibility of attenuating this senescent effect upon Sirt1 was addressed. In fact, it was demonstrated that resveratrol supplementation attenuated copper-induced appearance of some typical senescence features. In addition, the mechanism behind such anti-senescence effect of resveratrol was shown to involve the modulation of cellular proteostasis either by the protection of proteins from oxidative damage or by the induction of protein degradation processes.

The effect of resveratrol on cellular senescence has been investigated, but the results are contradictory: while some authors reported resveratrol ability to attenuate cellular aging (Demidenko & Blagosklonny 2009; Giovannelli *et al.* 2011; Ido *et al.* 2015), others showed that it induced the appearance of senescence (Rusin *et al.* 2009; Gao *et al.* 2011; Luo *et al.* 2013; Kilic Eren *et al.* 2015). In either case, the molecular mechanisms involved in such effects were not fully clear. We believe that this discrepancy is explained by

the different experimental conditions utilized in these studies: resveratrol ability to induce cell senescence was often reported using tumor cell lines (Rusin *et al.* 2009; Gao *et al.* 2011; Luo *et al.* 2013) treated with high concentrations of the compound (above 25 µM), that in some cases resulted in pro-apoptotic effects (Kilic Eren *et al.* 2015); in turn, anti-aging effects were described in non-tumor cell lines, incubated with lower doses of resveratrol (Giovannelli *et al.* 2011). In line with these evidences, herein, the administration of 5 or 10 µM resveratrol immediately after copper sulfate removal was able to attenuate the induction of WI-38 fibroblast cellular senescence, as the percentage of SA beta-gal positive cells was decreased, the typical

