

Chapter 2

Senescence Regulation by mTOR

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Abstract

The senescence program is activated in response to diverse stress stimuli potentially compromising genetic stability and leads to an irreversible cell cycle arrest. The mTOR pathway plays a crucial role in the regulation of cell metabolism and cellular growth. The goal of this chapter is to present evidence linking these two processes, which have one common regulator—the tumor suppressor p53. While the role of mTOR in senescence is still controversial, recent papers have shed new light onto this issue. This review, far from being exhaustive given the complexity of the field, will hopefully stimulate further research in this domain, whose relevance for ageing is becoming increasingly documented.

Key words: AKT, CDK, CKI, Cell cycle exit, D-type cyclins, mTOR, pRB, Quiescence, Senescence

Abbreviations

ARF	Alternative reading frame
ATM	Ataxia telangiectasia mutated
ATR	ATM and Rad3-related kinase
Bmi1	B lymphoma Mo-MLV insertion region 1
Cdc25	Cell division cycle 25 (CDK-activating phosphatase)
CDK	Cyclin-dependent kinase
Chk1/2	Checkpoint kinase 1/2
CKI	CDK inhibitor
ERK1/2	Extracellular regulated kinase (also called MAPK)
FOXO	Forkhead box protein O
FKBP12	Peptidyl-prolyl <i>cis/trans</i> isomerase that forms a complex with rapamycin
HES1	Hairy enhancer of Split1
IGF	Insulin-like growth factors
IRS	Insulin receptor substrate
INK4A	Inhibitor of Cdk4 A
MAPK	Mitogen-activated protein kinase
mTOR	Mechanistic (mammalian) target of rapamycin
TORC1/2	TOR complex 1 and 2
PcG	Polycomb group

PDK1	3-Phosphoinositide-dependent kinase 1
PI3K	Phosphoinositide 3-kinase
PIKK	Phosphoinositide 3-kinase (PI3K)-related protein serine/threonine kinase family
PML	Promyelocytic leukemia protein
PTEN	Phosphatase and tensin homologue
pRb	Retinoblastoma protein
RHEB	Ras homologue enriched in brain
SAHF	Senescence-associated heterochromatin foci
SASP	Senescence-associated secretory phenotype
SGK	Serum- and glucocorticoid-regulated kinase
TSC1/2	Tuberous sclerosis 1,2 GTPase
TGF β	Transforming growth factor β

1. Introduction

Cellular senescence was originally described by Leonard Hayflick, who showed that, after a finite number of population doublings (mitotic clock), primary human cells irreversibly cease to proliferate in vitro (1). This so-called Hayflick limit was later shown to be provoked, at least in part, by telomere erosion (2), the gradual loss of DNA at the ends of chromosomes, a consequence of the “replication problem” predicted by Olovnikov in the early 1970—hence “replicative senescence” (3). It has been therefore proposed that telomere erosion could activate a DNA damage response (DDR) and cause G1 cell cycle arrest similar to the one elicited by ionizing radiation (4). Moreover, following an excellent intuition, Hayflick associated cellular senescence with both cancer and ageing—with cancer, because cells must acquire certain characteristics of tumor cells to escape senescence, and with ageing, because accumulation of senescent cells could contribute to the global deterioration of an organism. We now know that these original hypotheses were fundamentally right, and since then, senescence, from a “tissue culture artifact,” became an ever-expanding field of intense research largely due to its tumor suppression potential and contribution to age-related pathologies. In addition to telomere dysfunction, senescence can be induced by a large variety of stress stimuli, including strong mitogenic signals generated by hyperactivation of certain oncogenes (Ras, Raf, Myc), irreparable DNA damage, or oxidative stress. This invariably inhibits cyclin-dependent kinases (CDK), key cell division regulators, leading to the cell cycle arrest that requires activities of two major tumor suppressors, p53 and pRb (Fig. 1).

As a number of excellent recent reviews extensively covered this topic (5–7), we shall here briefly outline the aspects of cellular senescence and the pathways implicated in its implementation that are relevant for this chapter.

