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Review

Cell fate determination during G1 phase progression

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Abstract. During the cell cycle, a cell may encounter one of five different fates: it can proliferate, differentiate, become quiescent or senescent, or go into apoptosis. The initiation of such fates is often seen in the G1 phase. The aim of this review is to describe an integrative model of G1 phase progression and cell fate determination. Along the G1 phase, the cell will encounter an early checkpoint after which apoptosis can result. For a quiescent state and for differentiation, the cell will exit G1 before the restriction point and a subsequent differentiation checkpoint will decide the fate of the cell, quiescence or differentiation. After the restriction point, the cell can be arrested in response to stress stimuli, such as telomere depletion, and a decision between senescence and apoptosis occurs.

Keywords. Cell cycle, quiescence, differentiation, senescence, apoptosis, G1 phase.

Introduction

During the development of an organism, its cells will proliferate, differentiate into specialized cells, rest and even die for morphological or homeostatic reasons. A proliferating cell is undergoing a process referred to as the cell cycle. The cell cycle has four main phases, namely the G1 phase (or Gap 1), the S phase, the G2 phase, and the M phase. Once a cell has divided by mitosis it will enter the G1 phase. In this phase, a cell will typically grow and prepare for the S phase. During the latter phase, the chromosomes will be duplicated. After this process, the cells will enter the second Gap phase in which the cell continues to grow and prepares for mitosis (M) during which the cell will actually divide.

However, not every cell in an organism is constantly proliferating. As a matter of fact, most cells in our body remain in a reversible resting state, often referred to as quiescence or G0. These cells have taken a break from the cell cycle, but can be stimulated to reenter the cycle and proliferate. Another cell fate that is frequently encountered is differentiation. During this process, cells exit the cell cycle and change into more specialized non-proliferating cells. Unlike quiescence, which is a temporary non-proliferating state, cells can also become senescent, a state characterized by an inability to return into the cell cycle. Once a cell has become senescent it will remain metabolically active, but it will be permanently retired [1]. Lastly, there is apoptosis, or programmed cell death. An apoptotic cell will die in an ordered fashion and this process is often observed during the development of, for example, the separate digits of our hands and feet [2].

Interestingly, many of the cells that undergo such states withdraw from the cell cycle during G1 (Fig. 1), since these cells often contain a G1 amount of DNA. It has long been assumed that during G1 phase progression, and cell cycle progression in general, check-

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points are encountered, and depending on the ability to pass such points, cell fate will be determined. Checkpoints have already been proposed for quiescence [3], differentiation [4], and apoptosis [5]. However, the chronological order in which the four cell fates, quiescence, senescence, apoptosis, and differentiation, are induced remains unclear. Therefore, the mechanisms and pathways behind these four cell fates will be reviewed in order to propose a temporal model for G1 progression and cell fate determination. The scope of this review will be limited to G1 progression under physiological conditions. Before elaborately reviewing the cell fates, a brief summary of regular G1 progression for a proliferating cell will be presented.

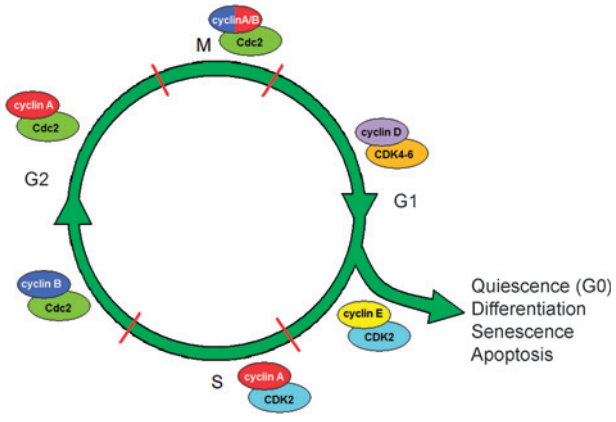

Figure 1. Diagram depicting the four phases of the cell cycle and the cyclin/CDK complexes that are crucial for progression. After dividing, a cell will enter the G1 phase during which it will grow. Subsequently, in the S phase, the chromosomes will be replicated and another phase of growth will follow (G2), until the cell divides again in mitosis (M).