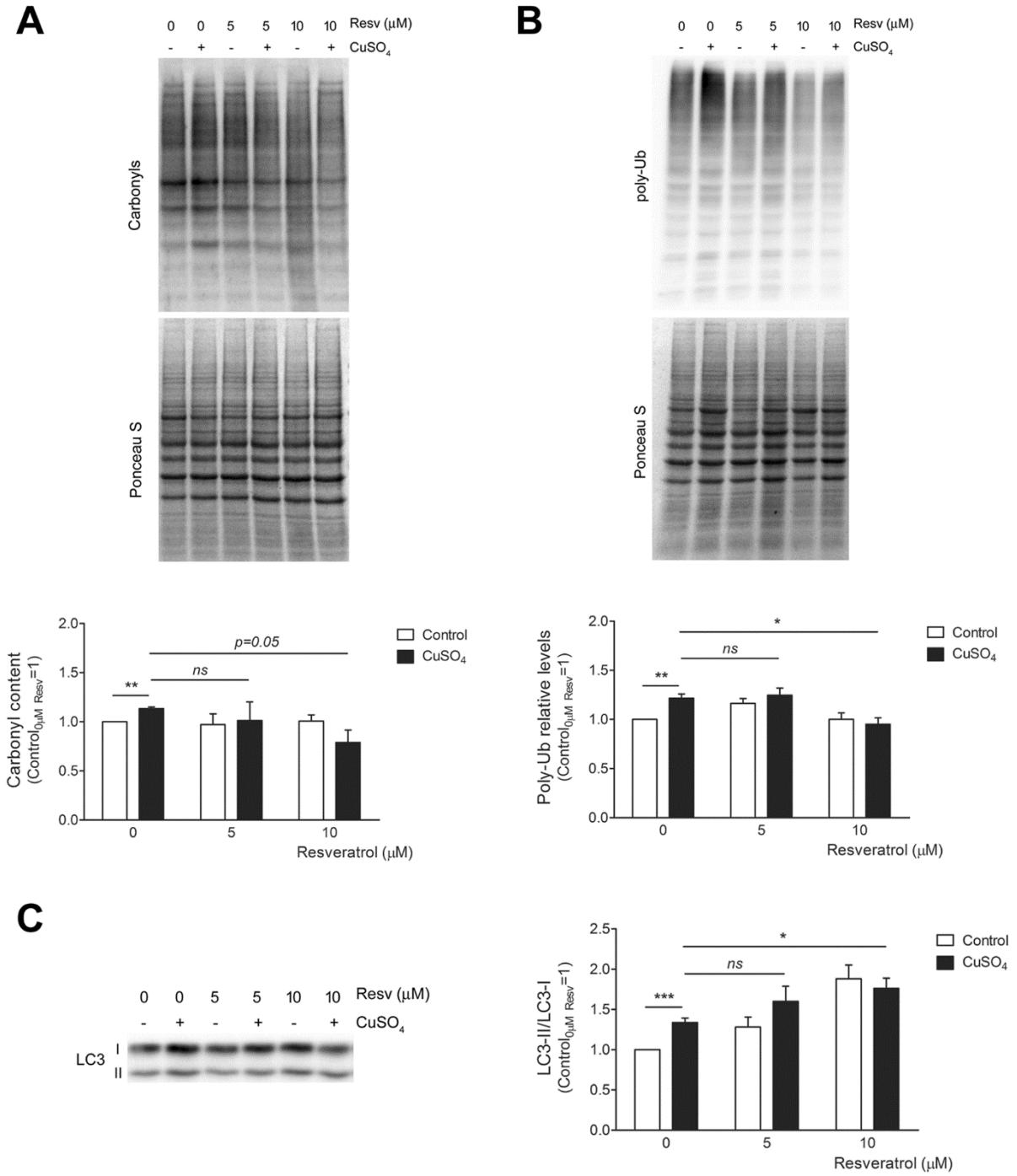

Figure 6: $CuSO_4$-induced accumulation of carbonylated and poly-ubiquitinated proteins are reduced by resveratrol, through lysosomal autophagy induction. (A) Protein carbonyl content and **(B)** poly-ubiquitinated (poly-Ub) proteins were evaluated in fibroblasts submitted to the indicated conditions by western blot. Representative blots are depicted and densitometric quantification was normalized by attributing the value 1 for control cells in the absence of resveratrol. Ponceau S staining was used as protein loading control. **(C)** Lysosomal autophagy was studied by the conversion of LC3-I to LC3-II, a critical step for autophagosome formation. LC3-II/LC3-I ratio was calculated upon densitometric quantification and plotted assuming that control cells without resveratrol present a value of 1. Data represent mean ± SEM of at least three independent experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ and ^{ns} non-significant, for the comparisons between the indicated groups.

morphological alterations were less evident and the blockage of cell cycle was alleviated. However, in this study, resveratrol was not able to attenuate copper-induced upregulation of senescence-associated molecules, such as p21, ApoJ and TGFβ1. This indicates that the mechanism behind the positive anti-senescence effects of resveratrol does not involve the inhibition of copper-induced expression of such senescence-associated genes.

It was recently reported that both RS and $CuSO_4$-SIPS models exhibit altered expression of several ER molecular chaperones and enzymes and activated ER UPR pathways (Matos *et al.* 2014). Here, $CuSO_4$-SIPS fibroblasts exhibited greater total protein content, increased expression of BiP, HSP70 and HSP90 molecular chaperones, a rise on the levels of carbonylated proteins and higher amount of poly-ubiquitinated proteins, adding further evidence to the occurrence of proteostasis disruption during senescence. In contrast, similar cells that were allowed to recover in the presence of resveratrol presented improved cellular proteostasis, as their total protein levels were similar to controls, BiP chaperone expression was attenuated and poly-ubiquitinated proteins level was reduced. Altogether, these data demonstrated that, in the presence of resveratrol, cells were able to circumvent copper-induced disruption of cellular proteostasis, intimately related to the appearance of the typical senescent phenotype.

The well documented antioxidant properties of resveratrol are the likely contributors to this cell proteostasis maintenance effect, as it can protect proteins from being oxidized in a concentration and time-dependent manner. In fact, using *in vitro* oxidative stressed erythrocytes, resveratrol ability to prevent protein oxidation reaches a maximum protective effect between 30 and 60 minutes after polyphenolic compound addition and is then slightly reduced with time (Pandey & Rizvi 2009). In the current study, resveratrol supplementation for 72h attenuated the amount of carbonylated proteins on copper-treated cells in a variation that was close to reach statistical significance. A time-course evaluation of protein carbonylation along these 72h should add further information on the existence of time-dependent variations on resveratrol efficiency to protect proteins from oxidation.