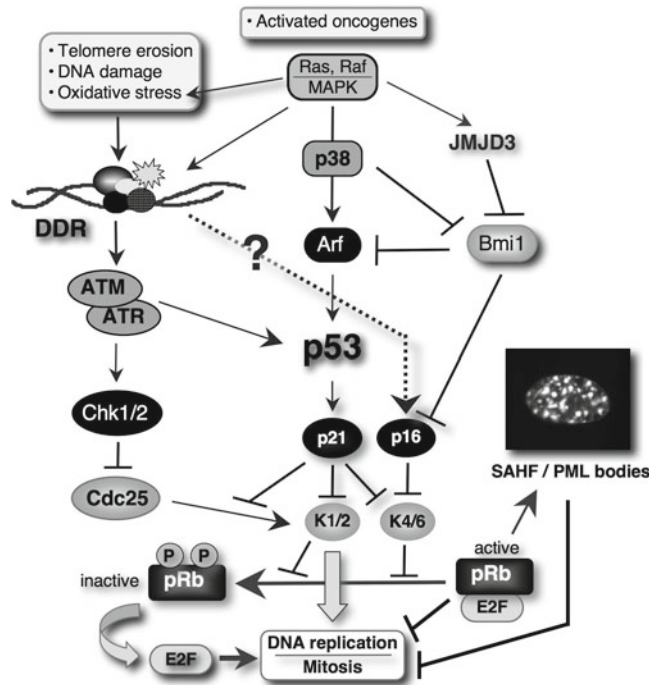


Fig. 1. Molecular pathways leading to senescence-associated irreversible cell cycle arrest. Pro-senescence stimuli activate DNA damage response pathway (DDR) leading to permanent inactivation of cyclin-dependent kinases (CDK) that control DNA replication and mitosis (K1/2). In addition, together with Cdk4/6 (K4/6), they keep inactive tumor suppressor pRb (and related pocket proteins p107 and p130). Genotoxic stress and activated oncogenes induce expression of CDK inhibitors p21^{Waf1/Cip1} (p21) and p16^{Ink4A} (p16) that block cell cycle progression and pRb inactivation. Active pRb blocks expression of genes controlling cell cycle (in part by sequestering E2F family of transcription factors) and contributes to chromatin reorganization in the form of SAHF or PML nuclear bodies. The pathways leading to induction of p16 are not entirely elucidated but, in the case of OIS (oncogene-induced senescence), it has been shown that demethylase JMJD3 blocks repression of Ink4A locus by PcG protein Bmi1. Arf (p19^{ARF}), another product of this locus, plays a major role in senescence in mice, while p16 is more important in humans. Figure adapted from ref. 119.

2. How is Permanent Cell Cycle Arrest Achieved?

Senescence is a viable and metabolically active state, which is characterized by a virtually permanent (irreversible) cessation of cell division. It is associated with dramatic changes in cell morphology (large flat cells), metabolism, gene expression and secretion patterns (senescence-associated secretory phenotype or SASP), originally described by Campisi and colleagues (8). Senescence has recently been shown to play a crucial role in age-related pathologies associated with accumulation of senescent cells (9, 10). Both in vitro and in vivo, senescent cells can be detected by virtue of β -galactosidase activity (β -gal) (11), a lysosomal enzyme, which probably does not play a direct role in senescence (12), but serves as a reliable biomarker

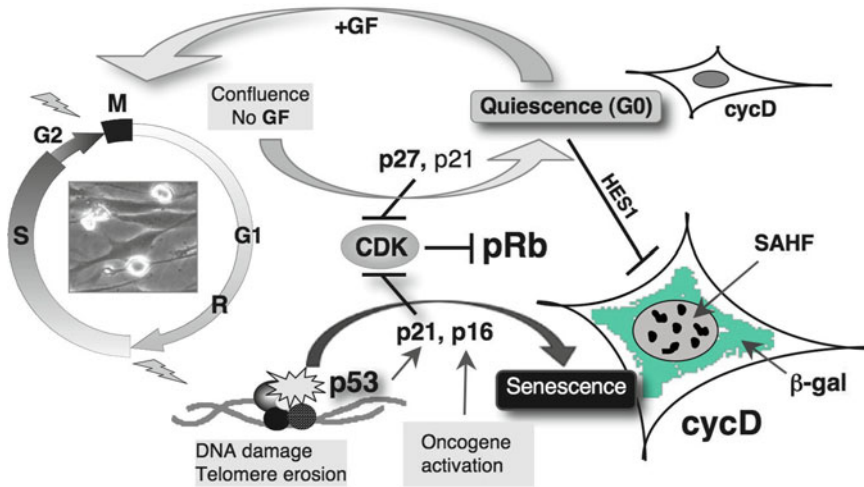


Fig. 2. Senescence versus quiescence. Quiescence (or G0) is reversible and hypotrophic cell cycle exit that occurs in G1 prior to restriction (R) point. It is mediated preferentially by p27-dependent CDK inactivation and cyclin D1 downregulation. Some results suggest that genes expressed in quiescent cells can block senescence (HES1). By contrast, senescence is irreversible and hypertrophic cell cycle exit that takes place either in G1 or G2, depending on the activated checkpoint. Cell cycle arrest is mediated by p16/p21-dependent CDK inactivation (Fig. 1) and senescent cells contain high levels of G1 cyclins (cyclin D and cyclin E) associated with inactive CDK. While the cytoplasm could be frequently stained for β -galactosidase (see Chapters 4, 8 and 9), senescent nuclei are often large and sometimes binuclear (due to endoreplication or defective cytokinesis) and could contain heterochromatin in the forms of SAHF (see Chapter 12) or PML bodies. Both events require active pRb family pocket proteins but in senescence only pRb is essential (ref. 19). GF growth factors.

(see Chapter 4 and Fig. 2). In addition, senescent cells often exhibit dramatic rearrangement of chromatin structure, in the forms of PML (promyelocytic leukemia protein) nuclear bodies (13, 14) and senescence-associated heterochromatin foci (SAHF) (15). These heterochromatin structures, whose formation requires active pRb, are thought to play an important role in conferring irreversibility of the cell cycle arrest by repressing the genes controlling cell proliferation (Figs. 1 and 2). However, SAHFs are not observed in all cell types and in all forms of senescence (15), and recent work associates SAHF formation with oncogene induced senescence and repression of DNA damage signaling (16).

The pro-senescence stimuli all converge on one major pathway to permanently arrest the cell cycle (Fig. 1). The senescence program is initiated by inactivation of CDK (17), the key cell cycle regulators. CDKs play two distinct roles. First, they control the onset and progression of DNA replication and mitosis, thus enabling and orchestrating the cell cycle. Second, CDKs, particularly Cdk4/6 associated with D-type cyclins, phosphorylate, thus keeping inactive, the retinoblastoma (pRb) tumor suppressor and related “pocket-proteins” (p107, p130). This is essential for cell cycle progression, since active (hypo-phosphorylated) pocket proteins drive cell cycle exit by sequestering E2F family transcription factors and by repressing the genes required for cell division (18).

