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Mechanisms of oncogene-induced genomic instability

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Abstract

Activating mutations in oncogenes promote uncontrolled proliferation and malignant transformation. Approximately 30% of human cancers carry mutations in the *RAS* oncogene. Paradoxically, expression of mutant constitutively active Ras protein in primary human cells results in a premature proliferation arrest known as oncogene-induced senescence (OIS). This is more commonly observed in human pre-neoplasia than in neoplastic lesions, and is considered a tumor suppressor mechanism. Senescent cells are still metabolically active but in a status of cell cycle arrest characterized by specific morphological and physiological features that distinguish them from both proliferating cells, and cells growth-arrested by other means. Although the molecular mechanisms by which OIS is established are not totally understood, the current view is that OIS in human cells is tightly linked to persistent activation of the DNA damage response (DDR) pathway, as a consequence of replication stress. Here we will highlight recent advances in our understanding of molecular mechanisms leading to hyper-replication stress in response to oncogene activation, and of the crosstalk between replication stress and persistent activation of the DDR. We will also discuss new evidence for DNA repair deficiencies during OIS, which might increase the genomic instability that drives senescence bypass and malignant transformation.

Graphical abstract

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Introduction

Cellular senescence was first described by Hayflick as the limited “lifetime *in vitro*” of human diploid fibroblasts [1], and refers to permanently growth-arrested cells that retain metabolic activity. This peculiar cellular state, known as “replicative senescence” (RS) is triggered by telomere attrition/dysfunction [2]. Other types of senescence include “stress-induced senescence”, which is caused by a variety of exogenous stresses [3] and “oncogene-induced senescence” (OIS), induced by aberrant activation of oncogenic signaling pathways [4]. Senescence is at the crossroads of aging and cancer, acting as a physiological barrier to malignant transformation at the expense of cellular aging [5, 6]. In fact, senescent cells are more often observed in pre-malignant lesions than in transformed cells [7, 8]. At the same time, senescence can promote tumorigenesis by chronic secretion of pro-inflammatory factors into the cellular milieu, which compromise the homeostasis of surrounding healthy tissues [3, 9]. The establishment of senescence and its effector programs is temporally regulated. Therefore, depending on the stage of OIS, anti- or pro-cancerous factors can be secreted by senescent cells into the surrounding environment. A recent study reported that OIS also allows clonal selection of cells carrying secondary hits in the genome, thus promoting malignant transformation [10].

Although the majority of studies investigate the senescent phenotype in a pathological context, this complex “growth-arrest program” plays also fundamental physiologic roles *in vivo*. There is evidence that cellular senescence is also part of the normal embryonic developmental program in mice. Embryonic senescent cells are distributed throughout the embryo [11–13], including the apical ectoderm ridge and the neural roof plate, two main signaling hubs in embryonic development [12]. Unlike OIS and RS, mouse embryonic senescence is independent of DNA damage and appears to be regulated by the TGF- β /SMAD and PI3K/FOXO pathways [13]. It shares with OIS and RS some typical features of senescence, such as activation of the senescence-associated secretory pathway (SASP) (discussed below), and increased expression of p21. Importantly, p21-null mice have impaired embryonic senescence and exhibit abnormal development, thus suggesting that developmentally programmed senescence fosters tissue remodeling during morphogenesis [11–13]. Some studies support the idea that senescent cells also mediate adult tissue remodeling by promoting wound healing through secretion of specific SASP factors [3, 14–16]. Specifically, matrix metalloproteinases secreted by senescent cells seem to help the resolution of the fibrotic scar resulting from healing processes in the liver [15] and cutaneous wounds [16]. These studies suggest that senescence has a broad spectrum of functions, from counteracting/promoting malignant transformation to mediating tissue repair [3]. Despite the increasing understanding of the physiological significance of senescence, some aspects of this complex process remain obscure. Here we discuss recent advances in our knowledge about the foundations of cellular senescence with particular emphasis on the molecular mechanisms having a causative role in the establishment of oncogene-induced senescence.

