## Cellular senescence and protein degradation

## Breaking down cancer

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utophagy and the ubiquitin-proteasome pathway (UPP) are the major protein degradation systems in eukaryotic cells. Whereas the former mediate a bulk nonspecific degradation, the UPP allows a rapid degradation of specific proteins. Both systems have been shown to play a role in tumorigenesis, and the interest in developing therapeutic agents inhibiting protein degradation is steadily growing. However, emerging data point to a critical role for autophagy in cellular senescence, an established tumor suppressor mechanism. Recently, a selective protein degradation process mediated by the UPP was also shown to contribute to the senescence phenotype. This process is tightly regulated by E3 ubiquitin ligases, deubiquitinases, and several posttranslational modifications of target proteins. Illustrating the complexity of UPP, more than 600 human genes have been shown to encode E3 ubiquitin ligases, a number which exceeds that of the protein kinases. Nevertheless, our knowledge of proteasome-dependent protein degradation as a regulated process in cellular contexts such as cancer and senescence remains very limited. Here we discuss the implications of protein degradation in senescence and attempt to relate this function to the protein degradation pattern observed in cancer cells.

## Introduction to Cellular Senescence

The long lifespan and constant cell turnover of complex organisms pose the

challenge of dealing with the inevitable accumulation of DNA damage and gene mutations that drive carcinogenesis. Fortunately, multiple mechanisms have evolved to detect DNA aberrations and oncogenic stress and protect against the initiation and progression of neoplastic growth. Among these, cellular senescence is a stable cell cycle arrest triggered by a variety of insults including short telomeres, activated oncogenes, DNA damage, and reactive oxygen species.<sup>1</sup> However, how these stresses converge to regulate a common cellular state is not currently well understood. Senescence is a complex multifaceted cellular phenotype, without an exclusive hallmark, with a broad range of proposed effector mechanisms, and, still, with an ambiguous definition. Indeed, different senescent cells are characterized by a wide range of biomarkers (reviewed in refs. 1 and 2), many of which are neither exclusive to senescence nor universally present in senescent cells. Because of this phenotypic heterogeneity and often imprecise definition, the assessment of senescence should be carefully addressed and should attempt to rigorously define a combination of senescence-associated features. Moreover, it needs to be recognized that this diversity in the phenotypic traits could reflect a concomitant heterogeneity at the level of the effector programs.

At the molecular level, senescence triggers important changes in gene expression patterns, but there is little overlap between different cell types.<sup>3</sup> For example, a comparison between young and senescent human fibroblasts and mammary epithelial cells (HMEC) revealed a transcriptional fingerprint unique to senescence, but limited similarity between the 2 cell lineages.<sup>4</sup> Other gene expression analyses have revealed a proinflammatory gene profile in senescent cells under the regulation of the NF-KB transcription factor<sup>5-7</sup> or a downregulation of E2F target genes under the regulation of the retinoblastoma tumor suppressor (RB).<sup>8,9</sup> However, cells with inactivation in NF-KB or RB can senesce in response to multiple stressors,<sup>5-9</sup> indicating that the programs they control are not essential for the initiation of the process. Several target genes of the tumor suppressor p53 (TP53) were also reported to mediate senescence, such as p21 (CDKN1A),<sup>10-12</sup> the tumor suppressor promyelocytic leukemia (PML),<sup>13,14</sup> the plasminogen activator inhibitor-1 (PAI-1),15 DEC1, and E2F7.16 Again, a p53-dependent transcriptional pattern is not a prerequisite for senescence, and its relative contribution to the process depends on the cell type and the status of the p16<sup>INK4A</sup>–RB pathway.<sup>17</sup> Our current knowledge thus suggests that senescence is consistent with distinct gene expression profiles and a variety of effector mechanisms, depending on the triggers, cell types, and cellular context.

Beyond transcriptional regulatory networks that characterize senescence, direct control of protein levels also appears strikingly affected. This involves the regulation of mRNA translation and protein stability of specific senescence mediators, such as p5318-20 and PML.21-23 In addition, it is thought that a global upregulation of translation may contribute to senescence, since the key regulator of protein synthesis, mTOR, has been shown to favor senescence in different contexts1 and total protein synthesis is increased in Rasinduced senescent cells.<sup>24</sup> Similarly, a more general function of protein degradation now emerges as critical to reorganize the proteome of cells undergoing senescence. Here, we will discuss the impact of protein degradation on the senescence-associated proteome and how this mechanism could contribute to the onset of cellular senescence. Thus, we will effectively address the question: how does a pre-neoplastic cell destroy the machinery required for its subsequent progression to a cancer?

## Protein Degradation and Senescence

The lysosomal degradation pathway is the principal system used by eukaryotic cells to degrade and recycle cytosolic components and organelles. A cytoplasmic cargo is engulfed into vesicles and delivered to the lysosome by the process of autophagy, which can be divided into 3 classes: (1) chaperone-mediated autophagy; (2) microautophagy; and (3) macroautophagy.<sup>25</sup> The latter is mainly a nonspecific cytoplasmic degradation mechanism that has been shown to support tumorigenesis in Ras-expressing cancer cells,<sup>26</sup> pancreatic tumors,<sup>27</sup> lymphomas,<sup>28</sup> and breast cancer.29 Macroautophagy is required to eliminate abnormal mitochondria, reduce the production of reactive oxygen species and replenish tricarboxylic acid (TCA) cycle metabolites.<sup>26,27</sup> Given its catabolic capacity, macroautophagy improves the survival of both normal and cancer cells under metabolic stress by maintaining the availability of building blocks in order to preserve essential cellular functions.<sup>30</sup>

It is now appreciated that in addition to supporting cell viability in established tumors, macroautophagy has context-specific tumor-suppressor functions. The first evidence of such a function came from the discovery that the haploinsufficiency of the autophagy-related gene Beclin1 (BECN1) leads to cancer predisposition in mice.<sup>31</sup> Moreover, many effectors of macroautophagy, including Atg5,32 Atg7,32,33 Atg4C,<sup>34</sup> Bif-1<sup>35</sup>, and UVRAG,<sup>36,37</sup> have been linked to tumor suppression, further supporting its importance in anticancer mechanisms. Mechanistically, macroautophagy may circumvent malignant transformation by inducing autophagic cell death<sup>38</sup> or cellular senescence<sup>24</sup> in the context of oncogenic stress. Despite the demonstration that chaperone-mediated autophagy is downregulated in senescent cells,<sup>39</sup> and that macroautophagy may prevent senescence in some contexts,<sup>40</sup> a growing number of observations show a correlation between markers of autophagy and the senescence phenotype.<sup>41-43</sup> Also, numerous studies have now demonstrated the critical role of macroautophagy during the establishment of senescence triggered by various stresses.<sup>24,44-49</sup> Interestingly,

some recent work suggests an intimate relationship between macroautophagy and the senescence-associated secretory phenotype (SASP).<sup>50,51</sup> These studies propose that macroautophagy is required to attenuate the proteotoxic stress induced by the high protein synthesis rate involved in the SASP and to supply the process with building blocks and energy.<sup>25,52</sup> The SASP has been linked to the deleterious effects of senescence,<sup>7,53</sup> but also to the auto/paracrine reinforcement of the phenotype<sup>5,54-56</sup> and to the immune clearance of senescent cells,57-60 thereby suggesting that autophagy might play a central function to explain the pathophysiological relevance of senescence.