G1 phase progression

The primary regulatory proteins behind cell cycle progression are the cyclins and the cyclin-dependent kinases (CDKs). The CDKs belong to the class of protein serine-threonine kinases and depend on the binding of a cyclin in order to become activated by a CDK-activating kinase [6]. Once activated these cyclin/CDK complexes are responsible for phosphorylation of downstream targets, such as cell cycle repressors. During cell cycle progression, the expression levels of cyclins vary over time. For example, cyclin D and cyclin E are crucial in the G1 phase, cyclin A is crucial in the S phase, and both cyclin B and A are also important for the G2 phase and mitosis as shown in Figure 1 [7]. However, the cyclin family is not limited to these four cyclins. At least 16 eukaryotic cyclins have been discovered and up to nine CDKs [8].

Cyclin D is one of the major players for G1 phase progression. After stimulation by mitogens, cyclin D is upregulated early after a cell enters G1. This protein associates with CDK4 or CDK6, depending on the cell type. The active cyclin D/CDK4/6 complexes will phosphorylate the retinoblastoma protein, Rb [9]. Rb is one of the pocket proteins (together with p107 and p130) and functions as a tumor suppressor. In other words, in its hypophosphorylated form it is active and represses cell cycle progression by inhibiting E2F transcription factors, which are necessary for S phase entry.

Later in the G1 phase, cyclin E is induced, which associates with CDK2 to form active complexes that again target the pocket proteins for phosphorylation. Once Rb is phosphorylated over a certain threshold, it is deactivated and the bound transcription factors are now released, facilitating the transcription of late G1/S phase genes [9]. There are assumed to be six of these proteins and some of these will act as cell cycle activators, while others act as inhibitors [10].

The key players in the negative regulation of the cell cycle are the cyclin-dependent kinase inhibitors (CKIs). As the name implies, these proteins are responsible for inhibiting the cyclin/CDK complexes, thus effectively preventing Rb hyperphosphorylation and preventing G1 progression. Two families of CKIs exist: the INK4 family and the Cip/Kip family. The former family is primarily responsible for the inhibition of cyclin D/CDK4/6 complexes, while the latter CKI family exerts its effects mainly on cyclin E/CDK2 complexes, although it can also inhibit other cyclin/CDK complexes [11]. Interestingly, at physiological concentrations, the Cip/Kip family members $p21^{Cip}$ and $p27^{Kip}$ facilitate the formation of active cyclin D/CDK4/6 complexes [11]. The expression of INK4 CKIs results in binding to the cyclin D/CDK4/6 complexes and displacement of the Cip/Kip family, allowing the latter to inhibit the cyclin E/CDK2 complexes [11]. Similarly, upregulation of Cip/Kip CKIs can overcome the cyclin D/CDK4/6 buffer, and the free CKIs will then inhibit the cyclin E/CDK2 complexes.

The interaction between Rb and cyclin/CDK complexes leading to Rb hyperphosphorylation and G1 phase progression is often referred to as the restriction point (R). After this point, the cell is no longer under the influence of growth factors and will enter the S phase even if deprived of mitogens [3]. Before R, mitogenic stimulation results in cyclin D expression which is essential for the initial Rb phosphorylation. In addition to the proteins discussed above, another important regulator of cell cycle arrest is p53. p53 is present at basal levels in cycling cells and has a short half-life due to ubiquitination by its negative regulator

MDM-2 and subsequent degradation [12]. It is stabilized and becomes activated after exposure to stress stimuli, including DNA damage, pH changes, heat shock, and oncogene activation [12]. Once activated it enforces a G1 arrest by acting as a transcriptional activator of $p21^{Cip1}$ [12]. In addition, p53 can also enforce cell cycle arrest in other phases of the cell cycle; in G2 also through the upregulation of $p21^{Cip1}$ among others [12]. Note that $p21^{Cip1}$ can also be upregulated through p53-independent pathways [13]. Furthermore, p53 plays a regulatory role in DNA repair, senescence, apoptosis, and possibly even quiescence [12, 14].

Quiescence

In an adult organism, most cells have stopped proliferating and linger in a reversible non-proliferating state referred to as quiescence or the G0 phase. These cells will generally display no DNA synthesis, have a smaller cell size, and a lower metabolism [15], but under the right conditions, they can reenter the cell cycle and divide. Under laboratory conditions, this phenotype is achieved by, for example, serum starvation, contact inhibition, and loss of adhesion [16]. Although the phenomenon of quiescence *in vitro* is readily produced and observed, its role and mechanism *in vivo* is quite unclear. The occurrence of growth factor deprivation, for example, is unlikely under normal physiological circumstances; nevertheless, many cells still appear to remain in this state.