Activation of the DNA damage response during replicative and oncogene-induced senescence

Replicative senescence (RS)

Mammalian telomeres consist of repetitive sequences 5'-TTAGGG-3' that are bound by a protein complex named Shelterin (comprising TRF1, TRF2, POT1, TPP1, TIN2 and Rap1), which is essential for the formation of a T-loop structure that “caps” the end of the chromosomes and protects them from nucleolytic attack [17]. Shelterin coating of telomeres prevents activation of the DNA damage response (DDR) checkpoint protein kinases ATM and ATR [18, 19], thus protecting functional telomeres from end-to-end fusion and/or recombination events mediated by the DNA repair machineries. Because human somatic cells express very low levels of Telomerase, the enzyme committed to the replication of chromosome ends, telomeric DNA repeats are progressively lost during proliferation. Telomere shortening below a critical threshold length prevents proper capping, triggering the activation of the DDR that culminates with the establishment of senescence [20–23]. Interestingly, the onset of senescence does not require massive telomere attrition, being induced by the presence of only a few dysfunctional telomeres [20, 22, 24], which engage the DDR at chromosome ends leading to the formation of telomere dysfunction-induced foci (TIF) [23]. Chromatin immunoprecipitation studies have shown that repair factors such as 53BP1, ATM, MRE11, MDC1 and NBS1 localize to TIF [20, 22, 23]. These telomeric foci signal through ATM and p53 that in turn activate the cyclin-dependent kinase inhibitor p21, causing the G1 phase arrest typical of replicative senescence [20]. Importantly, inactivation of the main DDR protein kinases ATM-Chk2 and ATR-Chk1 abrogates replicative senescence and restores cell cycle progression [20, 22]. Therefore, the current view is that activation of the DDR has a central role in the initiation and maintenance of replicative senescence [25].

Oncogene-induced senescence (OIS)

Unlike replicative senescence, OIS is a very rapid and dynamic process independent of telomere shortening/dysfunction. Typically, the expression of an oncogene such as H-Ras^{V12} (a constitutive active form of Ras), Mos, Cdc6 or Cyclin E [8], induces aberrant activation of growth signaling pathways that are counteracted by establishing a growth arrest. During the initial “mitotic phase” cells experience DNA hyper-replication stress and cellular hyper-proliferation [26]. This is followed by a “transition phase” in which the DDR is activated, and finally the “senescent phase” is established [27]. OIS is tightly linked to persistent activation of the DDR [8, 10, 26, 28–31]. In fact, cells expressing oncogenic Ras (H-Ras^{G12V}) but unable to activate DDR due to depletion of either Chk2 or ATM, do not enter senescence [8, 26]. Similarly, oncogene expression in non-replicating cells, either growth-arrested or treated with DNA polymerase inhibitors, does not trigger DDR [8, 26]. These studies point to DDR as a major player in the establishment of RS and OIS.

Exceptions to activation of DDR during senescence

Although persistent activation of the DDR represents a central dogma of cellular senescence, a number of exceptions to this model have been reported. Ablation of the tumor suppressor

gene PTEN in mouse embryonic fibroblasts [32], or constitutive activation of either the MAPK p38 [33] or the kinase ATR [34], result in premature senescence in absence of cellular proliferation and activation of DDR. In addition, an elegant study of senescence *in vivo* suggests that OIS in mice can occur without a detectable activated DDR [35]. In addition, the authors showed a limited role for murine ATM in OIS. These data suggest that fundamental differences exist between senescence mechanisms in human and mouse cells, which remain to be fully understood. They also alert us to use caution when extrapolating findings about OIS among species.

Hallmarks distinguishing proliferating from senescent cells

DDR activation is only one of multiple effector pathways that cooperate in reprogramming metabolism and gene expression of mitotic cells into those of senescent cells. Some of these effector programs are initiated during the early phases of growth arrest while others are ascribed to the late stages of senescence. The concomitant presence of multiple of those morphological and physiological markers, distinguish senescent from proliferating cells [9, 36] (Figure 1).

Senescence-associated β -galactosidase (SA- β -gal) activity

Within a few days from the activation of the senescence program cells change morphology and appear flat and enlarged, suggesting high hypertrophy with respect to proliferating cells [1]. They present with increased senescence-associated β -galactosidase (SA- β -gal) activity [37], considered a hallmark of senescence that is not required for the growth arrest, being simply the reflection of increased lysosomal mass during senescence. This is in part due to the activation of autophagy [38] that is clearly visible by the abundance of cytoplasmic vacuoles in senescent cells. Interestingly, the activation of oncogenic Ras in proliferating cells is associated with repression of autophagy via the induction of the mTOR pathway [39, 40], while it exerts the opposite effect in senescent cells by up-regulating the expression of ATG5 and Beclin-1, two key regulators of autophagy [38, 41–43]. In addition, over-expression of the autophagy-related gene ULK3 is sufficient to induce senescence of human primary cells, while inhibition of autophagy delays senescence occurrence [27]. These studies suggest that autophagy plays a role during the metabolic transition from the mitotic state to senescence, at least in response to oncogenic Ras activation.