During the molecular characterization of the role of the ERK kinases in Rasinduced senescence in human fibroblasts, our group discovered that senescence depends on high-strength ERK signals. In this context, we serendipitously found that some ERK targets were degraded. This initial observation lead to the identification of multiple actively degraded phosphoproteins during Ras-induced senescence.<sup>61</sup> Consistent with increased macroautophagy during senescence, we observed an increase in overall protein degradation in oncogenic Ras-expressing senescent cells (Fig. 1A and B) but no increase in the total amount of polyubiquitinated conjugates (Fig. 1C) or upregulation of the proteasome activity as measured with the proteasome activity probe Me4BodipyFL-Ahx3Leu3VS (Fig. 1D).<sup>62</sup> However, by in-depth characterization of an array of proteins that we found to be degraded, we discovered that the degradation process involved ubiquitination and the proteasome. This senescence-associated degradation program was conserved in multiple contexts, including mouse fibroblasts and human mammary epithelial cells expressing oncogenic Ras and in human fibroblasts with short telomeres. Thus, the second major degradation system used by eukaryotic cells, the selective degradation by the ubiquitin-proteasome pathway (UPP), is also engaged in senescent cells and allows the degradation of specific proteins. We called the process senescence-associated protein degradation or SAPD.<sup>61</sup> Although its exact contribution to senescence needs further

study, depletion of some individual SAPD targets was sufficient to trigger senescence, thereby illustrating the relevance of this mechanism for the onset and/or maintenance of senescence. We hypothesized that under mitogenic stress, such as A conferred by hyperactivation of the ERK/ MAPK pathway, the downstream effectors of mitogenic signaling undergo proteasome-dependent degradation, and that their depletion accounts for different characteristics of senescent cells.63 Consistent with this model, a phosphoproteomic analysis of Ras-expressing senescent cells treated with the proteasome inhibitor MG132 revealed many proteasome targets whose downregulation can contribute to senescence (Fig. 2; Table 1). We will thus discuss the features of senescence that are В most likely to be induced or affected by SAPD and the Senescent

## Mitochondrial dysfunction

Phenotype

the SAPD.

Mitochondria are dysfunctional in senescent cells,64,65 but the mechanism explaining their alterations is unknown. The ATP synthase enzyme uses the proton gradient generated by the electron transport chain in inner mitochondrial membrane to catalyze ATP production.<sup>66</sup> The ATP synthase subunit ATP5B is degraded by the proteasome in Rasinduced senescence (Table 1), and an increase of its turnover might explain the drop in ATP levels in senescent cells reported in some studies.<sup>65,67,68</sup> This might contribute to senescence, since inhibition of ATP synthase with oligomycin has been shown to induce a partial senescence phenotype.65

The signal transducer and activator of transcription 3 (STAT3) is a transcription factor activated by the JAK kinases in response to cytokines. However, a pool of this protein has been shown to be imported into mitochondria and incorporated to complex I via GRIM-19.69 Mitochondrial STAT3 modulates respiration, mainly by promoting the activity of complex I and II of the electron transport chain.<sup>70</sup> This function of STAT3 appears to support Ras-driven transformation

and ensures the proper functioning of mitochondria.71 Indeed, impaired levels or regulation of STAT3 have been shown to induce mitochondrial dysfunction

and ROS production.72,73 Interestingly, STAT3 is a confirmed SAPD target, and its degradation may thus link senescence to mitochondrial dysfunction (Table 1).<sup>61</sup>



Figure 1. Oncogenic Ras increases overall protein degradation, but does not increase proteasome activity. (A) Normal human fibroblasts (IMR90; from ATCC) cultured in Dulbecco modified Eagle medium (DMEM; Wisent) and expressing oncogenic Ras (R) or an empty pWZL vector (V), 10 d after retroviral infection. Total protein extracts after a pulse with 0.5 µCi [355]-methionine for 2 h, followed by a treatment with 75 µg/mL cycloheximide (CHX; Sigma-Aldrich) for the indicated times. (B) Bands were quantitated using Image Lab 4.0 (M = slope). An immunoblot for  $\alpha$ -tubulin (1:5000; clone B-5–1-2, T6074, Sigma-Aldrich) was used for normalization. (C) Immunoblots for HRas (1:250; clone F235, Sc-29, Santa Cruz), α-tubulin and mono-polyubiquitylated conjugates (1:1000; clone FK2, BML-PW8810, Enzo Life sciences). Protein extracts from IMR90 cells as in (A), but treated with DMSO or 20 µM MG132 (Sigma-Aldrich) for 18 h. (D) Fibroblasts as in (A) were treated with 500 nM of the proteasome activity probe Me4BodipyFL-Ahx3Leu3VS (Boston Biochem, I-190) for 1 h. Total protein extracts were subjected to SDS-PAGE, and fluorescence was analyzed on a ChemiDoc<sup>™</sup> MP System (Bio-Rad). Multiple catalytic subunits are visible ( $\beta$ 1, 2, and 5).

In addition, 3 components of the TOM complex were found to be unstable during Ras-induced senescence: HSP70 (HSP1A1), TOMM70A, and TOMM34 (**Table 1**).<sup>61</sup> The TOM complex is responsible for the import of matrix mitochondrial

proteins involved in the TCA cycle and  $\beta$ -oxidation.<sup>74</sup> This complex is assisted by the chaperone ATPase HSP70, which is very unstable in senescent cells.<sup>61</sup> It is thus possible that defects in mitochondrial protein import due to degradation of



**Figure 2.** Senescence-associated phenotypes likely regulated by SAPD targets. Normal human fibroblasts, 10 d after infection with H-RasV12, were treated 18 h with DMSO (control) or 20  $\mu$ M MG132 (Sigma-Aldrich). Then, cells were harvested, and protein extracts were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) for phosphoproteomics. Almost 3000 phosphopeptides from 1018 proteins were enriched. A FatiGO single enrichment analysis with the Babelomics 4.3 platform was perform in order to identify *Gene Ontology* (GO), *Kyoto Encyclopedia of Genes and Genomes* (KEGG), and *Reactome* terms significantly enriched. The terms related to a senescence phenotype and their associated peptides (398 proteins) were grouped into the indicated phenotypes.



**Figure 3.** The proteins corresponding to the genes downregulated by RB1 in Ras-induced senescent fibroblasts are also unstable. Unbiased Gene Set Enrichment Analysis (GSEA) of the proteomic data as in **Figure 2**. The gene set CHICAS\_RB1\_TARGETS\_SENESCENT (Systematic name: M2125) was the second most significant result among the proteins stabilized by MG132 in Ras-induced senescent cells. The normalized enrichment score (NES), the nominal *P* value determined by an empirical phenotype-based permutation test procedure and the false discovery rate (FDR; *Q* value) are shown. TOM complex components contribute to the mitochondrial dysfunction observed in senescent cells. It is known that the TOM complex is regulated by phosphorylation,<sup>75</sup> and we found phosphorylation of serine 91 of TOMM70A and serine 186 of TOMM34 in Ras-induced senescence. It will be of considerable interest to address whether these sites are phosphorylated by the ERKs or other kinases and mediate recognition of E3 ligases. Of note, HSP70 regulates oncogene-induced senescence (OIS), and knockdown of this protein is associated to an increase in ERK activity,<sup>76</sup> perhaps creating a positive feedback loop that plays a role in maintaining OIS.