It is often assumed that G0 arrest can only be achieved before the restriction point is reached [3, 17]. After cells pass the restriction point, they will be resistant to serum starvation and will most likely progress into the S phase [3, 17]. The molecular mechanism behind this is often assumed to be the phosphorylation of Rb over a certain threshold.

When scrutinizing the molecular hallmarks of quiescence, one of the first salient findings is the decrease in cyclin D1 in these cells [17–19]. The lack of cyclin D1/CDK complexes implies the possibility that Rb in quiescent cells is not phosphorylated to the same extent as in proliferating cells. This is indeed consistent with recent findings that suggest that Rb is unphosphorylated in G0, hypophosphorylated during G1 progression, and hyperphosphorylated in late G1 when passing the restriction point [20]. Furthermore, using coimmunoprecipitation, it was shown that unphosphorylated Rb does not associate with the transcription factor E2F4 [20].

Even though Rb does not appear to play an imminent role in maintaining quiescence, its pocket protein family members have often been implicated in this process. P130, especially has been shown to be expressed in high amounts in quiescent cells [10, 21, 22]. Furthermore, p130 is associated with the E2F repressor proteins (especially E2F4) during quiescence [22, 23], which prevent the expression of proliferative genes. These repressor E2Fs function in complex with the Rb family members p130 and p107 as active repressors of the E2F target genes [24, 25], i.e., not just by sequestering E2F proteins. The Rb family proteins exert their full effect not only by binding to the E2F activator or repressor proteins, they are also believed to function in conjunction with histone deacetylases and other transcription factors in order to achieve the required cell fate [23, 26, 27]. In summary, the lack of active cyclin D/CDK complexes leads to an accumulation of unphosphorylated Rb, which in turn has lost affinity for the E2F transcription factors. In this case, p130 will bind E2F4, or possibly other E2Fs, and repress the expression of genes that are responsible for S phase entry, thus resulting in a quiescent state. The importance of p130 for quiescence is confirmed by its phosphorylation by CDK4/6 and subsequent degradation during reentry into the cell cycle [28]. However, this does not mean that the often assumed key player in the restriction point, Rb, is dispensable. Although Rb knockout mice have a fairly normal phenotype due to compensation by the other pocket proteins, an acute loss of function in quiescent cells leads to immediate reentry into the cell cycle [29]. This indicates that although p130 is important in the regulation of quiescence, Rb still plays a role.

Even though p130/E2F4 complexes seem to be reliable indicators of cellular quiescence [22], the precise upstream pathways remain unclear. It is known that the pocket proteins are phosphorylated by cyclin D/CDK4/6 complexes, and these in turn are inhibited by CKIs. It would appear that cyclin D/CDK4/6 is responsible for the initial phosphorylation of Rb and then cyclin E/CDK2 is necessary for hyperphosphorylation of Rb and facilitation of G1 progression [30]. Since Rb is not phosphorylated in quiescent cells, one would expect both CDK4 and CDK2 activity to be inhibited.

The CKI that has been rather consistently shown to be active during quiescence is the Cip/Kip family member $p27^{Kip}$. A high concentration of $p27^{Kip}$ in peripheral blood T cells was measured during quiescence, which declined as G1 progressed [30]. Furthermore, an increase in $p27^{Kip}$ was found in murine fibroblasts that were serum deprived, and, interestingly, the inhibition of $p27^{Kip}$ by antisense oligonucleotides resulted in increased proliferation [31]. Apparently $p27^{Kip}$ is essential for the organization of the restriction point. This is confirmed by experiments using cDNA antisense to suppress $p27^{Kip}$ in serum-deprived Chinese

hamster lung fibroblasts [32]. Again, $p27^{Kip}$ inhibition resulted in increased cell proliferation under serum-deprived conditions. Similarly, these findings showed that $p27^{Kip}$ levels decreased when serum was added to the medium.