Senescence-associated protein degradation (SAPD) pathway

Another important feature of senescence metabolism is the activation of the senescence-associated protein degradation (SAPD) pathway. This is initiated and maintained through the ERK/MAP kinases signaling cascade involving ERK1 and ERK2 [44]. Importantly, genetic or pharmacological inhibition of these kinases in oncogenic Ras-expressing cells not only abolishes SAPD pathway activation and senescence, but also facilitates the transformation process of normal cells [44]. This study identified ERK/MAP kinases, central hubs of the cellular signaling network, and the SAPD pathway as essential machineries involved in the metabolic reprogramming of senescent cells. In fact, through the SAPD pathway proteins involved in cell cycle progression, RNA metabolism, mitochondrial function, cell migration, and cell signaling are degraded [44]. Thus, the SAPD is a selective macromolecules-

recycling network that runs in parallel to the broad-spectrum autophagic degradation pathway.

Senescence-associated secretory pathway (SASP)

Senescent cells secrete a complex mixture of factors including chemokines, cytokines, and proteases with a wide range of functions, known as the senescence-associated secretory pathway (SASP). Persistent activation of the DDR has been proposed as one of the main regulators of the senescent secretome [45, 46]. In particular, genetic ablation of the kinase ATM or of two of its substrates Chk2 or Nbs1, a component of the MRN complex, abolishes the secretion of IL-6 and IL-8, two major cytokines of the senescent secretome [46]. The same study also showed that senescence induced via over-expression of the CDK inhibitors p16 or p21 is not characterized by the secretion of pro-inflammatory factors [46], suggesting that activation of SASP occurs upstream of the factors ultimately imposing the permanent growth arrest. The senescent secretome (SASP) can have autocrine and paracrine effects, mediate pro- and anti-inflammatory signaling, and promote or counteract malignant transformation [47]. Importantly, the SASP has been linked to the low-grade, chronic inflammation that occurs as a consequence of normal aging, known as “inflammaging” [48].

Senescence-associated heterochromatic foci (SAHF)

Along with the complex metabolic reprogramming, a profound re-organization of constitutive and facultative heterochromatin, as well as of euchromatin occurs during senescence [36, 49–51]. As a consequence, the nucleus of senescent cells shows dense heterochromatin regions that can be visualized cytogenetically as foci intensely stained by DAPI. These senescence-associated heterochromatic foci (SAHF) are enriched in repressive epigenetic marks such as H3K9me3, HP1, and macroH2A [52–54]. Specifically, these foci appear as multilayered chromatin regions with a core of constitutive heterochromatin surrounded by facultative heterochromatin [55]. SAHF are also characterized by the presence of non-canonical histone proteins particularly by high-mobility group A (HMGA) proteins, and by the lack of the linker histone H1 [56]. Importantly, genome-wide analysis showed that SAHF are not formed *ex-novo* but rather they result from a spatial re-organization of pre-existing heterochromatin [56], and they embed genes that regulate cell cycle progression [25, 52]. Although SAHF are considered a hallmark of senescence, Kosar et al. have shown these structures manifest during senescence in a cell type-specific manner, unless oncogenic Ras is expressed. SAHFs are formed in a cell type- and stress-dependent fashion, and strongly depend on the activation of p53-p21 and p16^{ink4a}-pRb pathways [36]. These two pathways are triggered by persistent activation of the DDR, and SAHF formation is impaired in cells expressing oncogenic Ras where key DDR genes are inactivated [25, 57], thus reinforcing the idea of the DDR as master regulator of the senescent program.

Senescence-associated distension of satellites (SADS)

Other alterations of chromatin are represented by large-scale distension of pericentromeric satellites upon expression of oncogenic Ras, oxidative stress, and replicative senescence [50, 51]. Super-resolution microscopy revealed that these pericentromeric satellites are globular domains interspaced by linker regions. In cycling cells, these domains are compacted, while in senescent cells appear as more extended linear conformations [51]. Unlike SAHF, the

presence of SADS is more consistent and ubiquitous across different cells, it is not dependent on p16 or p21 pathways, nor characterized by changes in canonical histone marks or in DNA methylation status [50, 51]. Rather, SADS seems to be tightly dependent on nuclear structure, and therefore on the expression of nuclear lamins (type A and B) which are components of the nucleoskeleton. In fact, pericentromeric heterochromatin is enriched in lamin-associated domains (LADs) [58], and therefore defects in nuclear architecture can affect satellite organization [59]. A whole body of evidence demonstrates extensive reduction of lamin B1 during the early stages of different types of senescence [60–65], and single cell analysis suggests that loss of lamin B1 facilitates the formation of SADS [50, 51]. Although controversial, a number of studies link lamin B1 expression to the amount of reactive oxygen species (ROS) in senescent cells, and propose lamin B1 as a regulator of ROS [47, 60, 61, 64–68]. The opening of centromeric and pericentromeric regions in senescent cells increases transcription of satellite DNA, with the consequent mobilization of the major known retro-transposon classes Alu, SVA and L1, which can cause DNA damage and alterations in gene expression as observed during cellular aging, and in cancer cells [69]. Although SADS occur early during senescence, whether or not any of these chromatin re-arrangements contribute to the initial replication stress remains un-explored. A recent study reported that senescent cells also exhibit leaking of chromatin fragments enriched in γ H2AX and in H3K27me3 to the cytoplasm that are processed by the autophagy/lysosomal pathway, thus leading to a reduction in the total histone content [49]. Importantly, this chromatin leaking is strongly associated with down-regulation of lamin B1, and loss of integrity of the nuclear envelope [49]. Although growth-arrested, senescent cells exhibit intense metabolic activity, and while some of these players might be simply recycled through the autophagy-lysosome and SAPD pathways, some of them need to be specifically expressed. Thus, the profound re-organization of chromatin occurring during senescence might represent an intrinsic mechanism to globally shift the gene expression profile from that of a mitotic cell into that of a senescent cell.