#### Proteotoxic stress

Accumulation of damaged and misfolded proteins leads to chronic proteotoxic stress, which is intimately linked to organismal aging and associated pathologies.<sup>77</sup> The oxidative stress resulting from either mitochondrial dysfunction65,78 or upregulation of oxidative metabolism<sup>51,79</sup> can promote protein oxidation,<sup>80,81</sup> thereby leading to protein misfolding.82,83 Also, it is proposed that the high production of secreted cytokines in the SASP overcomes the cellular capacity for accurate protein synthesis and thus produces improper proteins and proteotoxic stress.<sup>51</sup> Interestingly, we found that the main housekeeping system to maintain protein homeostasis, the heat-shock proteins (HSPs), is also downregulated in senescence.<sup>61</sup> Indeed, the proteasomal degradation of HSP70 has been confirmed, and an impressive number of HSP proteins are unstable in Ras-induced senescence (Table 1). This is consistent with the demonstration that chaperonemediated autophagy, but not macroautophagy, is downregulated in senescent cells.39 Further supporting our observations, several reports have shown either a decrease in HSPs during senescence or a direct function of these proteins in opposing the induction of senescence.76,84-89 Conversely, high levels of HSPs support tumorigenesis by circumventing a toxic accumulation of misfolded proteins in cancer cells that frequently experience proteotoxic stress, suggesting a widespread vulnerability that can be targeted therapeutically.<sup>82,90-93</sup> Taken together, the observations discussed above strongly suggest that a breakdown of protein

homeostasis is an important feature of cellular senescence and therefore of tumor suppression.

Beyond these correlative findings, we are tempted to speculate that downregulation of HSP activity might have a primary and critical role during the establishment of a senescent program. First, the reduction of protein refolding might stimulate abnormal protein clearance by degradation, either by macroautophagy or proteasomal-dependent degradation.<sup>25,94-96</sup> Notably, a decrease in HSP levels correlates with an elevated activity of the CHIP ligase during senescence, suggesting that this E3 ligase could play a pivotal role in targeting misfolded proteins to the UPP.<sup>97</sup> Somehow, the directed degradation of HSPs could reinforce the main cellular protein degradation mechanisms in order to eliminate dysfunctional proteins instead of investing energy in protein repair. Senescent cells use energy to support production of signaling molecules and secretion products. Protein degradation produces amino acids used as building blocks and substrates to feed the TCA cycle, thereby supporting metabolite synthesis and energy production.<sup>25</sup> Perhaps protein degradation is a better investment

Table 1. Potential S	APD targets and the sene:	scence-associated ph	enotypes they	could regulate

Phenotypes	Proteins	Functions	
Mitochondrial dysfunction	ATP5B	$\beta$ -Subunit of the ATP synthase catalytic core (F1); ATP synthesis <sup>66</sup>	
	STAT3	Modulates respiration via complex I and II <sup>69,70</sup>	
	HSP1A1 (HSP70)	Component of the TOM complex; import of matrix mitochondrial proteins <sup>74</sup>	
	ТОММ70А	Component of the TOM complex; import of matrix mitochondrial proteins <sup>74</sup>	
	TOMM34	Component of the TOM complex; import of matrix mitochondrial proteins <sup>74</sup>	
Proteotoxic stress	HSPA1A (HSP70)	Protein refolding under stress conditions; supports oncogenesis <sup>82,83,89</sup>	
	HSPA5,7,8 and 9	Protein refolding under stress conditions; supports oncogenesis <sup>82,83,89</sup>	
	HSPB1 (HSP27)	Protein refolding under stress conditions; suppresses cellular senescence <sup>82,83,89,95</sup>	
	HSPD1 (HSP60)	Protein refolding under stress conditions <sup>82,83,89</sup>	
	HSP90AB1	Protein refolding under stress conditions; supports oncogenesis <sup>82,83,89</sup>	
DNA damage response	CCDC6	DNA damage checkpoints and DNA repair <sup>109,110,116</sup>	
	SOD1	Superoxide detoxification113,114,120	
	TOP2	Relaxes topological constraints during DNA replication; chromosomes segregation <sup>117</sup>	
	TERF2IP	Component of the telosome; tethering telomeres to the nuclear envelope; protect telomere ends from NHEJ and HDR <sup>124-126,128,267,268</sup>	
Nucleolar and ribosome biogenesis dysfunction	NOLC1	Polymerase I coactivator; scaffold protein for nucleolar assembly <sup>137,138</sup>	
	NOP56 and 58	Components of the box C/D snoRNPs <sup>139</sup>	
	DDX51	RNA helicase; processing of the 3' end of the 28S rRNA <sup>140</sup>	
	NOL6	Processing of the 18S rRNA <sup>141</sup>	
	NOC2L	Processing of the 18S, 28S and 5.8S rRNAs <sup>142</sup>	
	NCL	Polymerase I transcription; rRNA processing; ribosome assembly and transport <sup>143</sup>	
	RPLP1	Translational elongation; overexpression bypasses replicative senescence <sup>144</sup>	
	RSL1D1	Regulates the nucleolar localization of nucleostemin; rRNA processing <sup>146,149</sup>	
	NPM1	Processing of the 32S pre-rRNA to the mature 28S rRNA <sup>153,154</sup>	
Cell cycle arrest	YAP1	Regulates apoptosis; regulates organ size; liver oncogene <sup>167,269</sup>	
	MCM2	Initiation and progression of DNA replication <sup>172</sup>	
	LRWD1 (ORCA)	stabilizes the origin recognition complex (ORC) to chromatin <sup>173</sup>	
	МҮС	Promotes DNA replication <sup>177</sup>	
	JUN	Promotes G <sub>1</sub> -to-S-phase progression <sup>189</sup>	
	KAP1 (TRIM28)	Destabilized the tumor suppressor p53 <sup>192-194</sup>	
	TBX2	Repression of P19ARF; repression of the CDK inhibitors p21 and p27 <sup>199-201</sup>	
Impaired mRNA metabolism and translation	YB-1 (YBX1)	mRNA stability, mRNA packaging, splicing and translational initiation; oncogene <sup>204,207</sup>	
	SRm160 (SRRM1)	Coactivator for exonic splicing enhancers and for 3'-end processing of specific pre-mRNAs <sup>210-213</sup>	
	SRm300 (SRRM2)	Coactivator for exonic splicing enhancers and for 3'-end processing of specific pre-mRNAs <sup>210,211,213</sup>	

than protein repair to support the SASP. This could be particularly true if lowcost degradation processes are favored during senescence. In this regard, it has been reported that ubiquitin and ATPindependent proteasomal degradation, accomplished by the 20S proteasomes, is the predominant mechanism to remove damaged proteins in oxidative contexts,<sup>98</sup> as is the case in senescent cells. If this speculation proves true, this may be the designated route to optimize production of building blocks and energy saving.