Another important resveratrol contribution for the modulation of cellular proteostasis is its ability to regulate protein degradation mechanisms, such as the UPS (Sato *et al.* 2013; Regitz *et al.* 2015) or lysosomal autophagy (Duan *et al.* 2013; Suzuki & Bartlett 2014). Both mechanisms were shown to be intimately related as autophagy is activated to compensate UPS inhibition (Wojcik 2013). In brief, autophagy is crucial to degrade dysfunctional organelles and damaged protein aggregates and involves the formation of autophagosomes that are targeted to lysosomes for the degradation of their inner content. Autophagosome formation occurs in successive stages that depend on the concerted action of several proteins (Tanida 2011). The cytosolic soluble protein LC3-I is particularly important in this process because it is lipidated to originate LC3-II, which integrates the autophagosome membrane. As such conversion is essential for elongation and maturation of the autophagosomes, LC3-II/LC3-I ratio is usually used to detect autophagy activation. Here, $CuSO_4$-SIPS cells exhibited an increase in LC3-I to LC3-II conversion; when allowed to recover in the presence of resveratrol LC3-II/LC3-I ratio was even higher, indicating an enhanced induction of autophagy. These results are in agreement with previous *in vitro* (Lv & Zhou 2012) and *in vivo* (Duan *et al.* 2013) studies demonstrating that oxidative stress conditions promote LC3-II/LC3-I ratio increase, further enhanced in the presence of resveratrol. Moreover, resveratrol has recently been described as able to promote the flux of proteins through the autophagosomal-lysosomal pathway, thus attenuating the dysfunctional effect of intracellular accumulation of damaged or defective proteins (Regitz *et al.* 2015). This promotion is in agreement with the results of the current study that favor resveratrol anti-senescence effect consequent to its ability to improve cellular proteostasis through autophagy induction. However, given the proven crosstalk between autophagy and proteasomal degradation (Ding *et al.* 2003), we cannot exclude, in addition, the beneficial effects resulting from resveratrol ability to modulate the ubiquitin-proteasome system.

In summary, this study demonstrates that resveratrol is able to attenuate the induction of cell senescence consequent $CuSO_4$ exposure. Such effects result from resveratrol ability to promote cellular adaptive mechanisms, as autophagy upregulation, that sustain cellular proteostasis and confer cellular resistance to stress. Cellular proteostasis maintenance was found to be crucial to prevent the development of the senescent phenotype. These data also uncovers molecular targets whose modulation is likely to prevent age-associated cell and tissue function deterioration and improve human healthspan.

EXPERIMENTAL PROCEDURES

Cell culture

WI-38 human fetal lung fibroblasts were purchased from The European Collection of Cell Cultures (ECACC) and were cultivated in complete medium composed of Basal Medium Eagle (BME) supplemented with 10% fetal bovine serum, at 37°C in a 5% CO_2 humidified atmosphere. WI-38 cells are considered to be young below 30 population doublings (PDs) and enter senescence at 45 PDs or above. For the induction of SIPS with copper sulfate ($CuSO_4$-SIPS), subconfluent young WI-38 fibroblasts were exposed to 350 µM $CuSO_4$ (Na_2SO_4 for controls) for 24h. Then, cells were washed once with phosphate buffered saline (PBS) and replaced with fresh complete medium containing 5 or 10 µM of resveratrol (R5010-Sigma Aldrich®) for an additional 72h period. Control cells

were submitted to a final concentration of 0.1% DMSO for the same period.

Cell morphology and SA beta-gal detection

Cell morphology evaluation was performed 72h after copper removal by optical inspection using an inverted microscope. To assess the presence of senescent cells, SA beta-gal was detected 72h after copper removal as already described (Matos *et al.* 2012). The percentage of SA beta-gal positive cells in each condition was determined by microscopically counting 400 total cells/well from at least three independent experiments.