The importance of $p27^{Kip}$ in regulating quiescence is also supported by the observations that the activation of *myc* induces cyclin D2 expression, which in turn can form complexes with CDK4/6 to sequester $p27^{Kip}$ and induce proliferation [33]. It has indeed been shown that the Cip/Kip family of CKIs bind to cyclin D/CDK complexes and even stabilize rather than inhibit these complexes [34] under certain concentrations [35]. However, ectopic cyclin D1 expression alone in quiescent NIH 3T3 cells cannot induce proliferation, but it does so in combination with antisense inhibition of $p27^{Kip}$ [18]. These results suggest that $p27^{Kip}$ sequestration by cyclin D1 is not sufficient for reentry but that downregulation induced by mitotic factors is crucial as well. The downregulation of $p27^{Kip}$ is due to the activity of the PI 3-kinase pathway [36, 37], as demonstrated by translation inhibition and posttranslational modification [36]. The posttranslational modification includes phosphorylation, which enables $p27^{Kip}$ export from the nucleus [38] where it is normally active, and subsequent ubiquitination leading to proteasomal degradation [39]. These findings strongly suggest the involvement of $p27^{Kip}$ in the regulation of quiescence, by preventing Rb phosphorylation over the necessary threshold for cell cycle progression. However, in certain cells, another member of the Cip/Kip family seems to be important, namely $p21^{Cip}$. In mouse hematopoietic stem cells, $p21^{Cip}$ is an integral part of quiescence [40] and the depletion of $p21^{Cip}$ in human foreskin fibroblasts has a larger influence on reversing quiescence than depletion of $p27^{Kip}$, even though the levels of the latter are higher [19]. Furthermore, the expression of antisense $p21^{Cip}$ RNA in human fibroblasts leads to increased proliferation and resistance to G0 [41]. However, since the two proteins are somewhat homologous and can even compensate to a certain extent for the loss of function of the other [42, 43], they may function in a similar, albeit cell-specific, fashion in the regulation of quiescence. This assumption is supported by the observation that $p27^{Kip}$ is highly expressed in normal quiescent murine hepatocytes, but in $p27^{Kip}$-deficient cells, $p21^{Cip}$ levels increase and take over this function [42]. The proliferative tendencies of $p27^{Kip}$ deficient hepatocytes were quenched by the presence of an intact $p21^{Cip}$ gene.

Although the involvement of Cip/Kip family members is quite definite, it remains disputed whether the INK4 CKIs play a role in regulating cellular quiescence. As mentioned earlier, hyperphosphorylation of Rb by cyclin E/CDK2 is necessary for progression through R and cyclin D/CDK4/6 is important in the initial phosphorylation of Rb. Since Rb is largely unphosphorylated during quiescence, one could hypothesize that INK4 proteins might be induced that specifically inhibit the cyclin D/CDK4/6 complexes, thus inhibiting this initial step and, furthermore, displacing $p27^{Kip}$ and $p21^{Cip}$ from these complexes, so that they in turn can inhibit CDK2 [20]. This is supported by the observation that artificial $p16^{INK4a}$ expression in T cells prevents reentry into the cell cycle [44]. In addition, forced expression of $p16^{INK4a}$ in hematopoetic cells also induces a G0-like state [45]. Thus the involvement of an INK4 family member does not seem far-fetched, but in general $p21^{Cip}$ and $p27^{Kip}$ are assumed to play a larger role in cellular quiescence. Although $p16^{INK4a}$ levels are comparable to those of $p21^{Cip}$ in human foreskin fibroblasts, the RNA interference of $p16^{INK4a}$ does not induce cell cycle reentry, unlike RNA interference of $p21^{Cip}$ [19]. The finding that $p16^{INK4a}$-expressing cells fail to reenter the cell cycle [44] might be consistent with findings that this protein is responsible for permanent or prolonged cell cycle arrest (see senescence), rather than the induction of quiescence or cell cycle arrest. Another explanation might be that INK4 family expression during quiescence is dependent on the cell type [45].

So, if the artificial inhibition of $p21^{Cip}$ or $p27^{Kip}$ leads to cell cycle reentry, does artificial expression of these CKIs automatically lead to quiescence? Recent research suggests the contrary [16]. Using a DNA microarray, the changes in gene expression were monitored in cells that had been exposed to three quiescence-inducing stimuli, i.e., serum deprivation, contact inhibition (high cell density), and lack of adhesion. Interestingly, the three stimuli induced three different quiescence programs, each program with a distinct gene expression pattern. In addition, over time (after 4 days), the appearance of a uniform set of expressed genes became apparent. This included genes involved in suppression of apoptosis and differentiation, as well as genes involved in cell division and growth [16]. This led to the formulation of a hypothesis that, although the three quiescent states remain somewhat different, there may be a general quiescence program for cells expressing a set of antiproliferative genes. However, when the Cip/Kip proteins $p27^{Kip}$ and $p21^{Cip}$ were overexpressed, the gene expression program did not closely resemble the quiescent state induced by any particular extracellular signal [16]. It was concluded that, although the quiescence programs and CKI overexpression arrest have an approximate 50 % overlap when scrutinizing the downregulated genes, the upregulation of genes is more specific for quiescence (approximately 12 %