### DNA damage response

Cellular senescence induced by various stresses is characterized by an inability to properly repair DNA breaks and thus by a permanent DNA damage response (DDR).99-102 The latter is thought to contribute to both the induction and maintenance of senescence.<sup>103-108</sup> The coiled-coil domain containing protein 6 (CCDC6) is a component of the DNA damage checkpoint machinery, and its corresponding gene is rearranged in 20% of papillary thyroid carcinomas.<sup>109</sup> During DDR, CCDC6 is stabilized by ATM and contributes to proper DNA repair.<sup>110</sup> Interestingly, we found that CCDC6 is unstable in Ras-induced senescence (Table 1), and its degradation may thus contribute to the persistent DDR observed in senescent cells.<sup>100-102,104-108</sup> Moreover, this protein is a target for the tumor suppressor E3 ligase SCF-FBW7, suggesting a role of this E3 ligase in SAPD.<sup>110</sup>

In addition to limiting proper DNA repair, the SAPD could itself promote DNA damage. The DNA breaks that underlie senescence can be triggered by different stresses. One of these is the increase in reactive oxygen species (ROS),111-113 resulting from abnormal mitochondrial activities during senescence as discussed previously. Surprisingly, we found that the copper zinc superoxide dismutase 1 (SOD1) is unstable in Rasdependent senescent cells (Table 1). Since this enzyme metabolizes superoxide radicals to molecular oxygen and hydrogen peroxide, and therefore is a major component of the antioxidant defenses within the cell,113,114 SOD1 depletion could cooperate with mitochondrial generation of ROS to increase the total amount of these reactive ions and concomitant

DNA damage. Further supporting this conjecture, SOD1 deficiency has been shown to induce persistent DNA damage in mice<sup>115</sup> and senescence in human fibroblasts.<sup>116</sup> In addition to increased ROS levels, oncogenic activation drives an initial phase of DNA hyper-replication, leading to premature termination of replication forks, thereby producing DNA damage that triggers senescence.<sup>106,107</sup> Intriguingly, another candidate target of SAPD identified by proteomics is TOP2 (Table 1), which is known to relax topological constraints during DNA replication and to allow chromosome segregation.<sup>117</sup> Accordingly, a deficiency in this topoisomerase could increase fork collapses by preventing their progression, causing aberrant replication intermediates and the activation of DDR.<sup>118</sup> Also, a lack in TOP2 can impair completion of DNA replication by interfering with the proper resolution of replication forks at chromosomal termination regions (TERs),<sup>119,120</sup> thus generating DNA damage at TERs and even more when cells undergo mitosis.<sup>120-122</sup> Furthermore, TOP2 has been shown to play an architectural function at intergenic regions adjacent to transcribed genes during S phase, and this seems to protect against collisions between replication forks and transcription sites. This role appears critical to avoid replicationinduced DNA damage, since cells deficient in TOP2 experience DNA breaks at normally TOP2-bound regions.<sup>123</sup> Taken together, the functions of TOP2 suggest that it plays a critical role to maintain genome integrity in cycling cells, and that its depletion in cells experiencing hyper-replication is likely to trigger DNA damage-promoted senescence. Finally, another proposed source of DNA damage leading to senescence is telomere dysfunction, which leads to telomere dysfunctioninduced foci (TIF).99,105 The shelterin complex (telosome) associates with telomeres and protects chromosome ends.<sup>124</sup> The human ortholog of the yeast telomere binding protein Rap1, TERF2IP,125 is part of the complex and has been shown to play a role in tethering telomeres to the nuclear envelope<sup>126</sup> and to protect telomere ends from non-homologous end joining (NHEJ).<sup>127,128</sup> TERF2IP downregulation may trigger telomere dysfunction-induced

DNA damage (Table 1), which contributes to DDR in OIS.<sup>100,129,130</sup> Interestingly, TERF2IP interacts with PML,<sup>131</sup> a protein forming PML nuclear bodies (PML-NBs) during senescence and that has been implicated in protein degradation.<sup>132-135</sup> This suggests that senescence-associated PML-NBs could be a specialized compartment where nuclear proteins are degraded during SAPD.

Dysfunction in nucleolar and ribosome biogenesis

The nucleolus is the principal site of ribosome synthesis, where RNA polymerase I (PolI) transcribes rRNA genes (rDNA) to produce the 47S rRNA (rRNA) precursor. The 47S precursor is cleaved and modified by 2'-O-methylation and pseudouridylation of specific nucleotides to form the mature 18S and 28S rRNAs. These processes are guided by small nucleolar RNAs (snoRNAs) assembled into RNA/protein complexes called small nucleolar ribonucleoproteins (snoRNPs). Mature rRNAs are assembled with ribosomal proteins (RPs), inside the nucleolus, to produce the 40S and 60S ribosomal subunits, which then migrate toward the cytoplasm.<sup>136</sup> Approximately 50% of the energy of proliferating eukaryotic cells is dedicated to ribosome biogenesis, and the process requires approximately 200 snoRNAs, more than 80 RPs, and hundreds of accessory proteins.136 We found that many proteins implicated in rRNA transcription and maturation are unstable in Ras-induced senescence. They include NOLC1,137,138 NOP58, NOP56,139 DDX51,140 NOL6,141 and NOC2L.61,142 We also found unstable proteins which are implicated in late steps of ribosome synthesis, such as nucleolin (NCL),143 the ribosomal protein P1 (RPLP1),144 and the ribosomal protein L23 (RPL23) (Table 1). Although we do not know yet whether the instability of the proteins discussed above causes a decrease in their levels in senescent cells, such a reduction may lead to defects in ribosome biogenesis or may simply be part of a compensatory mechanism that degrades these proteins when ribosome biogenesis is reduced.

We confirmed the proteasome-dependent degradation of the ribosomal L1 domain-containing 1 protein (RSL1D1 or CSIG) and its decrease in Ras-induced senescence (Table 1).<sup>61</sup> Interestingly, this protein was previously found downregulated in senescent cells.145 RSL1D1 regulates the nucleolar localization of nucleostemin (NS), which, in turn, regulates the nucleolar localization of DDX21.146 Nucleostemin and DDX21 have been shown to be important for the processing of pre-rRNA.147,148 Using RNAi screening, a role in rRNA processing was also shown for RSL1D1 together with NOP56, DDX51, NOL6/NRAP, NOC2L/NIR, and nucleolin (NCL).149 We knockeddown the expression of this protein in normal human fibroblasts, and this resulted in the induction of the senescent phenotype.<sup>61</sup> Hence, a reduction in RSL1D1 can be causative for senescence, and its role in ribosomal biogenesis suggests that defects in this process may be another effector mechanism of senescence.

It has been shown that the alternative reading frame protein (ARF, also known as p19ARF), a well-known inducer of senescence, stabilizes p53 by inhibiting the E3 ubiquitin ligase MDM2 (HDM2).<sup>150-152</sup> However, ARF also inhibits cell proliferation by targeting nucleophosmin (NPM1/B23) for sumovlation and degradation and in this way regulates the processing of the 32S prerRNA to the mature 28S rRNA.153,154 Furthermore, ARF and NPM1 control the sub-nuclear localization of the transcription termination factor I (TTF-1), which has been shown to regulate PolI transcription initiation/ termination and rRNA processing.155 Therefore, in addition to inducing the senescence phenotype through the MDM2-p53 axis, ARF affects ribosome biogenesis, and we can hypothesize that this function may also reinforce the senescence program. Supporting this, we found that NPM1 is unstable in Ras-induced senescence (Table 1).