Cell proliferation and total protein content

To assess the effect of the different treatments on cell proliferation and total protein content, cell number determination and SRB assay (Vichai & Kirtikara 2006) were performed along time after copper removal. Briefly, 3000 cells/well were seeded in 96-well culture plates, treated for 24h with $CuSO_4$ (or Na_2SO_4 for controls) and then were analyzed at different time points (0, 24, 48 and 72h) while recovering in the presence or absence of resveratrol. For cell number determination, cells were tripsinized, stained with Tripan Blue, and the viable cells were microscopically counted in a Neubauer chamber. The total number of cells per well for each condition at the different time-points was calculated and plotted, assuming that at t=0, for each condition, cell number equals 1. For the total protein content determination, cells were treated with 10% trichloroacetic acid (TCA), 1 hour at 4°C. The TCA-precipitated proteins fixed at the bottom of the wells were stained for 30 minutes with 0.057% (w/v) SRB in 1% acetic acid solution and then washed four times with 1% acetic acid. Bound dye was solubilized with 10 mM Tris base solution and the absorbance at 510 nm of each well was recorded using a microplate reader (Infinite 200 - TECAN).

Real time PCR

Gene expression experiments were performed 72h after copper sulfate treatment by real time quantitative PCR (qPCR). Total RNA extracted (PureLink® RNA Mini Kit, Ambion) from cells derived from at least three independent cultures from each condition was converted into cDNA by reverse transcription reaction. Amplification reaction assays contained SYBR Green Mastermix (SYBR® Select Master Mix, Applied Biosystems®), 50 ng cDNA and primers (STAB VIDA, Lda.) at optimal concentrations. The primer sequences were: p21, 5'-CTGGAGACTCTCAGGGT CGAA and 5'-CCAGGACTGCAGGCTTCCT; ApoJ, 5'-GGATGAAGGACCAGTGTGACAAG-3' and 5'-CAGCGACCTGGAGGGATTC-3'; TGFβ1, 5'-AGG GCTACCATGCCAACTTCT-3' and 5'-CCGGGTTA TGCTGGTTGT ACA-3'; and TATA box binding protein (TBP), 5'-TCAAACCCAGAATTGTTCTCCT TAT-3' and 5'-CCTGAATCCCTTTAGAATAGGG TAGA-3'. The protocol used for qPCR was: 95°C (3 min); 40 cycles of 95°C (15 sec) and 60°C (1 min). qPCR was performed in the StepOnePlus™ thermal cycler (Applied Biosystems™). TBP was the selected housekeeping gene when calculating relative transcript levels of the target genes.

Western blot

Protein levels were assessed 72h after copper sulfate exposure by Western blot analysis. WI-38 cells submitted to the different treatments were washed with PBS and scrapped on ice in a lysis buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0,1% Triton X-100) supplemented with protease inhibitors cocktail (Sigma-Aldrich®). Upon Bradford assay, 20 µg (or 10 µg for de detection of carbonylated or poly-Ub proteins) of protein from each cell extract were resolved by SDS-PAGE. Proteins were blotted into a nitrocellulose membrane and, after blocking with 5% non-fat dry milk diluted in Tris-buffered saline- 0,1% tween 20 (TBST), were probed with specific primary antibodies (anti-HSP90 ab13495, abcam®; anti-LC3 NB100-2220, Novus Biologicals; anti-ubiquitin PW0930, Enzo® Life Sciences; anti-p21 #2946, anti-phospho-eIF2 #3398, anti-HSP70 #4876 and anti-BiP #3177, Cell Signaling Technology®) overnight at pre-determined optimal dilutions. For the specific detection of carbonylated proteins, immediately after protein transfer, the nitrocellulose-bound proteins were treated as described elsewhere (Castro *et al.* 2012). Briefly, the membranes were equilibrated in 20% methanol in TBS, washed for 5 min with 10% trifluoroacetic acid (TFA), derivatized with 5 mM 2,4-dinitrophenylhydrazine (DNPH, Sigma-Aldrich®) diluted in 10% TFA for 10 min (protected from light), washed with 10% TFA to remove the excess of DNPH, and finally washed with 50% methanol. Following this procedure, the membranes were blocked with 5% bovine serum albumin in TBST and incubated with primary anti-DNP antibody (D9656, Sigma-Aldrich®). From here on, the western blot procedure was similar to all antibodies: after TBST washing, immunoblots were incubated with the appropriate peroxidase-conjugated secondary antibodies for 1h, detected using ECL western blotting substrate (Pierce™ - Thermo Scientific) and visualized in ChemiDocTM XRS (BioRad Laboratories). Results were quantified by densitometry using the Image Lab® software. Protein loading was normalized using Ponceau S protein staining, but similar data were also obtained using tubulin detection (data not shown).