overlap). Furthermore, human dermal fibroblasts that have initiated a quiescence program are resistant to differentiation triggered by MyoD activation, while cells that are arrested by CKI overexpression are not [16]. CKI-overexpressing arrested fibroblasts transfected with a MyoD-estrogen receptor fusion protein showed muscle-specific protein expression, while quiescent cells did not [16]. This is consistent with the upregulation of antidifferentiation genes observed in quiescent cells. Although differentiation will be discussed in detail further on, this already suggests that quiescence and differentiation are regulated in a different way. More importantly, this research introduces the concept that quiescence is different from a normal cell cycle arrest; it is an actual separate, though reversible, cell fate.

As mentioned before, much of the research on quiescence used serum starvation to induce the state. However, loss of adhesion and contact inhibition have also been studied as quiescence-inducing stimuli [16]. CHO and N2A cells in suspension (no adhesion) exhibit downregulated cyclin E, much like the serum-starved cells that downregulate cyclin D [46]. Considering that cyclin E/CDK2 is required for phosphorylation of Rb over the threshold value, the cells that lost adhesion will not progress to S phase. Interestingly, $p21^{Cip}$ and $p27^{Kip}$ levels were not increased in these cells [46]. This might be explained by the fact that the Cip/Kip family CKIs exert their influence primarily on cyclin E/CDK 2 [47], and if these complexes are not present, such as in adhesion-deprived cells, $p21^{Cip}$ and $p27^{Kip}$ may not be needed to attain cell cycle arrest [46]. However, in NIH 3T3 cells, p27 is upregulated in response to loss of adhesion [48, 49]. Furthermore, the pocket protein p107 seems to form complexes with the E2F transcription factors in response to loss of adhesion [49], although p130 seems to be upregulated as well [50].

Contact inhibition appears to have a link with cyclin D, although not by actively downregulating cyclin D but, instead, by inhibiting the activation of cyclin D/CDK4/6 complexes [51]. Contact-inhibited rat fibroblasts lack phosphorylation of CDK4 on thr^{172}. Phosphorylation by a CDK-activating kinase (CAK) was sufficient to reactivate cyclin D/CDK4 complexes *in vitro* [51]. Furthermore, increased levels of $p27^{Kip}$ were observed in contact-inhibited cells [51]. Similarly, human thyroid anaplastic cancer cells are not prone to contact inhibition due to the inability to upregulate $p27^{Kip}$ in response to such stimuli [52]. In addition, upregulation of p130 is observed in response to contact inhibition [53].

Even though serum starvation is the primary means to invoke quiescence, one must wonder whether this reflects accurately a condition in an intact organism. One would expect that *in vivo* growth factors and mitogens are present, since diffusion and blood flow do not facilitate the localization of small molecules. Nerve growth factor (NGF) serum levels in adult mice, for example, are 10–50 pM [54]. Similarly, epidermal growth factor (EGF) levels in human serum have been reported to be around 2–4 ng/ml [55]. However, most cells in an adult body are assumed to be quiescent. This paradox challenges the assumption of the importance of growth factor deprivation in quiescence, and one might therefore be propose that this observed state is merely a form of cellular stress in response to a rare condition. Contact inhibition and cell adhesion, on the other hand, might represent more natural forms of quiescence. As tissues reach confluence, the cell cycle is halted and quiescence is initiated. Upon trauma to the specific tissue, contact inhibition may be lost, leading to reentry into the cell cycle and subsequent proliferation which may result in tissue regeneration.