DNA hyper-replication stress activates DDR during OIS

There is compelling evidence that initiation and maintenance of oncogene-induced senescence is strikingly dependent on the DDR pathway [8, 10, 25, 28, 57, 70, 71]. A number of studies point to DNA hyper-replication stress as a cause of persistent DDR activation, following the initial proliferative burst induced by activation of oncogenes in human normal cells [8, 10, 25, 70, 72]. Di Micco et al. showed that a significant fraction of oncogenic Ras expressing cells arrest with partially replicated DNA content, and display altered signals for known DNA replication origins (lamin B2 and β -globin) when inquired by fluorescence *in situ* hybridization (FISH) [25]. Importantly, oncogene activation in cells unable to replicate DNA does not activate DDR, thus providing strong evidence about a causative role of DNA replication stress in DDR activation [25]. Accordingly, Bartkova et al. reported that over-expression of the oncogenes Cyclin E in osteosarcoma cells (U2OS), and Mos or Cdc6 in normal foreskin fibroblasts (BJs), results in accumulation of ssDNA marked by the activated form of replication protein A (phosphorylated RPA), and that in these cells DNA damage foci co-localize with sites of DNA replication stained by PCNA (proliferating cell nuclear antigen) [8]. In addition, both studies showed that expression of oncogenes

Ras^{V12}, Cyclin E or Mos in normal cells results in impaired DNA replication dynamics. In particular, DNA combing analysis revealed smaller distance between origins, discontinuous fork advancement, and premature termination of DNA replication forks. This suggests that in the earlier stages of OIS increased number of active replicons, impaired fork stability, and extensive fork pausing might occur [8, 25]. This impairment of DNA replication results in activation of ATR and ATM, and subsequent engagement of an intra-S phase checkpoint that culminates in DDR activation and S-phase arrest.

Recent studies propose that depletion of deoxyribonucleotide (dNTP) pools is a main cause of replication stress during OIS. Specifically, Aird et al. reported that the ribonucleotide reductase (RR) subunit M2 (RRM2), the rate-limiting enzyme in dNTP synthesis, is downregulated prior to growth arrest in oncogenic Ras-expressing cells [30]. An independent study also reported depletion of dNTP pools in oncogene-induced senescent cells due to down-regulation of both the RR1 and RR2 subunits of RR enzyme and of thymidine kinase 1 (TK1) enzyme, involved in *de-novo* and salvage dNTP synthesis pathways, respectively [31]. In both studies, depletion of either one of these enzymes was sufficient to induce senescence of human normal cells, while ectopic expression of these factors or supplementation of dNTPs reverted the senescent phenotype [30, 31]. The decrease in dNTP pools leads to increased expression of two important mediators of growth arrest during OIS, p53/p21 and p16/Rb, which in turn inhibit the transcription activator E2F1. Interestingly, chromatin immunoprecipitation (ChIP) analyses showed increased occupancy of the repressive transcription factor E2F7 and a simultaneous reduced E2F1 factor binding, at the proximal promoter of RRM2 prior to cell cycle exit in Ras-expressing cells [30]. The same group also showed that down-regulation of RRM2 activates p53, which is known to increase E2F7 expression, thus suggesting a crosstalk among these pathways in maintaining the stable growth arrest. In addition, Mannava et al. reported that these regulatory regions are enriched in the heterochromatic marker H3K9me3 during the growth arrest, suggesting their incorporation into SAHF structures [31]. Thereby, binding of repressive transcription factors to *in cis* regulatory regions of key genes early during OIS, flags those loci for recognition by repressive chromatin remodeling complexes that mediate the global heterochromatinization characteristic of the late senescence stages.