Statistical analysis

Student's t-test was used to compare the means between two different conditions. A p-value lower than 0.05 was considered statistically significant.

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AUTHOR CONTRIBUTIONS

HA and AMG designed the study, analyzed the data and reviewed the manuscript. LM designed and performed the experiments, analyzed the data and wrote the manuscript.

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III

Discussion

The involvement of copper in ageing has long been suggested, since alterations on copper homeostasis leading to the increase of its levels have been described along ageing and in age-associated diseases (Harman, 1965, Sohler and Pfeiffer, 1987, Jimenez-Jimenez *et al.*, 1992, Brewer *et al.*, 2010). However, the actual role of copper in the age-associated progressive deterioration has remained unclear, although it was believed to mediate ROS generation, resulting in increased oxidative stress and its harmful impacts on cellular function.

Departing from this idea, it was hypothesized that copper-induced oxidative stress would be able to induce premature senescence in human fibroblasts in culture. In fact, for the first time, it is shown here that WI-38 fibroblasts exposed to a subcytotoxic concentration of copper sulfate exhibit a set of typical senescence features that resulted in the establishment and characterization of a novel SIPS model, the $CuSO_4$-SIPS (**Publication I**). In addition, this cellular model was used to unravel molecular events behind copper contribution for cellular senescence; these were shown to involve proteostasis disruption (**Publication II and III**) and the activation of UPR pathways (**Publication II**). Further supporting these findings, the induction of autophagy, and consequent improvement of cellular proteostasis by resveratrol, was found to attenuate copper-induced cellular senescence and provided further insights on cell response to proteostasis impairment (**Publication III**).

MAJOR GOALS IN AGEING RESEARCH

Ageing has been subjected to extensive research in an attempt to clearly determine its underlying molecular and cellular events. As it is characterized by a progressive physiological decline and increased susceptibility to the establishment of chronic diseases, understanding the ageing process will certainly have profound implications for the quality of human health. Indeed, the current paradigm of ageing research emphasizes that instead of trying to find illusory anti-ageing treatments to prolong longevity, it essentially aims at developing realistic methods to extend healthspan, i.e. to ameliorate health throughout lifespan and delay the development of diseases. Generally, efforts to improve healthspan implicate the attenuation of biological processes known to contribute to ageing, such as mitochondrial dysfunction, increased oxidative stress, impaired proteostasis and increased senescence.

Given the proved contribution of increased oxidative stress to ageing, the use of antioxidants has been tested in an attempt to increase healthspan and delay ageing. However, evidence from studies using several organism models from yeast to rodent mammals are diverse and often contradictory (*as reviewed in:* Sadowska-Bartosz and Bartosz, 2014). In addition, a meta-analysis of randomized trials involving more than 230,000 participants consuming diverse antioxidants, namely vitamin A, vitamin C, vitamin E, beta-carotene or selenium, either individually or in combination, failed to uncover longevity benefits from such supplementation and led authors to conclude that some antioxidants could actually be related to increased mortality (Bjelakovic *et al.*, 2007). The ability of dietary antioxidant supplementation to delay or prevent the appearance of age-associated disorders, such as age-related macular degeneration, atherosclerosis and Alzheimer disease, was also investigated but resulted in contradictory findings as well (Chong *et al.*, 2007, Fusco *et al.*, 2007). Altogether, existing data do not support the recommendation of diet supplementation with antioxidants as a tool to delay or prevent the appearance of age-related pathophysiological alterations and improve healthspan. Moreover, beyond the logical concerns about their actual efficacy, it is also critical to assure their safety.