Despite often-overlapping functions between different members of the pocket protein family, pRb was shown to play a unique and nonredundant role in senescence by repressing E2F target genes involved in DNA replication (19) (Fig. 1). Recent data suggest that this process requires a recruitment of promoter-bound pRb and E2Fs to PML nuclear bodies (14). More recently, p130/E2F4 complex was also implicated in senescence by repressing, via PML, T-box protein 2 (TBX2) frequently overexpressed in cancer (20).

The presence of diverse genotoxic stresses that induce senescence activates a DDR network, controlled by ATM/ATR kinases, which blocks CDK activation via CDC25 family phosphatases, thereby temporarily halting cell cycle progression (Fig. 1). While not required for this transient arrest, p53 and its transcriptional target p21^{Waf1/Cip1/Sdi1} (p21), a CDK inhibitor (CKI), are essential parts of irreversible cell cycle arrest by the senescence program (21, 22). Permanent CDK inactivation by p21 stably blocks DNA synthesis and mitotic entry and, via activation of pRb, drives exit from the cell cycle (19, 23) (Fig. 1). Another CKI, p16^{Ink4A} (p16), which targets specifically pRb kinases Cdk4/6, also plays an important role in senescence and, along with β -gal, it is often used as a biomarker both in vitro and in vivo (9, 24). However, unlike p21, p16 does not seem to be involved in the cell cycle arrest associated with replicative senescence (21) and its late induction, which occurs after p21-mediated cell cycle arrest, might promote a stabilization of senescent state (25, 26). Nevertheless, p16 is a key regulator of oncogene-induced senescence (OIS) (27) and recent work in a mouse model strongly implicated this inhibitor in acquisition of age-related pathologies (9). Although p16 is widely considered as an essential part of the senescence program, the pathways regulating its induction in response to senescence-promoting stimuli are still not entirely elucidated. It has been suggested that, in the case of OIS, activation of histone demethylase JMJD3 suppresses the repression of the INK4A-ARF locus by Polycomb group proteins (PcG), Bmi1; (5). Another protein encoded by this locus is tumor suppressor p19^{ARF} (ARF) that plays a prominent role in senescence in mice but not in humans (27). ARF stabilizes p53 by inhibiting MDM2 and, via its effector p21 blocks cell cycle. Thus, ultimate targets of both products of the INK4A-ARF locus are CDKs that inactivate pRb, and their dysregulation invariably compromises genome integrity (Fig. 1).

3. Quiescence Versus Senescence

What distinguishes senescence from another non-proliferating state, quiescence, also referred to as G0 or G0/G1 phase (28)? In contrast to senescence, quiescence is a *reversible* cell cycle arrest,

which is induced by the absence of mitogens or growth factors, nutrient starvation, or increasing cell density (confluence) (Fig. 2). Typical examples of quiescent cells are lymphocytes, whose activation is part of the immune response, adult stem cells, or dermal fibroblasts, which actively participate in wound healing (29). In general, quiescence is characterized by low metabolism and protein synthesis, lack of cellular growth, and, the absence of global heterochromatin structures such as PML bodies (30) or SAHF (15). The latter feature is probably responsible for the reversible nature of quiescence. However, much against the prevailing view, recent results showed that fibroblasts rendered quiescent by contact inhibition exhibit comparable metabolic activity to actively proliferating cells. In addition to promote recycling of damaged macromolecules via autophagy, high metabolic activity might serve for biosynthesis and secretion of extracellular matrix proteins (31). Importantly, while downregulating expression of genes involved in cell division, quiescent cells upregulate genes, such as HES1, that inhibit senescence, differentiation and apoptosis (32, 33). Unlike senescence, which can occur either in the G1 or G2 phase of the cell cycle (34), depending on when the damage is detected and the efficiency of the checkpoints (23, 35), quiescence essentially takes place in G1, prior to the restriction (R) checkpoint (36). A teleological explanation for this observation could be given by the unidirectionality of the cell cycle—G0 state always precedes DNA replication (S phase). Therefore, if cells became quiescent in the G2 phase, cell cycle entry upon stimulation would result in genome reduplication giving rise to undesirable tetraploidy.

Like senescence, quiescence is characterized by CDK inactivation, the absence of S-phase- and Mitosis-promoting cyclins and the presence of hypo-phosphorylated pocket proteins. However, unlike senescence, where CDKs are inhibited by p21 or/and p16, the major CKI involved in quiescent arrest is p27^{Kip1} (p27) (37–39). Unlike p21, p27 induction is independent of the p53 pathway (39) and its levels/activity is primarily regulated by translation, phosphorylation, and Skp2-mediated degradation (40). Although p53 and p21 were also implicated in the cell cycle arrest following growth factor removal (41, 42), p53 integrity does not seem to be essential for quiescence (43) (Fig. 2). However, a redundancy of p27 with p21 might explain why contact inhibition is not impaired in p27^{-/-} cells (44). Interestingly, the expression of D-type cyclins, regulatory subunits of Cdk4/6 kinases and key signal integrators, strikingly differs between quiescence and senescence (Fig. 2). Downregulation of cyclin Ds is a hallmark of quiescence and their rapid mitogen-dependent induction is invariably associated with cell cycle entry (42, 45, 46). In sharp contrast, D cyclins are stabilized (cyclin D1) and even overexpressed (cyclin D2) in senescent cells (17, 35, 43, 47). In conjunction with the large-cell phenotype that is observed in senescent cells, these results suggest that the

mitogenic pathways involved in cell growth are active in senescent cells (see below) and might even have a positive role in senescence regulation. Indeed, several recent publications have connected the “hypertrophic” phenotype of senescent cells with activity of mTOR, a master cell growth regulator (48), although this topic is still controversial (49).