It has been proposed that ATM, a sensor of DNA replication stress, is the main upstream mediator of nucleotide metabolism suppression. Specifically, ATM depletion rescues dNTP levels and ameliorates DNA replication stress in cells depleted of RRM2, prior to senescence establishment [73]. This increased substrate availability is due to up-regulation of glucose-6-phosphate dehydrogenase (G6PD), the rate limiting enzyme in the pentose phosphate pathway. This pathway is the main source of metabolites for dNTP synthesis starting from glucose and glutamine, the uptake and metabolism of which was remarkably enhanced [73]. In addition, a coordinated loss of p53 and up-regulation of c-Myc was observed downstream of ATM ablation. Therefore the authors propose that ATM status is a central mediator in coupling replication stress with metabolic reprogramming during senescence. The current model is that replication stress activates ATM that in turn induces p53, which inhibits G6PD and decreases dNTP pools. In the context of ATM inhibition, replication stress activates c-Myc, which increases the uptake of glucose and glutamine and the amount of available

dNTPs, thereby bypassing growth arrest. This mechanism is proposed to facilitate the metabolic reprogramming of senescent cells toward that of a transformed cell.

Importantly, DNA replication stress and DDR markers have been observed also in tissue sections of different types of pre-cancerous lesions enriched in cells positive for markers of OIS [30, 70]. The observation that OIS relies on the same mechanisms *in vivo*, reinforce the idea that DNA replication stress activate DDR that acts as anti-cancer barrier in early human tumorigenesis [70].

DNA damage repair during OIS

Despite recent advances in our understanding of the mechanisms involved in OIS establishment, fairly few studies have attempted to shed light on the ability of oncogene-expressing cells to repair DNA damage. Oncogene-expressing cells accumulate unrepaired DNA damage, as evidenced by the presence of DNA damage foci. In particular, oncogene-expressing cells display aberrant DNA replication dynamics that result in accumulation of ssDNA, which eventually leads to the formation of double strand breaks (DSBs) [8, 25]. Unrepaired DSBs represent a big threat for genome stability, and thus cells have in place two complex mechanisms for the repair of DNA DSBs: non-homologous end-joining (NHEJ) and homology-directed repair (HDR) [74]. In mammalian cells, NHEJ seems to act independently of the cell cycle phase and is considered an error-prone mechanism of DSB repair, in which the two broken DNA ends are joined together with some loss of sequence in the process. HDR is mainly active during S/G2 phases of the cell cycle, requiring resection of the broken DNA ends and the presence of a sister chromatid as template to initiate recombination [75]. Although HDR is considered an error-free mechanism of repair that generally utilizes a high fidelity gene-conversion (GC) reaction, cells can also perform a type of HDR known as single-strand annealing (SSA), which is highly mutagenic [76]. The ability of oncogene-induced senescent cells to utilize these mechanisms to repair DSBs remains poorly understood.

Two DNA repair factors, BRCA1 and 53BP1, play a decisive role in the competition between DNA DSB repair pathways [77, 78]. Elegant studies showed that while BRCA1 promotes DNA end-resection, a pre-requisite for RAD51-mediated HDR by GC, 53BP1 inhibits end-resection and thus facilitates NHEJ [79–81]. However, recent studies have challenged the simplistic idea that 53BP1 always antagonizes HDR. Ochs et al. showed that the extensively resected DSBs that result from 53BP1 deficiency promote the mutagenic SSA-HDR over the conservative GC-HDR [76]. These results suggest a more complex function of 53BP1 in DNA DSB repair, whereby in addition to the classical role in NHEJ, 53BP1 promotes GC-HDR and antagonizes SSA-HDR, at least in late S/G2 phases of the cell cycle. Although more studies are needed to fully understand the implications of this competition between BRCA1- and 53BP1-mediated DNA repair, it is clear that a balance between both pathways is critical for the maintenance of genome stability [82, 83]. As such, the imbalance generated by either loss of BRCA1 or 53BP1 has been associated with genomic instability and cancer susceptibility, while loss of both proteins rescues at least partially these phenotypes. For instance, loss of 53BP1 alleviates HDR defects in BRCA1-deficient cells and rescues the embryonic lethality that results from genomic instability in

BRCA1-deficient mice [81]. These studies demonstrate that 53BP1 loss is synthetically viable with BRCA1 loss [77, 84, 85]. This functional relationship between 53BP1 and BRCA1 is important in the context of cancer. Reduced 53BP1 levels have been reported in triple negative breast cancers (TNBC) and BRCA1-mutated breast tumors, suggesting that 53BP1 loss contributes to the survival of BRCA1-deficient tumor cells [79, 86]. Moreover, 53BP1 loss confers resistance of tumor cells to replication inhibitors such as cisplatin and PARP inhibitors, and thus strategies that stabilize 53BP1 levels in the context of BRCA1-deficiency could be used to improve the response to these therapeutic agents. Although the interplay between BRCA1 and 53BP1 has been extensively characterized in a variety of contexts, it remains to be characterized in the context of OIS.