An alternative to circumvent the lack of consistency obtained from the studies with antioxidants may be the use of more specific and site-directed approaches, aiming at preventing the age-related deterioration of cellular and tissue function. The strategy is to foster the knowledge on mechanisms and molecules that contribute to age-related functional decline, to identify specific targets and to develop suitable pharmaceutical compounds.

The accumulation of senescent cells *in vivo* is believed to add to the age-related functional deterioration, as they may affect tissue homeostasis and function. In contrast, the selective elimination of *in vivo* accumulating senescent cells has been described to delay the progression of age-associated disorders and extend healthspan in mice (Baker *et al.*, 2011). More recently, a new class of drugs termed senolytic agents, i.e. compounds that specifically eliminate senescent cells *in vivo*, have been investigated. Actually, the use of desatinib and quercetin in combination was able to reduce the appearance of senescent cells and to improve healthspan in chronologically aged and a progeroid strain of mice (Zhu *et al.*, 2015). Briefly, in this latter study, desatinib and quercetin were selected from a pool of several candidate drugs that target anti-apoptotic molecules that were shown to be upregulated in senescent cells, such as the ephrins (EFNB1 or 3) and phosphoinositide 3-kinase delta catalytic subunit (PI3Kδ). Desatinib is an inhibitor of various tyrosine kinases that is used for treating cancers (Montero *et al.*, 2011), and interferes with EFNB-dependent suppression of apoptosis (Chang *et al.*, 2008), while quercetin is a natural flavonol known to inhibit PI3K and other kinases (Bruning, 2013). In combination, these two drugs proved to be effective to eliminate senescent cells and delay the appearance of age-related deterioration symptoms and pathological conditions in mice.

Thus, as eliminating senescent cells *in vivo* is a promising approach to improve human healthspan, finding molecules that actively intervene in cellular senescence induction is crucial to develop specific and site-directed senolytic compounds. As an example, an exhaustive proteomic study of the $CuSO_4$-SIPS model or other, should add valuable information on the differential protein expression profile of senescent cells and provide hints on the cellular pathways involved. In fact, previous studies in RS fibroblasts identified expression changes in components of the cytoskeleton, modulators of energy production and metabolism, and proteostasis regulators (Trougakos *et al.*, 2006, Aan *et al.*, 2013). However, recent technologic advances, employing other proteomic approaches, allowed a deeper characterization of cellular proteome (Calabrese *et al.*, 2015), that in RS endothelial cells, yielded a significantly higher amount of proteins differentially expressed in senescence (Yentrapalli *et al.*, 2015).

A similar proteomic study to compare results obtained with RS cells and $CuSO_4$-SIPS fibroblasts might bring further insights on this issue. These include the identification of senescence pathways and the evidence of proteins that are exclusive to senescent cells membranes. In addition, considering the altered ER proteostasis that accompanies cellular senescence (**Publication II and III**), the study may be used in the assessment of likely glycosylation errors occurring in the ER or the Golgi complex, purposed to identify proteins with altered glycan branches, having potential biomarker properties. This possibility is being explored for tumor cells that secrete or express proteins with aberrant glycosylation patterns at their surface (Tuccillo *et al.*, 2014).

COPPER AND AGEING

Copper levels have been repeatedly shown to increase with ageing (Harman, 1965, Sohler and Pfeiffer, 1987) and in age-associated diseases (Jimenez-Jimenez *et al.*, 1992, Brewer *et al.*, 2010). Moreover, tissues from aged individuals or from patients with age-associated diseases have been shown to accumulate senescent cells (Dimri *et al.*, 1995, Paradis *et al.*, 2001, Minamino *et al.*, 2002, Sone and Kagawa, 2005, Gorgoulis *et al.*, 2005, Ressler *et al.*, 2006, Erusalimsky, 2009, Babizhayev *et al.*, 2011, Benisch *et al.*, 2012, Markowski *et al.*, 2013, Malavolta *et al.*, 2013). Such cells remain metabolically active and, as they persist and accumulate along time, can affect their surrounding environment and lead to tissue dysfunction (Parrinello *et al.*, 2005, Coppe *et al.*, 2008, Acosta *et al.*, 2013). In the present work, copper is shown to promote cellular senescence in human fibroblasts (**Publication I**) and foster UPR response through molecular mechanisms similar to RS (**Publication II**).