4. The mTOR Pathway Essentials

Originally identified in budding yeast mutants conferring resistance to a potent antifungal metabolite (50), the target of rapamycin (TOR) is at the core of a vast signaling pathway regulating cell growth and metabolism in virtually all eukaryotes. The complexity of this pathway, which couples energy and nutrient abundance to the execution of cellular growth and division, relies on the fact that TOR simultaneously senses energy (ATP state), nutrients, stress, as well as growth factors (51, 52). In addition to its growth-related functions, the TOR pathway is also wired to the regulators of cell cycle machinery such as G1 cyclins (D-type and E-type) controlling G1/S-phase progression (see below and Fig. 3). It is therefore not surprising that TOR is essential for many developmental and physiological processes (53) while deregulation in its signaling has been implicated in a wide variety of diseases (52). mTOR (“m” stands for mammalian or, more recently, mechanistic), which belongs to the phosphoinositide 3-kinase (PI3K)-related protein serine/threonine kinase family (PIKK), forms two distinct multi-protein complexes, having distinctive physical structures and functions: mTOR complex 1 (mTORC1), which is sensitive to rapamycin owing to association with the peptidyl-prolyl *cis/trans* isomerase FKBP12 (50), and mTORC2, which is not sensitive to rapamycin in most cases. Their assembly, substrate specificity, and regulation are defined by specific accessory proteins: Raptor (regulatory associated protein of mTOR) being the most prominent for mTORC1, and Rictor (rapamycin-insensitive companion of mTOR), which is specific for mTORC2. Since the regulatory inputs and the cellular actions of mTOR are far too complex for the scope of this chapter (and are reviewed in ref. 53, 54), I outline only some “essentials” focusing mainly on mTORC1, the better characterized of the two.

mTORC1, which is activated by nutrients, growth factors, and cellular energy status, regulates temporal aspects of cellular growth including protein synthesis, ribosome biogenesis, lipid synthesis, nutrient import, and autophagy. Best-known substrates of mTORC1 are p70 ribosomal protein S6 kinases (S6K1 and S6K2) and the eIF4E binding proteins (4E-BP1 and 4E-BP2) by which it controls protein synthesis. The positive and negative control of

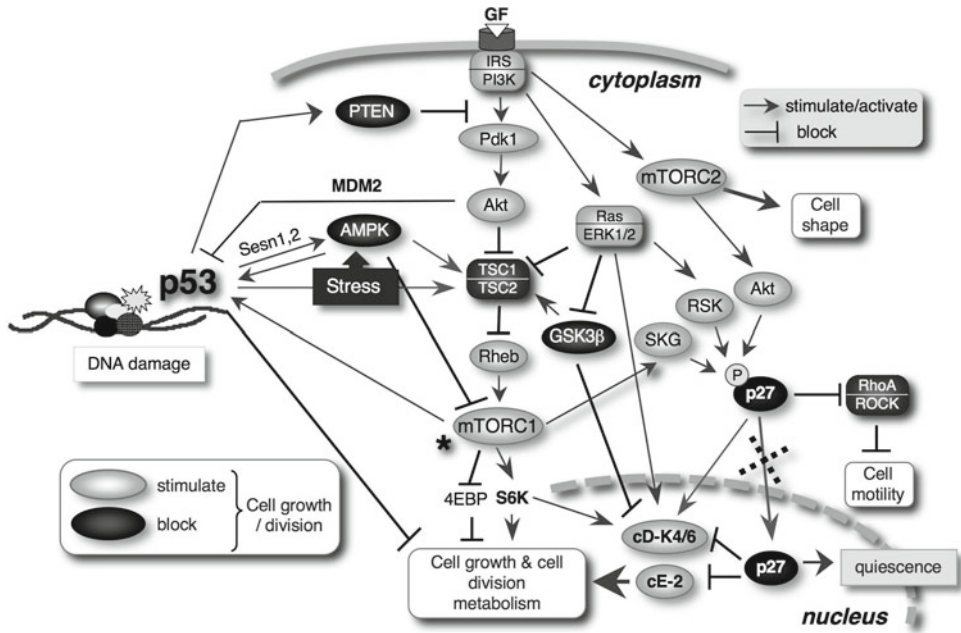


Fig. 3. Interactions between mTOR signaling, p53 and cell cycle regulators. While mTORC1 controls cell growth/division and metabolism, TORC2 mainly controls cell shape and activates Akt. TSC1/TSC2 complex plays a pivotal role as a hub for both positive and negative cues that control mTORC1 activity via signaling kinases (see ref. 53). mTOR controls cell division by regulating synthesis of cyclin D and activity/localization of CDK inhibitor p27. While in the nucleus, p27 inhibits CDKs (cD-K4/K6 and cE-K2) thereby blocking cell cycle progression (like in quiescence). Growth factors stimulate mTOR and ERK1/2 that, via Akt, RSK and SGK, mediate p27 phosphorylation leading to its cytoplasmic sequestration. Cytoplasmic p27 promotes proliferation by stabilizing cyclin D-Cdk4/6 assembly and cell motility by inhibiting RhoA/ROCK (see ref. 71). In response to DNA damage or other stresses, p53 negatively regulates mTORC1 by activating AMPK and PTEN and inducing TSC2. Constitutive activation of mTOR (*asterisk*) could induce p53 translation (see ref. 86). Figure adapted from ref. 119. GF growth factors (insulin, IGF1); Sesn, sestrin.