However, it might be too early to altogether dismiss the involvement of growth factors in physiological quiescence. One can speculate, for example, about possible growth factor receptor desensitization or downstream pathway regulation which potentially results in a phenotype similar to growth factor deprivation. When serum-starved cells are prompted to reenter the cell cycle by addition of serum, the mitogen-activated kinases (MAPKs) $p42^{MAPK}$ and $p44^{MAPK}$ (or ERK1 and ERK2) are quickly phosphorylated by a MAPK kinase $p45^{MAPKK}$ and translocated to the nucleus [56]. Here they have been shown to stimulate the upregulation of cyclin D and degradation of $p27^{Kip}$ [57], which in turn facilitates cell cycle reentry. Interestingly, during normal cell cycle progression, MAPK phosphorylation and nuclear translocation occur independently of each other [58]. Using CHO cells synchronized by mitotic shake-off, which allows the study of cycling cells, it was shown that the phosphorylation of $p42/p44^{MAPK}$ occurred early in G1, while the nuclear translocation occurred only in late/mid G1 [58]. Although the phosphorylation of $p42/p44^{MAPK}$ is dependent on growth factors, the actual import into the nucleus is not dependent solely on growth factors, since these were present during the entire G1 phase and this translocation occurred only in mid/late G1. This raises the possibility of physiological quiescence as a result of inhibition of $p42/p44^{MAPK}$ nuclear import, theoretically a phenotype similar to serum-starved cells due to the lack of cyclin D upregulation, and the upregulation of $p27^{Kip}$ can be achieved. It has indeed been shown that inhibition of MAPK nuclear translocation results in a lack of DNA replication [59]. The prevention of nuclear translocation might be a direct

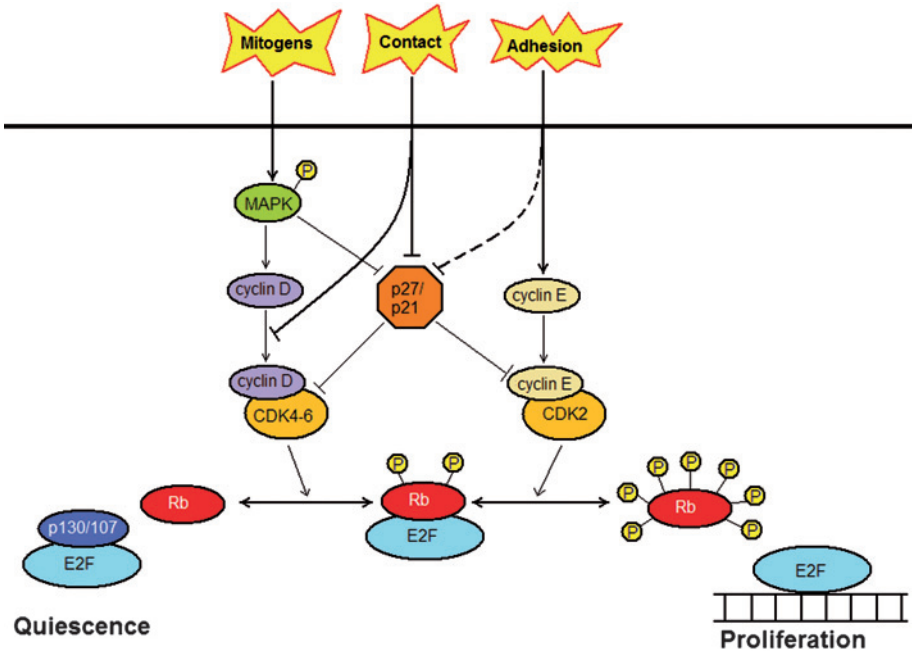

Figure 2. Proposed model for quiescence. The classical and presumably non-physiological form of quiescence is achieved by deprivation of mitotic factors, leading to a lack of MAPK nuclear translocation and subsequent lack of stimulation of cyclin D and lack of degradation of $p27^{Kip}$. This results in a failure to phosphorylate Rb, leading to association of p130 with E2F and the initiation of a quiescence program. The lack of cell adhesion will result in downregulation of cyclin E, association of E2F with p107, and possibly an upregulation of p27. Contact inhibition leads to decreased activity of cyclin D/CDK4/6 complexes as well as induction of $p27^{Kip}$ and E2F-p130 association. In addition, the specific quiescence-inducing stimuli promote the initiation of distinct quiescence programs.

interference with this process or the prevention of phosphorylation of p42/44 by MAPKK inhibition or phosphatase activation. The latter process has indeed been observed for human neural progenitor quiescence induced by chemokines activating CXCR4 and CCR3 [60]. Interestingly, although cell adhesion stimulates cyclin E expression, it also induces MAPK activation [61]. This indicates at least some overlap between mitogen and adhesion pathways. Whether the exit from the cell cycle for the different quiescence programs occurs at the same point in time (i.e. at the restriction point) remains a question.