Changes in BRCA1 and 53BP1 expression in growth-arrested cells

Studies in our laboratory revealed a correlation between 53BP1 and BRCA1 in growth-arrested cells. Primary human fibroblasts arrested by contact inhibition and breast cancer cells (MCF7) arrested by serum deprivation show a profound reduction in BRCA1 and 53BP1 protein levels [87]. Growth-arrested cells downregulate BRCA1 transcripts, resulting in reduced protein levels, and upregulate the protease cathepsin L (CTSL), which in turn mediates the degradation of 53BP1 protein. Accordingly, depletion of CTSL prevented the decrease in 53BP1 protein levels during growth arrest, without any effect on BRCA1 levels [87]. In addition, we found that in breast tumor cells (MCF7), depletion of BRCA1 activates CTSL-mediated degradation of 53BP1, allowing these cells to overcome the genomic instability and growth arrest caused by BRCA1 loss [86]. Importantly, inhibition of CTSL activity directly via CTSL inhibitors, or indirectly via the vitamin D/vitamin D receptor (VDR) axis, results in stabilization of 53BP1 in the context of BRCA1 deficiency. This imbalance between 53BP1 and BRCA1 increases genomic instability and sensitivity to DNA damaging strategies such as ionizing radiation and PARP inhibitors [86], providing a potential strategy to overcome drug resistance in tumors that lose 53BP1 function. Moreover, depletion of A-type lamins (lamin A/C), structural components of the nuclear lamina, results in reduced levels of BRCA1 transcripts and protein and activation of CTSL-mediated degradation of 53BP1 [88–90]. Altogether, these studies suggest that the levels of BRCA1 and 53BP1 are reduced in response to stresses that cause growth arrest. Of note, different reports have shown a marked upregulation of CTSL as well as its accumulation in the nucleus upon expression of oncogenic Ras [91–94], although the effect on 53BP1 was not tested.

Dissociation of BRCA1 from chromatin during OIS

A study recently reported that oncogenic Ras expression inactivates the BRCA1 DNA repair complex by dissociating BRCA1 from chromatin, prior to growth arrest and coinciding with the accumulation of DNA damage [10, 28]. Specifically, BRCA1 dissociation from chromatin seems to be dependent on Ras-mediated down-regulation of BRCA1-interacting protein 1 (BRIP1) [10]. Loss of BRIP1 occurs prior to Ras-induced cell cycle exit through reduced binding of B-Myb to the promoter of the BRIP gene, concomitant with upregulation of microRNA 29 (mir29), a known suppressor of B-Myb expression. Moreover, depletion of BRIP1 alone is sufficient to induce expression of senescence markers and to drive BRCA1 dissociation from chromatin. Importantly, oncogenic Ras-expressing cells subjected to DNA

damage by ionizing radiation were able to bypass senescence more efficiently than not irradiated cells, and formed colonies under anchorage-independent growth conditions (soft-agar), indicating that they were transformed [10]. Thus, oncogenic Ras is thought to predispose senescent cells to additional hits in the genome that facilitate senescence bypass and cellular transformation. The reduced binding of BRCA1 to chromatin during the initial hyper-replication phase of OIS could hinder HDR, contributing to DNA damage and accumulation of additional mutations that ultimately facilitate cellular transformation. Whether BRCA1 dissociation from chromatin during OIS affects 53BP1 expression or function was not tested, but it is conceivable that a reduction of 53BP1 levels, as in other types of growth arrest, could help senescence bypass and transformation.

Loss of BRCA1 and 53BP1 during OIS is regulated by vitamin D/vitamin D receptor axis

Our recent studies in primary human fibroblasts retrovirally transduced with oncogenic Ras (H-Ras^{G12V}) revealed a marked downregulation of BRCA1 transcript and protein levels during OIS (Figures 2A&B). In addition, 53BP1 protein levels and recruitment to DNA damaged sites are decreased in Ras-expressing cells, concomitant with the upregulation of CTSL expression [95] (Figures 2B&C). Importantly, CTSL depletion prior to oncogenic Ras expression prevents 53BP1 loss, indicating that CTSL-mediated degradation of 53BP1 is activated during OIS, as previously shown in other growth arrest contexts. In addition, we observed downregulation of vitamin D receptor (VDR) expression during OIS (Figures 2A&B). This is in line with previous reports showing reduced nuclear VDR activity in mouse fibroblasts expressing oncogenic Ras [96], instability of VDR transcripts in tumor cells [97], and stress-induced senescence in mouse vascular smooth muscle cells lacking VDR [98].