mTORC1 in response to intracellular and extracellular stimuli is mediated by the phosphorylation and subsequent inhibition of Tuberous sclerosis 1 (TSC1, hamartin)/TSC2 (tuberin) complex (Fig. 3). TSC2 subunit, a hub for a variety of cues impinging on mTORC1, serves as GTPase activating protein (GAP), which itself inhibits the small GTPase Rheb, a direct mTORC1 activator (51, 53, 55). TSC2 activity is regulated by several kinases: negatively, by Akt and ERK1/2 (activate mTORC1) and positively, by AMPK and GSK3 β (inhibit mTORC1). Importantly, the Wnt pathway also regulates TSC1/TSC2, via inhibition of GSK3 β (53) (see Fig. 4). mTORC2 is thought to regulate mainly cytoskeleton organization and cell survival by phosphorylating of AGC kinase family members, including Akt, SGK1, and PKC (52, 53) (Fig. 3). Although little is known about upstream regulators of mTORC2 (56), recent reports showing that its activation requires association with ribosomes suggest that it is active only in growing cells (57).

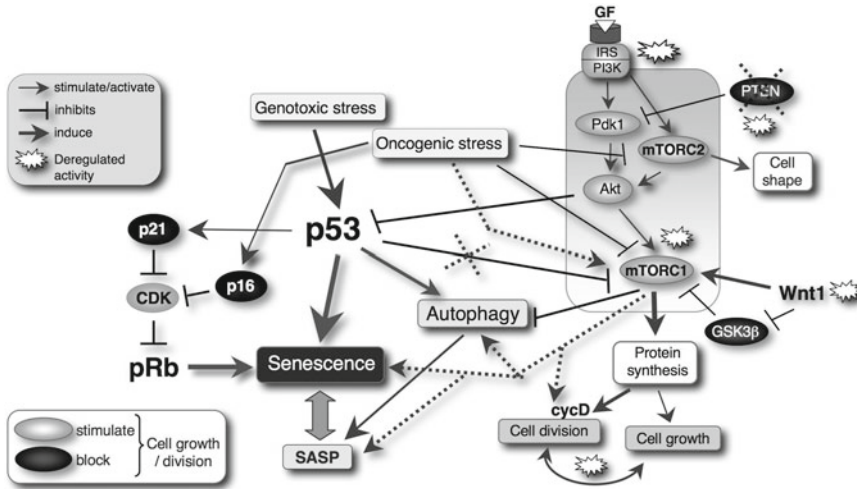


Fig. 4. Possible roles of p53 and mTOR in senescence. Genotoxic stress (via p53–p21) and activated oncogenes (via p16) induce senescence by inactivating CDK and activating pRb (see Fig. 1). Classically, p53 and activated oncogenes induce autophagy by inhibiting mTOR pathway (*grey box*). Autophagy is implicated in senescence by stimulating specific secretory phenotype (SASP) that also promotes senescence. However, recent data suggest that mTOR might positively regulate senescence (*grey dotted lines*) as in the case of persistent Wnt1 signaling or PTEN knockdown. Pro-senescence mTOR activity could be also activated via oncogenic stress—by inducing autophagy, or via low p53 levels (*grey dotted cross*)—by promoting cell growth and D-type cyclin synthesis. Hypertrophy of senescent cells could be explained by deregulated cell signaling (not responding to extracellular cues) resulting in permanent mTOR activation. This is in agreement with results showing that in senescent cells cell growth and cell division are uncoupled events. For more details regarding mTOR regulation, see Fig. 3 and ref. 53. Figure adapted from ref. 119. GF growth factors.

5. mTOR and Cell Cycle

As eluded above, mTOR pathway is also implicated in cell division, in part by controlling the synthesis of D-type cyclins (58, 59), major regulators of G1-S phase progression, whose induction in response to growth factors is regulated by the Ras-ERK1/2 pathway (60, 61) (Fig. 3). Recent data show that mTORC1 controls cell proliferation, but not growth, via its targets 4E-BPs, by stimulating the translation of proteins involved in cell cycle progression, including cyclin D3 (62). In addition, in the fission yeast model, TOR was also shown to control mitotic entry in response to nutrients. In this system, both rapamycin (by inhibiting TOR) and poor nitrogen sources stimulate the stress MAPK pathway (Spcl/Sty1), which, by activating Polo kinase and Cdc2 (Cdk1 ortholog, the main mitotic regulator) accelerated mitosis with reduced cell size as consequence (63). While no equivalent pathway was yet reported in other models, these observations are consistent with earlier results that linked mTOR signaling with cell-size control (64). Nevertheless, despite general downregulation of

protein translation in mitosis (65), several recent papers suggest that mTORC1 might also control protein synthesis in mitosis by regulating expression of human Cdk1, which phosphorylates eukaryotic elongation factor 2 (eEF2); (66). Moreover, it seems that, at G2/M transition, Cdk1 might also regulate mTORC1 activity by phosphorylating Raptor (67, 68).