In light of the results presented above, it is tempting to suggest that defects in ribosome biogenesis can be an important mediator of senescence. This is in agreement with recent



**Figure 4.** Modulation of protein stability for proteins regulated by phosphorylation-driven ubiquitination and proteasome-dependent degradation. (**A**) Under normal conditions, competition between the activity of kinases vs. phosphatases (PPases) and E3 ubiquitin ligases vs. deubiquitinases (DUBs) ensures the maintenance of appropriate levels of a specific protein. The turnover of this protein can be increased by (**B**) increasing the activity of its kinases; (**C**) increasing the activity of its E3 ubiquitin ligases; (**D**) both (**B and C**); (**E**) decreasing the activity of its PPases; (**F**) decreasing the activity of its DUBs; (**G**) both (**E and F**). Of note, different combinations of (**B**) to (**G**) can be involved. Also, a similar scenario can be applied for SUMO-dependent ubiquitination; kinases and PPases can be substituted by SUMO E3 ligases and deSU-MOylase. Ub, ubiquitin; P, phosphorylation.



**Figure 5.** The balance of oncogenic vs. tumor suppressor E3 ubiquitin ligases. The activities of oncogenic vs. tumor suppressor E3 ligases are in equilibrium to maintain cells in a normal state. Tipping the balance in one direction or the other can be critical for determining whether a cell under oncogenic stress will undergo tumor suppression or neoplastic transformation.

reports showing that CX-5461, an inhibitor of rRNA synthesis, induces cellular senescence in solid tumor cell lines.156,157 However, a defect in ribosome biogenesis may appear contradictory to the increased global translation reported in senescent cells,24 which could result from the activation of TOR signaling.<sup>158</sup> Indeed, this pathway has been shown important to convert cells from a reversible quiescent state to a permanent senescent phenotype,<sup>159-162</sup> a phenomenon called geroconversion by Blagosklonny and colleagues,<sup>163</sup> and this could be in part due to the translational effects of TOR.164 Although further work will be required to explain how senescent cells can increase translation despite less ribosome biogenesis, it is likely that ribosome turnover decreases in these cells forcing them to use "old" ribosomes to make proteins.

#### Cell cycle arrest

Impaired proliferation, mainly by an arrest in the  $G_1$  phase of the cell cycle, is an established senescence feature, and the SAPD may be an important player in this process. During  $G_1$ , the D-type cyclins bind the cyclin-dependent kinase (CDK) 4 and 6, and this stimulates

the progression toward initiation of S phase.<sup>165</sup> We found that the transcription coactivator Yes-associated protein YAP1 has a high turnover in Ras-expressing senescent cells (Table 1).<sup>61</sup> Later, it was found reduced by another group during replicative senescence as well.<sup>166</sup> YAP1 localizes to PML bodies and can regulate apoptosis via p73.167 In addition, YAP1 is the ortholog of Drosophila Yorkie that regulates organ size as part of the Hippo pathway and acts as a liver oncogene in mammals.<sup>168</sup> Interestingly, it appears that YAP1 can circumvent senescence in some contexts by inducing the transcription of CDK6.<sup>166</sup> Despite the fact that CDK4/6 have been shown not to be essential for proliferation, unlike CDK1,165 their downregulation in YAP1-deficient cells might interfere with cell cycle progression in a subset of specialized cells or YAP1 might play a more broad effect on CDKs. The identification of YAP1 as a potential SAPD target also suggests a role for more E3 ligases in this process. YAP1 degradation depends on a phosphodegron recognized by the E3 ligase SCF-B-TRCP169 and can also be triggered by the E3 ligase NOT4.170

The G<sub>1</sub>-to-S-phase transition is ensured by the formation of the prereplication complexes (pre-RCs) on chromatin, which depends on the sequential recruitment of the origin recognition complexes (ORCs), CDC6, and MCM proteins.<sup>171</sup> The DNA replication licensing factor MCM2 is an important component of the pre-RCs and was found unstable in Ras-induced senescence (Table 1). Accordingly, degradation of MCM2 could limit the initiation of DNA replication and the progression of the cell cycle.172 In addition, LRWD1/ORCA is a protein that stabilizes the origin recognition complex (ORC) on chromatin.173 LRWD1/ORCA degradation in Rastriggered senescence (Table 1) is likely to abrogate the binding of the ORC to chromatin, consequently arresting the cells in G<sub>1</sub>. Interestingly, this protein is suspected to be polyubiquitinated by the E3 ligase complex CUL4A-DDB1,174 which has already been linked to p16<sup>INK4A</sup> upregulation in senescence.<sup>175</sup> Hence, we can add CUL4A-DDB1 to the list of E3 ligase candidates that promote SAPD.

Since CUL4A-DDB1 has also been shown to promote proteasome-dependent degradation of MYC via the substrate receptor TRUSS (TRPC4AP),176 a role of this E3 ligase in cell cycle arrest and senescence is even more consistent considering our observation of MYC degradation in Ras-induced senescent cells (Table 1).61 MYC promotes DNA replication and is a master regulator of many cellular programs, including proliferation.<sup>177</sup> Its downregulation is reported to contribute to senescence,178,179 and its overexpression cooperates with different oncogenes to transform cells by inhibiting cellular senescence.179-181 The downregulation of MYC levels in order to shut down the cell cycle is thus possibly at the crossroads of several senescence-promoting pathways. This not only suggests a role for the CUL4A-DDB1-TRUSS ligase in senescence, but also supports the investigation of the multiple other E3 ligases reported to target MYC to the proteasome, such as SCF-FBW7,<sup>182,183</sup> SCF-SKP2,<sup>184,185</sup> CHIP,<sup>186</sup> the Mule complex (Mule/Huwe1/Arf-BP1),187 and the suggested CUL2/F-Box hybrid ElonginBC-CUL2-SKP2.188 complex Similar to MYC, JUN is another classic



**Figure 6.** Theoretical purpose of oncogene-induced senescence and contribution of protein degradation. Increasing evidence suggests that the destiny of senescent cells in many organs is clearance by the immune system. This implies a central role for the cytokine production characteristic of the SASP in the recruitment of immune cells (in red). Specific protein degradation (SAPD) may contribute directly and/or indirectly to the initial cell cycle arrest, but may also cooperate with macroautophagy to produce antigenic peptides and to support the SASP. Proteolysis may redistribute cellular energy to the SASP and may supply nutrient building blocks for biosynthetic reactions.

regulator of cell proliferation found unstable in Ras-induced senescent cells.<sup>189</sup> Its downregulation may contribute to a block in  $G_1$ -to-S-phase progression by decreasing the expression of cyclin D1<sup>190</sup> and elevating the expression of p53 and p21.<sup>191</sup>

The degradation of the KRABassociated protein 1 (KAP1, also known as TRIM28 or TIF1 $\beta$ ) (Table 1), a validated SAPD target,<sup>61</sup> may block the cell cycle by different mechanisms. First, KAP1 is known to destabilize p53, possibly explaining why high levels of KAP1 are associated with poor prognosis in gastric cancers.<sup>192</sup> KAP1 binds and cooperates with the E3 ligase MDM2 to drive p53 degradation.<sup>193</sup> Furthermore, the melanoma antigen (MAGE) proteins interact with KAP1 and stimulate its own E3 ligase activity to allow p53 ubiquitination and degradation in a MDM2-independent manner.<sup>194</sup> Accordingly, downregulation of KAP1 is likely to stabilize p53, allowing the expression of key cell cycle inhibitors.195 Second, the degradation of KAP1 may relieve its transcriptional repression functions, which have been shown to directly inhibit the transcription of p53-target genes, such as the CDK inhibitor p21.196,197 Third, KAP1 depletion increases the number of PML-NBs.<sup>198</sup> These senescence-associated nuclear structures inhibit E2F target gene expression. The latter are critical to initiate DNA synthesis, and inhibiting their transcription arrests cell proliferation.8