Treatment of Ras-expressing cells with 1,25 α -dihydroxy-vitamin D₃ (calcitriol), the most bioactive vitamin D metabolite, increased VDR levels, as expected. Unexpected however was the finding that vitamin D treatment restores BRCA1 and 53BP1 levels, as well as their recruitment to DNA repair foci (Figure 2C), revealing a role for the vitamin D/VDR axis in the regulation of these two important DNA repair factors during OIS [95]. Of note, the decrease in VDR and DNA repair factors BRCA1 and 53BP1 seems to be linked to the expression of oncogenic Ras, as other types of senescence did not show these deficiencies.

There is evidence that the vitamin D/VDR axis regulates indirectly BRCA1 expression [99], and that VDR interacts with BRCA1 [100]. However, the functional relationship between VDR and BRCA1 in DNA repair remains obscure. Our studies in normal human fibroblasts depleted of VDR showed a marked reduction of BRCA1 expression, when compared to VDR-proficient fibroblasts [95]. As a consequence, VDR-depleted cells are unable to recruit BRCA1 to sites of DNA damage (Figure 2D), leading to defects in HDR (Figure 2E), accumulation of unrepaired DSBs, and a fast onset of senescence. These data suggest that VDR deficiency could contribute to OIS by hindering DNA repair by HDR. Importantly, CTSL and 53BP1 protein levels were not affected by VDR depletion, indicating that activation of CTSL-mediated degradation of 53BP1 during OIS is independent of VDR and dependent on oncogenic Ras expression. Moreover, these data indicate that VDR deficiency causes an imbalance between 53BP1 and BRCA1, which in a variety of contexts causes

genomic instability and proliferation arrest. Consistent with this notion, depletion of 53BP1 allows VDR-deficient cells to bypass senescence [95]. These studies uncovered a critical role for 53BP1 in the establishment of senescence due to VDR loss, as previously shown in the context of BRCA1 depletion [86].

Altogether, these data demonstrate a functional relationship between the oncogene Ras, the vitamin D/VDR axis, and the expression of key DNA repair factors in the context of OIS (Graphical abstract). We propose a model whereby expression of oncogenic Ras activates CTSL-mediated degradation of 53BP1 and reduces VDR expression, which in turn causes a decrease in BRCA1 function. Vitamin D treatment inhibits CTSL-mediated degradation of 53BP1 and stabilizes VDR and BRCA1 levels. Although vitamin D treatment was not sufficient to prevent OIS, it improved DNA repair capabilities in senescent cells [95]. We speculate that vitamin D treatment might ameliorate the generation of secondary hits in the genome that promote senescence bypass and malignant transformation (Graphical abstract). However, it is possible that activation of vitamin D/VDR signaling affects additional cellular pathways, with consequences for proliferation, senescence, and potentially malignancy. Future studies are needed to understand in depth the consequences of VDR deficiency for the genomic instability that drives senescence, senescence bypass, and tumorigenesis.

Concluding remarks and future studies

There is extensive evidence that OIS is induced and maintained by hyper-replication stress and persistent activation of the DDR. Recent studies also show deficiencies in DNA DSB repair during OIS. Despite remarkable advances in the field, little is known the molecular mechanisms whereby the initial replication stress culminates in the accumulation of DNA damage over a certain threshold necessary to induce DDR-mediated growth arrest of cells carrying activated oncogenes. In particular, future studies need to address the possibility that cellular mechanisms that protect forks from translating into DSBs are hindered during OIS.

The extensive origin firing occurring during OIS could account, at least in part, for the rapid exhaustion of limited dNTPs [30, 31] and of replication protein A (RPA) [8], which would result in fork stalling and fork de-protection, respectively [101]. These results might explain the accumulation of stalled forks but do not provide any insights about their fate. Under normal conditions, stalled forks can be restarted or they can collapse into DSBs [102, 103]. In human cells, stalled forks are coated by a plethora of factors that cooperate in mediating replication fork restart, including SMARCAL1, PARP1, MRE11, XRCC3, RAD51, MUS81, and helicases BLM, WRN, and RECQ1 [102–105]. In particular, the structure-specific endonuclease MUS81 can mediate fork restart upon prolonged chemical depletion of dNTPs [106], a similar scenario to that observed during OIS. The fact that OIS cells accumulate partially replicated DNA [25] suggests that replication fork restart might be impaired during senescence, perhaps because of aberrant function of MUS81 or its interactors. Therefore it is possible that accumulation of stalled forks during OIS is due in part to suppression of nucleotide metabolism [30, 31, 107], and in part to the inability of cells to restart replication. In this picture, prolonged stalled forks could degenerate into collapsed forks leading to the generation of DSBs. These possibilities remain to be tested in the context of OIS.