The mTOR pathway also positively controls cell cycle by regulating the activity and subcellular localization of the CKI p27, negative regulator of G1/S progression (Fig. 3). In the presence of growth factors, Akt (activated by mTORC2), RSK (activated by ERK1/2), and SGK (activated by mTORC1, mTORC2 and Pdk1) phosphorylate p27, resulting in its cytoplasmic retention (69–71). This not only prevents p27 to exert its nuclear role as CDK inhibitor, by inactivating CDKs controlling DNA replication (as cyclin E-Cdk2), but also promote cell proliferation by stabilizing assembly of cyclin D-CDK4/6 complexes and increasing cell motility, via inhibition of RhoA signaling (71) (Fig. 3). In addition, mTORC2 regulates cell proliferation via AKT-mediated inhibition of forkhead box protein O (FOXO) transcription factors playing a key role in promoting apoptosis (72). Importantly, the FOXO family members were also shown to block cell cycle by inducing p27 (73) or repressing D-type cyclins (74).

6. TOR, p53, and Genotoxic Stress

In the light of increasing evidence that altered metabolism is not only a common feature of many cancer cells but that it can also greatly contribute to malignant transformation (75), the discovery that p53 also regulates metabolic pathways and interacts with mTOR/AKT pathways is not that surprising (reviewed in refs. 76, 77) (Fig. 3 and 4). Indeed, it has been shown that, in response to diverse stress signals, activation of p53 directly or indirectly inhibits mTOR activity, thus regulating its downstream targets, including those involved in the activation of autophagy, a recently described tumor suppression mechanism that is involved in senescence (54, 78) (see also Chapter 3). The role of autophagy in senescence is discussed in more detail in the chapter by M. Narita (see Chapter 1). In addition to its housekeeping role in the maintenance of energy homeostasis, autophagy, which is negatively regulated by mTOR, is also induced by a variety of stress stimuli, such as nutrient depletion (when mTOR is inhibited), playing a vital role in preserving cellular viability through the degradation of cellular proteins and organelles (53, 79).

The mechanism by which p53 negatively regulates mTOR involves, in part, the activation of AMPK and requires its target TSC1/TSC2 complex (Fig. 3), both of which respond to energy deprivation in cells (53, 80). In response to genotoxic stress caused by DNA damage, p53 inhibits mTORC1 pathway, by phosphorylating Sestrin 1 and 2 that activate AMPK (81), as well as the IGF-1/Akt pathway, by inducing transcription of PTEN (82) (Fig. 3). In addition, p53 can repress mTORC1 by inducing directly TSC2 (83) or AMPK, which suppresses translation by activating TSC2 (80) or by directly inhibiting mTORC1 (54, 84). However, it has been reported that AMPK might also control the p53 activity, like in the case of glucose starvation, which induces the transient AMPK-mediated phosphorylation and activation of p53 leading to reversible cell cycle arrest (85, 86). There are several examples showing that conversely, mTOR can also control p53. Upon glucose removal, mTOR negatively regulates p53 by activating PP2A (87). However, some results suggest that deregulated (constitutive) mTOR activity might, in response to nutrient or genomic stress, induce p53 translation, which in conjunction with activation by AMPK, induces apoptosis (88). This implies that, at least in certain biological contexts, mTOR downregulation/inactivation is required to prevent cell death caused by stress stimuli. Thus, p53 and mTOR signaling networks can cross-talk and coordinately regulate cell growth, proliferation and death.

7. mTOR' s Role in Senescence: Repressor or Inducer?

The mTOR pathway has been shown to have opposing roles on cellular senescence. On the one hand, by inhibiting autophagy (78, 86, 89), mTOR is considered as a negative regulator of senescence (Fig. 4), which is in agreement with its widely accepted negative role in organismal ageing (90). Moreover, as mentioned above, mTOR is inhibited by p53, a *bona fide* pro-senescence positive regulator (Figs. 3 and 4). On the other hand, the hypertrophic phenotype of senescent cells (Fig. 2) and increased production of secretory proteins (SASP; Fig. 4), which requires high metabolic activity, are in apparent contradiction with cellular shrinkage that is usually associated with autophagy and mTOR inhibition (as in quiescence). Indeed, in the case of oncogene or DNA damage-induced senescence, the onset of both senescence and autophagy correlates with inhibition of mTORC1 and mTORC2 activity (91). This might suggest a negative feedback between mTOR and senescence. These findings are also in agreement with earlier observations that activation of p53 by genotoxic stress inhibits mTOR pathway by activating both AMPK and PTEN (Fig. 3) (81, 86). Likewise, persistent

AMPK activation leads to senescence in mouse embryo fibroblasts (MEF) in the presence of functional p53 (85), which is consistent with earlier observation that AMP levels strongly increase in senescent cells (92).

However, it is also possible that mTOR activation via different stimuli could be involved in the induction of senescence. This pro-senescence role of mTOR was first uncovered by manipulation of its upstream regulators or downstream targets. Pandolfi and colleagues found that overexpression of an mTOR target eIF-4E induced senescence both in vitro and in vivo (93). More recently, activation of the PI3K/Akt and mTOR pathways by PTEN knock-down has been shown to induce senescence by promoting accumulation of p53 and p21 (94). Similarly, in an elegant in vivo skin model, Gutkind and colleagues showed that continuous mTOR activation induced by persistent Wnt1 signaling (via inhibiting GSK3; (95)) promoted epithelial stem cell exhaustion by provoking senescence (96). Importantly, senescence and hair loss were abolished by the presence of the mTOR inhibitor rapamycin suggesting that mTOR stimulation is a direct consequence of Wnt1 expression (96) (Fig. 4). These results connect persistent mitogen stimulation and mTOR activation with senescence. Moreover, they are in agreement with accumulated evidence connecting accumulation of senescent cells with ageing (7, 9). Indeed, increased mTOR activity was also observed in hematopoietic stem cells from old mice (97), whereas mTOR inhibition by rapamycin could extend lifespan both in invertebrates (98, 99) and in mice (97, 100, 101).