It is conceivable that the enhanced levels of copper along the organism ageing and the consequences on cell cultures here reported extrapolate to the *in vivo* condition, and thus contribute to the enhanced percentage of senescent cells within tissues. In turn, the accumulation of such copper-induced senescent cells would impact on tissue function and lead to the age-related gradual functional deterioration and increased susceptibility to

illness. Actually, there is some evidence supporting such hypothetical *in vivo* copper contribution to ageing.

Wilson disease (WD) is an autosomal recessive disorder characterized by an increased deposition of copper in the liver that is caused by mutations in ATP7B gene, which encodes a P-type adenosine triphosphatase (ATPase) that is responsible for moving copper across hepatocyte intracellular membranes and for its excretion to the bile. In case of mutations in ATP7B, these functions are affected and circulating copper levels are found decreased while copper accumulates within the hepatocytes. When the hepatocellular storage capacity is exceeded, copper is slowly released into circulation and deposited in multiple organs, particularly the brain, the eyes and the kidneys (De Bie *et al.*, 2007). Therefore, the clinical manifestations of WD may include hepatic abnormalities, ophthalmic changes, neurologic defects and psychiatric alterations (Ala *et al.*, 2007). Using liver biopsies of WD patients, the occurrence of premature, age-related mitochondrial DNA oxidation was reported as consequent to the pro-oxidant effect of accumulating copper within the hepatocytes (Mansouri *et al.*, 1997). A similar copper-induced ageing effect was verified at the organismal level, as flies chronically exposed to copper exhibited neurologic alterations and decreased lifespan when compared to controls (Arcaya *et al.*, 2013).

Adding support to copper contribution for age-related functional decline, the involvement of copper in age-associated diseases has also been explored. In fact, both type II diabetes and AD are conditions usually associated with systemic copper excess (Eaton and Qian, 2002, Squitti *et al.*, 2005, Brewer *et al.*, 2010), although the mechanisms causing such imbalance are not clear. Thus, the use of therapeutic strategies intended to reduce circulating copper levels was proposed to prevent or delay health deterioration in these two diseases (Cooper *et al.*, 2004, Cooper, 2012, Squitti *et al.*, 2014, Robert *et al.*, 2015).

Despite the well documented detrimental effects of excess copper, its depletion greatly impacts on cellular functions as well. In fact, copper is crucial for the activity of several enzymes with important functions, such as ceruloplasmin, copper and zinc containing SODs and cytochrome c oxidase (as reviewed in: Vest *et al.*, 2013). Menkes disease is characterized by systemic copper deficiency and usually results in severe neurological symptoms and early death. In these cases copper supplementation has been shown to be effective in extending lifespan although not always being able to avoid neurologic impairment (Christodoulou *et al.*, 1998, Munakata *et al.*, 2005, Lenartowicz *et al.*, 2010).

Altogether, these data bring relevance to the role of copper in the modulation of critical cellular functions, which may ultimately impinge on organismal healthspan and longevity. Thus, the novel $CuSO_4$-SIPS model here presented constitute a valuable experimental tool to characterize copper-mediated molecular mechanisms and to identify targets liable to be modulated hoping to prevent or attenuate age-associated cellular dysfunction.