The T-box protein 2 (TBX2) is linked to repression of p19ARF gene expression, thereby promoting the MDM2-mediated degradation of p53 and cellular senescence suppression.<sup>199</sup> TBX2 further antagonizes senescence by repressing the CDK inhibitors p21 and p27 (CDKN1B).<sup>200,201</sup> Finally, TBX2 is reported to be an E2Ftarget gene repressed by PML, and its repression stimulates the pro-senescence functions of PML.<sup>202</sup> Collectively, these results suggest that degradation of TBX2 (Table 1) could initiate the cell cycle arrest characterizing senescence and then reinforce the phenotype by activating a positive loop via the inhibition of its own transcription by PML.

According to a FatiGO single enrichment analysis of proteomics data with the bioinformatics platform Babelomics, the regulation of proliferation is one of the biological functions that is most enriched among unstable proteins in Ras-induced senescence (Fig. 2).61 Here, we have discussed the implication of just a few of the possible SAPD targets involved in cell proliferation. Surprisingly, after further analysis of the proteomics data, we found that the proteins corresponding to the genes identified by Chicas et al. (2010) as downregulated by RB1 in Ras-induced senescent fibroblasts are also unstable (Fig. 3).9 In this context, RB1 predominantly represses the E2F target genes implicated in DNA replication. Although this idea will need further investigation,

our results suggest that bulk degradation of the same E2F-induced proteins could cooperate with transcriptional repression to safeguard cell cycle arrest. Does the SAPD cooperate with RB1 and PML-NBs to ensure a rapid and potent shutdown of E2F target genes?

# Impaired mRNA metabolism and translation

The Y-Box binding protein 1 (YB-1 or YBX1) is also unstable in Ras-induced senescent cells (Table 1), and its downregulation has been linked to the senescence phenotype,<sup>203</sup> whereas its overexpression strongly correlates with aggressive tumors.<sup>204</sup> However, YB-1 is a multifunctional protein, and we are still far from understanding how its functions could oppose senescence. One hypothesis is that YB-1 could stimulate the transcription of E2F-target genes by binding to multiple E2F promoters.<sup>205</sup> Conversely, it could act as a transcriptional repressor of p53.206 These scenarios suggest that depletion of YB-1 could have a relatively direct effect on cell cycle as discussed in the previous section. Nonetheless, direct evidence also highlights critical functions in mRNA metabolism, including mRNA stability, mRNA packaging, splicing, and translational initiation.<sup>204,207</sup> Does the regulation of p53 and E2F-target genes result from these activities? Although the answer is not clear, this could be the case at least for p53. The DNA-damaging stresses prevent YB-1-mediated splicing of

MDM2, leading to an mRNA molecule lacking several exons, and resulting in a non-functional protein.<sup>208</sup> This regulation of MDM2 may contribute to the stabilization of p53 in senescent cells experiencing DDR. Because YB-1 is a putative general regulator of mRNA maturation and translation for mRNAs with YB-1 binding sites,<sup>207</sup> we hypothesize that suppression of these functions could promote senescence by affecting the expression of several proteins. In senescence, the YB-1 functions could be abrogated by its degradation, possibly catalyzed by the E3 ligase activity of RBBP6.<sup>209</sup>

Two other splicing regulators have an increased turnover in Ras-induced senescence (Table 1). SRm160 (SRRM1) and SRm300 (SRRM2) are splicing coactivators required for the functions of exonic splicing enhancers and for 3'-end processing of specific pre-mRNAs.210-213 These proteins are phosphorylated at multiple distinct S/T-P phosphorylation sites in senescent cells, suggesting that they may act as a sensor of ERK signaling strength.<sup>61</sup> Perhaps an accumulation of phosphorylated sites over a given threshold controls the interaction with E3 ligases, promoting the ubiquitination and degradation of hyperphosphorylated SRm160/300 in response to oncogenic stress. Such degradation could consequently promote senescence by impeding normal mRNA maturation of a specific set of genes, including critical regulators of normal cell functions.

#### **Key Remaining Questions**

### Targeting protein to SAPD: Where?

Proteasomes are widespread in cells, but can interact with some specific cellular structures. In the cytoplasm, proteasomes can bind the cytoskeleton, the outer surface of the endoplasmic reticulum, and the centrosomes.<sup>214-216</sup> They are also found throughout the nucleoplasm, but, interestingly, they have been shown to be concentrated in PML-NBs,<sup>214,217-220</sup> nucleoplasmic speckles,<sup>219,221</sup> and other focal subdomains.<sup>219,222</sup> In some particular contexts, proteasomes can also accumulate in nucleoli.<sup>222,223</sup> Thus, the degradation of SAPD targets could use specific

"proteolytic centers". For example, Wójcik and DeMartino (2003) proposed that cytosolic proteins targeted for degradation are delivered to a master proteolytic complex located at the centrosome via microtubule-mediated transport.<sup>214</sup> Similarly, PML-NBs and nuclear speckles could act as the proteolytic complexes for nucleoplasmic SADP targets. Speckles are enriched in splicing factors and may thus be the proteolytic center for these proteins we found unstable during Ras-induced senescence, including YBX1, SRm160, and 300 (Table 1).<sup>224,225</sup> Also, PML-NBs might be a specialized structure for short proteins destined to be degraded vs. these that should not, thereby representing the so-called clastosome previously described as nuclear bodies enriched in proteasome-dependent degradation effectors.<sup>226</sup> Consistent with this idea, several potential and validated SAPD targets colocalize with PML-NBs, including TERF2IP,<sup>131</sup> YAP1,<sup>167</sup> MYC,<sup>133,179,227</sup> and STAT3.<sup>228,229</sup> Furthermore, PML-NBs have been shown to be involved in the degradation of factors for which downregulation is known to mediate a senescence program, such as CREBBP (CBP)<sup>230,231</sup> and MYC.<sup>133,178,179</sup> Conversely, PML-NBs might also play an active role in protecting other proteins from degradation, like HIPK2,232 p73,233 TOPBP1,<sup>234</sup> and p53.<sup>235</sup> Thus, PML may be critical for the specificity of SAPD.