In the context of OIS, mTOR promotes lysosome biogenesis, which is required for autophagy and senescence-specific secretory phenotype, in a newly identified cytoplasmic compartment (termed TOR-autophagy spatial coupling compartment or TASCC) (102). While these results clearly indicate that mTOR has pro-senescence functions (Fig. 4), they are surprising in light of earlier report, connecting the induction of senescence by oncogenic ras with mTOR downregulation (91). However, in this previous report it was clearly shown that increased phosphorylation of both mTORC1 and mTORC2 substrates (S6K and FoxO3a, respectively) coincided more with the onset of cycle arrest (and senescence) than with ras-induced cell cycle entry, as proposed by the authors. Indeed, transient activation of mTOR was also observed in cells in which senescence was triggered by the genotoxic agent etoposide. Therefore, while the results showing that appearance of late senescence and autophagy markers coincided with downregulation of mTOR activity, overall this work supports a positive role of mTOR pathway in the induction of autophagy at the onset of senescence.

Although mainly focused on autophagy-related roles of mTOR, the above results are consistent with the model proposed by Blagosklonny and coworkers, whose recent experiments suggest

that cellular growth and persistent mTOR activity might be implicated in the onset of the senescence program. Consistent with their finding that serum stimulation is required for both the senescent phenotype (large flat cells) and irreversibility of the cell cycle arrest induced by overexpression of the CDK inhibitor p21 or DNA damage, they observed that a key mTOR target, S6K, is strongly phosphorylated in senescent cells (103). Moreover, in agreement with our earlier observation (17), senescent cells also accumulated another mTOR target, cyclin D1, but the biological significance of its presence is not clear (see below). Interestingly, while rapamycin, like serum starvation, prevented S6K phosphorylation and diminished the senescent marker β -gal staining, it failed to completely abolish large flat cell morphology and to inhibit cyclin D1 induction. While these results imply that mTORC1 is not essential for these events, it would be interesting to test whether they are controlled by mTORC2, which is less sensitive to rapamycin and which is implicated in both cell shape and, via its target Akt, in cyclin D1 stability (Figs. 3 and 4). In addition, rapamycin prevents the onset of senescence if added simultaneously with senescence inducers (p21 induction, genotoxic agents), but fails to revert the already established senescence (104).

Taken together, the above results suggest that (transient?) mTOR activity might positively control senescence but it is still unclear how DDR network controls activity of this pathway.

8. Connections Between mTOR and p53 in Senescence—Lessons from Nutlin-3A

How one can reconcile the data suggesting a positive role of mTOR in senescence with those showing that p53 inhibits the mTOR pathway (76, 77)? In the work of Narita's team the possible interactions between p53 and the mTOR pathway were not examined, because in OIS cell cycle arrest is mainly mediated by the CDK inhibitor p16, which is not regulated by p53 (91) (Fig. 1). In the quest to decipher the respective roles of mTOR and p53 in the senescence program, Blagosklonny and coworkers took advantage of nutlin-3A, a specific small-molecule MDM2 antagonist, which strongly induces p53 expression thereby triggering its transcriptional activity in a non-genotoxic manner (105). Surprisingly, while previous work has shown that nutlin-3A induces senescence in mouse fibroblasts (106), in human fibrosarcoma cells HT1080 and WI-38 human fibroblasts, nutlin-3A induces reversible cell cycle exit resembling quiescence (107). By exploiting a HT1080-derived cell line in which p21 is expressed from an inducible promoter, Demidenko et al. showed that nutlin-3A or p53 overexpression could “convert” p21-induced senescence into a quiescence-like state (108). Nutlin-3A was, however, much less efficient

in preventing senescence in the presence of DNA damage by H_2O_2 , which also stimulates the phosphorylation of mTOR (108). In addition, both nutlin-3A and rapamycin suppress senescence without interfering with cell cycle arrest, which led the authors to suggest that high p53 levels preclude the onset of senescence by inhibiting mTOR and inducing quiescence (108,109). Accordingly, knockdown of TSC2, a negative mTORC1 regulator (Fig. 3), partially compromises nutlin-3-induced quiescence, resulting in accumulation of senescent cells (110). In agreement with the positive role of mTOR in senescence, in cell lines in which nutlin-3A did induce senescence (termed “senescence-prone”), mTOR was not inhibited, whereas the quiescence was “restored” in the presence of rapamycin (110). Unfortunately, these studies did not provide insight into molecular mechanisms explaining differences between “senescence-prone” and “quiescence-prone” cells. In agreement with the above experiments, when exposed to etoposide, serum-deprived or rapamycin-treated fibroblasts (or epithelial cells) failed to become senescent despite p21 induction, as drug removal, concomitant with serum addition, enabled proliferation (111). In contrast, serum addition in the presence of etoposide induced senescence, presumably by activating mTOR. These results support the hypothesis that quiescence (or mTOR inhibition) compromises senescence. However, it is not clear why the checkpoints were not activated once cells exposed to etoposide reentered the cell cycle (assuming that the DNA damage had not been repaired) and how the cells got rid of high p21 levels.