LOSS OF PROTEOSTASIS AND THE AGEING PROCESS

Understanding the complex molecular events that intervene in the ageing process could have profound implications for the quality of human health. Both health- and lifespan depend on the ability of cells and tissues to adapt, resist and maintain their functions upon age-related stressful conditions. However, cellular stress responses are often compromised in ageing and so, they frequently associate with the onset of age-associated pathologies. Together, the occurrence of oxidative damage upon cellular proteins (Levine and Stadtman, 2001, Hipkiss, 2006) combined with the decreased efficiency of cellular chaperoning systems (Macario and Conway De Macario, 2002) result in loss of proteostasis, pointed out as a major hallmark of ageing and several age-associated diseases (López-Ótin *et al.*, 2013). In addition, it was suggested that proteostasis disruption represents an early molecular event in *C. elegans* ageing (Ben-Zvi *et al.*, 2009) and is part of a programmed proteostasis network remodeling, instead of being a purely stochastic phenomenon (Labbadia and Morimoto, 2015). Therefore, unraveling the specific molecular mechanisms that contribute to proteostasis disruption during ageing may shed light on potential therapeutic targets aiming to delay age-associated health deterioration.

The results here presented add information on the involvement of protein quality control cellular processes that result in cellular senescence establishment. In fact, cellular proteostasis is altered in $CuSO_4$-SIPS fibroblasts as they exhibit the activation of UPR pathways (**Publication II**) and the increase of total protein content (**Publication III**). Also shown is that the induction of cellular senescence requires copper-induced cell cycle arrest dependently on PERK activation (**Publication II**). In accordance, at the organismal level (*C. elegans and D. melanogaster*) it has been demonstrated that the specific regulation of UPR cellular pathways improves healthspan and leads to lifespan extension (Taylor and Dillin, 2013, Wang *et al.*, 2015). Moreover, the increased expression of molecular chaperones usually correlate with increased stress resistance and extended lifespan in worms (Walker and Lithgow, 2003, Hsu *et al.*, 2003) and flies (Morrow *et al.*, 2004), while the impairment of protein quality control systems result in accelerated ageing in mice (Min *et al.*, 2008). Likewise, improvement of proteostasis by the use of autophagy or proteasome activators was also able to extend longevity in yeast, worms and flies (Eisenberg *et al.*, 2009, Bjedov *et al.*, 2010, Liu *et al.*, 2011, Vilchez *et al.*, 2012) and to delay ageing functional failure in mice (Zhang and Cuervo, 2008).

In the current study, the use of resveratrol is shown to ameliorate cellular proteostasis in $CuSO_4$-fibroblasts by the activation of lysosomal autophagy, resulting in the attenuation of cellular senescence (**Publication III**). In line with these findings, there are studies reporting the ability of resveratrol to regulate protein degradation mechanisms, such as lysosomal autophagy (Duan *et al.*, 2013, Guo *et al.*, 2013, Suzuki and Bartlett, 2014, Dutta *et al.*, 2014) and the ubiquitin-proteasome system (Marambaud *et al.*, 2005, Niu *et al.*, 2011, Sato *et al.*, 2013, Chothe and Swaan, 2014, Regitz *et al.*, 2015). Understanding the exact mode of action of resveratrol on such protein quality control mechanisms is important

to identify specific ways to modulate the molecular targets involved in the ageing process. Here, the $CuSO_4$-SIPS model revealed to be of most value to unravel such molecular interveners, as it mimics the molecular events regarding the alterations on cellular proteostasis that occur in RS. In contrast, the H_2O_2-SIPS model, which is the most frequently utilized for these studies, was shown to exhibit a different molecular profile even though it presents senescent phenotype alterations that resemble RS (**Publication II**).

The occurrence of cellular proteostasis disruption in ageing and age-associated disorders has been repeatedly demonstrated and, in part, it may be a consequence of the age-related progressive loss of efficiency of molecular chaperoning and cellular protein degradation mechanisms. The work presented in this thesis confirms the involvement of proteostasis disruption in the induction of cellular senescence and shows that improving cellular proteostasis through autophagy activation attenuates the appearance of some typical senescence features (**Publication III**).

The findings support the view that activation of protein degradation mechanisms is a promising approach to delay ageing and improve healthspan (Rubinsztein *et al.*, 2011, Chondrogianni *et al.*, 2015). Such approach may lead to a general improvement of cellular and tissue proteostasis and, in line with the findings of the present work, it may be able to prevent the appearance and accumulation of senescent cells in organismal tissues.

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