#### Targeting protein to SAPD: How?

The pattern of proteins degraded by the proteasome seems dramatically changed during senescence, while there is no apparent modification in total proteasome activity (Fig. 1D). Also, even if there is a large amount of unstable proteins, other key senescence mediators are stabilized (e.g., p53). These observations suggest 2 principal mechanisms explaining the proteasome-dependent degradation of a large subset of specific proteins in senescence: the upregulation of specific E3 ligases activity and the targeting of specific proteins for SAPD. Previous work and our recent observations strongly propose that post-translational modifications of proteins play a central role in SAPD. Because PML-NBs could be involved in SAPD target degradation, sumoylation is a candidate modification of particular interest. Indeed, PML-NBs are among

the principal sites of sumoylation in cells, since they interact with many SUMO ligases and sumoylated proteins.<sup>220</sup> Furthermore, sumoylation is known to lead to the subsequent ubiquitination and degradation of particular proteins.<sup>236,237</sup> There is now accumulating evidence that sumovlation at PML-NBs is coupled with the UPP, the SUMO-dependent degradation of N4BP1135 and NRF2238 being examples. Of note, the degradation of the latter in PML-NBs could limit ROS detoxification, thereby contributing to the induction of senescence.239 Senescent cells experience oxidative stress, suggesting that protein carbonylation may serve as another modification to distinguish SAPD targets.<sup>240</sup> This modification marks oxidized proteins for degradation, mostly via the 20S proteasome and in an ATPand ubiquitin-independent manner.241 Despite the fact that carbonyl-mediated degradation exhibits a certain level of specificity, depending on the intrinsic susceptibility of a protein to oxidative carbonylation, this mechanism is rather unspecific and hardly explains the global proteome of senescent cells.<sup>240</sup>

Our group identified a remarkable number of phosphopeptides from proteins degraded by the proteasome in Ras-induced senescent cells, suggesting that phosphorylation is an important protein modification triggering SAPD.<sup>61</sup> Further strengthening this hypothesis, protein phosphorylation and ubiquitination-dependent degradation are tightly linked.242 Phosphorylation can drive ubiquitination either by regulating the subcellular localization of target proteins, thereby eliminating a spatial separation between the substrate and its E3 ligase, or by creating a docking site for an E3 ligase.<sup>242</sup> In replicative and Ras-induced senescence, hyperactivation of the ERK/ MAPK pathway is essential to mediate SAPD and to maintain the senescent phenotype.<sup>61</sup> This suggests a model where the hyperphosphorylated ERK targets are degraded, creating a negative feedback to mitogenic signaling that promotes senescence.<sup>63</sup> In this model, the sustained phosphorylation of ERK targets is suspected to increase the chance of an interaction with an E3 ligase or to activate a phosphodegron. However, we cannot exclude that

other kinases play a role in ERK-mediated SAPD. Such kinases could be either hyperactivated downstream of the ERK/ MAPK pathway or contribute to the full activation of phosphodegrons. The SAPD candidates MYC and JUN are 2 examples of proteins regulated by a phosphodegron implicating multiple kinases. Both are first phosphorylated by the ERK kinases, priming them for further phosphorylation by the GSK3 kinase, which is the final act in order to recruit the E3 ligase SCF-FBW7.189,242,243 Supporting an important role for GSK3 in mediating activation of phosphodegrons during SAPD, its inhibition leads to a reduction of MYC ubiquitination.<sup>61</sup> Furthermore, another SAPD candidate in Ras-induced senescence, namely β-catenin (CTNNB1), is a well-known protein undergoing degradation following GSK3-mediated phosphodegron activation.<sup>242</sup> Considering that GSK3 is inactivated by the PI3K/ AKT pathway,<sup>243</sup> buffering AKT activity is maybe an important strategy employed to promote SAPD in Ras-induced senescent cells. This could explain, at less in part, why activation of AKT contributes to circumvent RAF and Ras-induced senescence.<sup>244,245</sup> Nevertheless, we can speculate that hyperactivation of different kinases, including AKT, could also engage the degradation of their targets and thus promote a different pattern of SAPD, but with senescence as a common phenotypic output. SAPD could be a universal response to "phosphorylation stress" to avoid cellular transformation in the context of abnormal mitogenic signaling.

#### Kinases vs. phosphatases and E3 ligases vs. deubiquitinases: Different weapons, same fight?

When we address proteasome-dependent protein degradation, we naturally think of E3 ubiquitin ligases. However, the global picture is much more complicated and involves several players. Proteins can be dynamically ubiquitinated by E3 ligases and deubiquitinated by deubiquitinases. As we discussed in the previous section, ubiquitination can depend on phosphorylation.<sup>242</sup> In this situation, protein degradation is also regulated by kinases and phosphatases. We can thus simplify the situation by presenting kinases and E3 ubiquitin ligases as collaborating to favor protein degradation, whereas phosphatases and deubiquitinases are their respective opponents. A similar logic can be applied for SUMO-dependent ubiquitination; while SUMO E3 ligases cooperate with E3 ubiquitin ligases, deSUMOylases antagonize the process.237 In normal conditions, a subtle equilibrium between all the players impacting on protein stability ensures determined levels for a specific protein (Fig. 4A). During SAPD, the equilibrium is displaced to favor an increased turnover leading to reduced levels of the same protein. What exactly leads to the displacement of the equilibrium? For a given protein, the process can be mediated mostly by: (1) an increased activity of its kinases/SUMO E3 ligases (Fig. 4B); (2) an increased activity of its E3 ubiquitin ligases (Fig. 4C); (3) both 1 and 2 (Fig. 4D); (4) a decreased activity of its phosphatases/deSUMOylases (Fig. 4E); (5) a decreased activity of its deubiquitinases (Fig. 4F); (6) both 4 and 5 (Fig. 4G); (7) different combinations of 2 to 6. One challenge for the coming years will be to determine how these regulators interact to affect the steady state and what the resulting dynamic is. Is the equilibrium displaced linearly or does the collaboration between different SAPD mediators rather promote switch-like mechanisms? Such switch-like responses could point to competition between regulators with opposite effects on the substrate, which has been shown in the control of the orthologous yeast ERK/MAPK pathway.246 Finally, another challenge is to evaluate whether there are master regulators of protein degradation in SAPD, allowing opportunities to target the phenotype, or whether each protein or subset of proteins is regulated via distinct machinery.

Is there a master senescence-associated E3 ligase?

The specificity of the UPP is conferred by E3 ubiquitin ligases, a large and complex group of proteins, with an estimated 600 to 1000 members in the human proteome.<sup>247</sup> Based on the structure of their catalytic domain, the E3 ubiquitin ligases are generally classified into 4 main categories: the RING-finger type,<sup>248,249</sup> the HECT type,<sup>250</sup> the U-box type,<sup>251,252</sup> and the less characterized PHD domain-containing type.<sup>248,253</sup> The former is by far the most abundant and is further subdivided as single unit or multiple subunit RING-finger E3 ligases. The latter form complexes grouped into 2 principal families, the anaphase-promoting complex (APC) and the cullin-RING ligase (CRL) superfamily.<sup>248</sup> There are 7 cullins expressed in human cells (CUL1, 2, 3, 4A, 4B, 5, and 7) and they interact with specific receptor proteins which provide target specificity, including proteins harboring F-box, SOCS-box, VHL-box, and BTB domains.<sup>254,255</sup> These complexes are referred to by various names (reviewed in ref. 255), but the most common appellation is probably SCF for the classic complex containing CUL1 and SCF2-5 and 7 for complexes containing the corresponding cullins.