This work, however, did not address the role of the CKI p27 in p53-induced quiescence. This point is relevant for understanding the mechanism that induces quiescence, since mTOR inhibition by rapamycin or p53 overexpression might also block SGK/Akt-mediated p27 phosphorylation and cytoplasmic localization leading to its nuclear accumulation and activation (70,71) (Fig. 3). One could imagine that, in the presence of rapamycin or absence of serum, p27-dependent CDK inactivation together with cyclin D1 down-regulation could also contribute to quiescence. Reactivation of mTOR (by serum addition) would revert this process, enabling cell cycle entry and progression, probably by degrading p27 and p21 and inducing G1 cyclins (D- and E-type).

Overall, the results of Blagosklonny’s team are consistent with the idea that mTOR positively regulates senescence, which might be because p53 levels are not sufficient to inhibit mTOR-dependent cell growth. According to their hypothesis, the “true role” of p53 (when highly expressed) would be to induce quiescence by inhibiting mTOR, which in turn would block the onset of senescence. Consequently, senescence occurs in situations where “the conditions for quiescence are not met” (low p53 levels) and where p53 “fails” to suppress the mTOR pathway (108,112). This hypothesis is consistent with the data showing that quiescence prevents

p21-induced senescence or inappropriate differentiation due to expression of the transcriptional repressor HES1, playing a key role in the reversibility of this non-proliferative state (33) (Fig. 2).

There are, however, several caveats regarding the use of nutlin-3A as a tool to study the role of p53 and mTOR in senescence. For example, strong p53 induction by nutlin-3A is not usually observed in either quiescence or senescence and it is not clear which biological event produces equivalent amounts of p53. As a matter of fact, primary fibroblasts expressing the HPV16-E6 (hereafter E6) onco-gene that degrades p53 (113) can become quiescent in the absence of serum or confluence, whereas strong p21 induction in senescent cells occurs in the absence of significant increases in p53 protein levels (43). Therefore, the hypothesis that high p53 levels induce quiescence while low p53 levels lead to senescence (108, 109) should be verified in other experimental models. For example, our recent results showed that irreversible cell cycle arrest by genotoxic drugs in non-transformed human cells is independent on the degree of p53 activation (or levels) or even p21 levels but rather on the efficiency of p21 to inhibit different CDKs (Lossaint, 2011). Finally, as mentioned above, this model is in apparent contradiction with the results showing that in other cell lines nutlin-3A does not induce quiescence but instead elicits endoreduplication, giving rise to tetraploid cells (114), senescence—due to persistent p21 expression (106, 115), or even apoptosis (116). One explanation could be that nutlin-3A might have other targets than p53 (pRb?) or that its effects might be dependent on the experimental protocol and cell type.

9. mTOR and Senescence: Constitutive Activation or Deregulation?

The above-mentioned work mostly addressed the role of mTOR in premature (induced) senescence but less is known regarding its implication in replicative senescence. Several earlier observations, however, are consistent with the notion that the mTOR pathway might be active in senescent cells and that it is not controlled by p53 or affected by its status. In fact, in human fibroblasts aged in vitro a suppression of the p53/p21 pathway compromises cell cycle arrest, senescence-specific cyclin D2 induction (see below), as well as formation of SAHF, but it does not prevent large flat cell morphology or cyclin D1 accumulation (43). This implies that these two sets of key events associated with senescence, i.e. irreversible cell cycle arrest and cell growth, might be uncoupled, which can also explain the senescent phenotype (Fig. 4). In addition, some data suggest that mitogenic pathways in senescent cells might be also deregulated. Firstly, in sharp contrast to early passage fibroblasts, serum withdrawal in senescent fibroblasts does not

affect cyclin D1 mRNA or cyclin E1 expression or protein synthesis. The CDKs associated with these cyclins are, however, inhibited by p21, explaining the absence of DNA replication (17, 26) (Fig. 1). Interestingly, in agreement with other publications (35, 117), late senescence is associated with strong accumulation of cyclin D2, whose levels are not affected by serum withdrawal. The presence of G1 cyclins, and especially of cyclin D2, in senescent cells is intriguing and it is not clear whether they might play a role in senescence or they merely reflect deregulated mTOR activity. One possibility is that cyclin D1 is part of the DNA repair machinery, as suggested recently (118). Secondly, late-passage p53-deficient fibroblasts (E6) failed to become quiescent upon serum withdrawal, which is documented by both the absence of cyclin D1 or cyclin A down-regulation and p27 induction (or activation). Consequently, CDKs controlling DNA replication were highly active even in the absence of serum. However, these cells failed to proliferate due to deleterious effects of DNA damage resulting in aberrant mitoses, endoreplication, or cell death (43). It is therefore possible that, concomitant with increased population doubling, gradual deregulation of the mTOR pathway contributes to the senescent phenotype. In the absence of the p53/pRb safeguard system, this deregulation provides a fertile ground for tumorigenesis.

In conclusion, the role of the mTOR pathway in senescence is still controversial, partly due to the various models and experimental designs employed in different studies. Apart from the studies manipulating upstream mTOR regulators (PTEN, Wnt1) that reveal pro-senescence mTOR-functions, most of the work has focused on the autophagy-related aspects of senescence, which appeared to exclude mTOR as an important regulator of senescence. Few researchers, however, considered a hypertrophic phenotype of senescent cells and elevated levels of D-type cyclins as an evidence of mTOR activity. Future work will show whether or not this activity is actually required for the onset of senescence, as some investigators proposed, or it is merely the result of senescence-associated deregulation of the mTOR pathway. If the former is the case, it will be interesting to learn whether, and if so, how p53 and/or pRb networks contribute to its induction.

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