Another factor that might contribute to stalling of the forks is the global re-organization of nuclear architecture that takes place during the initial phase of OIS. The nuclear lamina is known to be essential for the proper spatial-temporal organization of the replication machinery [108–112]. In particular, lamin B1 normally co-localizes with PCNA during replication [112], and its loss induces a prolonged S-phase [113]. Depletion of lamin B1 was studied during the initial phase of OIS for his causative role in decondensation of chromosome territories [60–65], however no direct evidence is available to date supporting a role for lamin B1 loss in inducing stalled forks during OIS.

An additional point in the “dark road” from replication stress to DSBs generation, regards the protection of stalled forks during OIS. Replication fork protection is necessary to prevent the collapse of stalled forks and generation of DSBs. Human cells recruit a number of DNA repair factors such as BRCA2, BRCA1, RAD51, 53BP1, and Fanconi Anemia proteins to protect stalled forks from degradation mediated by the MRN-complex subunit MRE11 [114–117]. The finding that BRCA1 function is reduced during OIS [10, 95] suggests a possible mechanism contributing to deprotection of stalled forks, leading to DSBs. In addition, *in vivo* studies suggest that MRE11 complex is required for DDR activation upon NeuT oncogene expression [118]. An interesting idea is that dissociation of protective factors from chromatin during hyper-replication stress might induce extensive MRE11-mediated resection of stalled forks, which would activate DDR and growth-arrest. Further studies are needed to identify the sequential steps required to convert stalled forks into DSBs, during OIS.

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Highlights

- Oncogenic Ras expression causes replication stress and DNA damage response (DDR)-dependent entry into senescence.
- Senescent cells activate cathepsin L (CTSL)-mediated degradation of 53BP1 and downregulate expression of VDR and BRCA1.
- Treatment of senescent cells with vitamin D stabilizes VDR and DNA repair factors (53BP1 and BRCA1).
- Vitamin D improves DNA repair capabilities in senescent cells, which might reduce the extent of secondary hits in the genome that promote malignant transformation.

Figure 1. Hallmarks of senescence

Establishment and maintenance of both oncogene-induced senescence (OIS), and replicative senescence (RS) is dependent on DDR activation. While in RS the DNA damage is due to telomere erosion across multiple mitotic divisions, in OIS this is due to the initial hyper-replication stress. In both cases, ATM-Chk1 and ATR-Chk2 pathways signal to the downstream effectors p53 and pRb that cooperate in growth-arresting the cells. Senescent cells do not proliferate but retain intense metabolic activity as demonstrated by activation of multiple effector pathways: the senescence-associated secretory pathway (SASP) with

autocrine and paracrine functions; the senescence-associated protein degradation pathway (SAPD), important recycling network that along with autophagy provide essential metabolites to sustain the senescent hypertrophy. Senescent cells also undergo profound reorganization of chromatin as shown by the formation of senescence-associated heterochromatic foci (SAHF), and senescence-associated distension of satellite (SADS). This chromatin remodeling is thought to mediate the shift from the initial mitotic gene expression profile to that typical of a senescent cell.

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Figure 2. Loss of DNA repair factors during Ras-induced senescence

Human primary fibroblasts were infected with retroviruses encoding oncogenic Ras or an empty vector control (EV), and cells processed for different assays. **(A)** Graph shows the relative expression of transcripts of DNA repair factors BRCA1, RAD51 and 53BP1, as well as VDR and CTSL, as measured by qRT-PCR. Note the downregulation of HDR factors (BRCA1 and RAD51) and VDR, and the upregulation of CTSL, with no changes in 53BP1 transcripts. **(B)** Immunoblots showing reduced levels of BRCA1, VDR and 53BP1 proteins and increased levels of CTSL in response to oncogenic Ras expression. Vinculin was used as

loading control. **(C)** Graph shows the quantification of DNA repair foci labeled with 53BP1, γ H2AX, or BRCA1 in Ras-expressing cells or control cells, and in response to ionizing radiation (IR) and vitamin D (VD) administration. Note how VD improves the ability of Ras-induced senescent cells to recruit DNA repair factors to sites of DNA damage. **(D)** Graph shows quantification of immunofluorescence staining of BRCA1 at different times (hours) after ionizing irradiation in human normal cells depleted for VDR. Note how VDR-depleted cells lose the ability to recruit BRCA1 to sites of DNA damage (ionizing radiation-induced foci or IRIF). **(E)** HDR-reporter assay, showing impaired HDR efficiency in VDR-depleted cells, compared to control (EV) cells.