In the simplest scenario, one or few E3 ubiquitin ligases could be responsible for SAPD. Such a possibility would likely involve the regulation of the activity of specific E3 ligases. However, current evidence reviewed above points to the specificity being conferred by upstream steps targeting designated proteins for degradation. Considering these data as well as the complexity of the E3 ubiquitin ligase superfamily, we favor the view that SAPD is likely regulated by several E3 ligases, each catalyzing the ubiquitination of its specific targets. However, this more complex picture does not exclude the possibility that some E3 ligases could play a more critical role in the senescent phenotype. Indeed, as discussed previously, SCF-FBW7 is a well-known tumor suppressor and has been recently shown to contribute to senescence, 243, 247, 256 and correspondingly many FBW7 targets are degraded in SAPD. The CUL4A-DDB1 (SCF4) complex and its interacting receptor protein DDB2 are also strong candidates, since both have been shown to drive senescence.<sup>175,257</sup> Furthermore, the fact that phosphorylation could be a mark to distinguish SAPD targets highlights the interest in investigating the roles of the SCF complexes in senescence. Indeed, this subfamily of E3 ubiquitin ligases is primarily responsible for serine/threonine phosphorylation-dependent ubiquitination. Two classes of F-box proteins are specialized to recognize phosphodegrons, namely, the WD40 F-box proteins (e.g.,

FBW7 and  $\beta$ -TRCP1/2) and leucinerich repeat (LRR) F-box proteins (e.g., SKP2).<sup>242</sup> The proposed involvement of PML-NBs and SUMO-dependent degradation in SAPD also increases the interest in studying the contribution of the SUMO-targeted ubiquitin ligase (STUbL) family in senescence,<sup>258</sup> such as RNF4, which contains a SUMOinteracting motif (SIM).<sup>259</sup>

Although many E3 ubiquitin ligases have tumor-suppressive functions, including APC, SCF-FBW7, BRCA1, VHL, and FANC, several others are clearly oncogenic and can oppose senescence.<sup>248</sup> For example, MDM2 and MDMX are bona fide oncogenes and limit senescence by catalyzing ubiquitination and degradation of p53.<sup>152,248</sup> Senescence is also limited by the oncogene SCF-SKP2 that targets p27 and p21 in a p53-independent manner.<sup>260</sup> The potential role in cancer of E3 ligases that have a complex array of targets, including both tumor suppressors and oncogenes, is more difficult to ascertain. This is the case for SCF- $\beta$ -TRCP, functioning primarily as an oncogene by targeting apoptotic proteins, but showing tumorsuppressive activities in some contexts.<sup>248</sup> Since SCF-B-TRCP targets preferentially phosphorylated proteins,242 whether it acts as an oncogene or as a tumor suppressor may depend on the pool of phosphorylated substrates in a given context. In light of these dissimilar functions in tumorigenesis, it seems obvious that different members of the large family of E3 ubiquitin ligases use the UPP to compete in opposite directions. We can thus compare the effect of the E3 ligases on cell fate to a delicate balance, where the equilibrium between the activities of oncogenic vs. tumor-suppressive E3 ligases is critical to maintain cells in a normal state (Fig. 5). Under oncogenic stress, depending on whether the balance is tipped in one direction or the other, the UPP could favor transformation into cancer cells or tumor suppression, respectively.

## SAPD: A coordinated proteome reprogramming?

The answer to this question first depends on how senescence should be seen. Is it a totally abnormal and nonfunctional cellular state initiated in response to stress, which is basically avoided in normal organisms? Is it rather one of the fundamental tools that evolution has provided as a defense against the insults inherent to organismal life? The prevailing view at present favors that latter paradigm. Indeed, not only is senescence a gatekeeper response that is acutely triggered by stress stimuli, but it now appears that the process can have important functions in non-stressed conditions, namely in embryonic patterning.261,262 Hence, considering senescence as a "normal" adaptive state, the question is now: what is the fundamental role of SAPD in senescence, and why it was selected during evolution?

Cellular senescence was first thought to underlie organismal aging, and this hypothesis steadily gained experimental support.263 The deleterious effects of senescence are caused by the accumulation of senescent cells in aging organisms. However, it is possible that this accumulation is rather the result of an abnormal senescence program, and that evolution has selected a mechanism to avoid the accumulation of senescent cells. The recent literature suggests that this mechanism may be the clearance of senescent cells by the immune system.<sup>57-60</sup> The SASP seems critical to activate the immune response by signaling the presence of senescent cells and attracting destructive immune cells.5 If the ultimate destiny of senescent cells is their elimination, the production of signaling molecules during the SASP appears central to ensure a complete and effective senescence phenotype (Fig. 6). An abnormal SASP pattern or a defect in the capacity of the immune system to eliminate senescent cells could thus be the basis of an "abnormal" accumulation of senescent cells and age-related pathologies.<sup>263</sup>

The SASP is a costly anabolic process, and senescent cells have to deal with the limited availability of building blocks and energy to support the process. Thus, we can suppose that cells reorganize the distribution of these resources in order to favor the synthesis of cytokines. Does reorganizing the proteome mean reallocating resources? Such a link between autophagy and SASP has already been proposed.<sup>25,51,52</sup> Is the SAPD part of this reorganization? The degradation of specific proteins by the UPP could shut down highly energy-consuming functions, such as protein repair, DNA repair, synthesis of new ribosomes, and DNA synthesis. Since senescent cells are destined for clearance, these functions are dispensable for senescent cells, and their inhibition allows more resources to support the SASP (Fig. 6). Protein degradation by UPP consumes ATP, but the resulting amino acids can be used to obtain energy or supply building blocks for anabolic reactions. Overall, the SAPD could be a better investment for senescent cells whose final destiny is to be eliminated.

#### Breaking down cancer?

Although SAPD could be a powerful mechanism to mediate senescence and tumor suppression, it raises many new questions for further research. The exact contribution of protein degradation to senescence, including SAPD and autophagy, is still mostly speculative. Perhaps it simply brings a balance to cells unable to divide but making more proteins. However, catabolic processes may take a central place to induce cell cycle arrest of premalignant cells and to trigger their elimination by the immune system. Not only could proteolysis redistribute the resources to support the production of cytokines by oncogene-expressing cells, but it could also generate peptides for antigen presentation to ensure their specific recognition and destruction by immune cells (Fig. 6).<sup>264,265</sup> This may involve the production of an abnormal quantity of a self-antigen or the generation of abnormal antigens, such as pieces of activated oncogenes or damaged proteins.

A better understanding of the senescence degradome appears essential to have a more global picture of how anabolic and catabolic changes are linked together to trigger a complete senescence phenotype. This could provide insights into how cancer cells circumvent senescence and the role of metabolic changes in this process, thereby suggesting new therapeutic strategies. Targeting components of the UPP and autophagy with small-molecule inhibitors is an emerging area for the treatment of cancer.266 The clinical potential of this strategy has been highlighted by the success of the proteasome inhibitor bortezomib for the treatment of myeloma and lymphoma. Currently, most of the efforts

are invested in the development of proteasome inhibitors, which have a global, and thus non-specific, effect on the UPPmediated degradome. Such an approach can preferentially affect cancer cells where the pattern of E3 ubiquitin ligase activities and UPP-targeted substrates clearly support tumorigenesis and cancer progression (Fig. 5). However, the UPP has a fundamental role in normal cellular functions and in tumor suppression as well. This suggests caution in the clinical use of proteasome inhibitors and may explain the toxicity associated with these compounds.266 A better comprehension of SAPD and its dysfunction in cancer cells will certainly uncover new pharmacologic vulnerabilities to allow the rational development of new targeted therapies. Can we restore the advantages given by the SAPD, such as the elimination of precancerous cells by the immune system, and at the same time inhibit UPP-driven oncogenesis? In other words, can we tip the balance of protein breakdown to break down cancer?

#### **Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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