In summary, the depletion of Cip/Kip CKIs appears to be sufficient to make a quiescent cell reenter the cell cycle. However, simply overexpressing these proteins does lead to cell cycle arrest, but does not automatically lead to a sustained quiescence program as discussed above. The findings presented above suggest the following model (Fig. 2). Stimulation by mitogens leads to the upregulation of the D cyclins as well as the repression of $p27^{Kip}$ or $p21^{Cip}$. Thus, lack of mitogen stimulation will decrease cyclin D levels, leading to inactivation of cyclin D/CDK4/6 complexes, thus preventing phosphorylation of Rb. Furthermore, $p27^{Kip}$ or $p21^{Cip}$ is displaced from cyclin D/CDK complexes and can now inhibit cyclin E/CDK2, thus again preventing further phosphorylation of Rb. The unphosphorylated Rb loses affinity for E2F transcription factors and p130 takes over this role, possibly already transcribing non-proliferative genes. However, this classical quiescence state might not reflect a state activated by actual physiological conditions. A similar quiescence phenotype may possibly be acquired by inhibiting MAPK nuclear translocation or receptor modification or regulation. Different quiescence programs can be induced by contact inhibition and loss of adhesion. In the case of lack of adhesion, cyclin E levels will drop, leading to insufficient Rb phosphorylation. Lastly, in the case of contact inhibition, cyclin D/CDK4/6 complexes will be inactive, also decreasing the phosphorylation levels of Rb. Interestingly, the lack of the different types of stimulation also appears to dictate a differential type of quiescence, thus implying that either directly or indirectly these proliferative stimuli can activate a specific quiescence gene expression program in the absence of such stimulation.

Senescence

Another frequently encountered cell fate is senescence. Unlike quiescence, senescence is characterized by the fact that it cannot be reversed under physiological conditions [62]. It is highly related to cell aging [63] and can be referred to as a final resting state, since it denotes a permanent retirement out of the cell cycle. However, a senescent state is more than merely a prolonged and irreversible quiescent state. Whereas quiescent cells are, as discussed above, not metabolically active and rather small compared to dividing cells, for senescent cells, the opposite seems to be true. The cells are rather big and flattened and have a relatively high metabolism [64]. Furthermore, where-

as quiescent cells lack cyclin D, in senescent cells, this protein is still fairly abundant [65, 66]. Lastly, senescent cells often upregulate a lysozomal enzyme β-galactosidase [1], which is often used as a marker to identify such cells.

The senescent state is induced by several different stimuli, the most prominent being DNA damage, telomere depletion, and oncogenic stress [1]. This review will discuss the latter two, since our scope is limited to cell fate decision under physiological conditions, thus excluding chemical- or radiation-induced DNA damage. However, when considering these three senescent inducers, it is not far-fetched to conclude that it is a mechanism that prevents tumorigenesis, by preventing proliferation of cells at risk. Therefore, it is not surprising that the mechanisms of senescence are often mutated in cancers [67, 68]. A senescent state provides an organism with a metabolically active and functional cell, unlike apoptosis; the only catch is that it can no longer divide.

To understand the molecular mechanisms behind senescence, much of the research points to an important mediating role for both the Cip/Kip CDK inhibitors as well as the INK4 family, in particular $p21^{Cip}$ and $p16^{INK4a}$, respectively. Human diploid fibroblasts express $p21^{Cip}$ and $p16^{INK4a}$ [69]. These cells can divide a finite number of times and thereafter become senescent. The expression of $p21^{Cip}$ increased dramatically during the final two or three passages when the majority of cells lost their growth potential and approached senescence, but declined in senescent cells. [69]. In addition, the levels of $p16^{INK4a}$ increased when the levels of $p21^{Cip}$ were decreasing. The $p16^{INK4a}$ was primarily binding CDK4 and CDK6. Similar and complementary findings showed that the accumulation of $p16^{INK4a}$ was directly proportional to the senescence markers β-galactosidase and cell volume. It was suggested that $p21^{Cip}$ acts as the mediator of cell cycle arrest prior to senescence, while $p16^{INK4a}$ is responsible for induction and maintenance of the actual senescent state [65]. In WI-38 human fibroblasts, $p16^{INK4a}$ mRNA appears to accumulate in senescent cells and inhibition of the AUF1 protein, a protein responsible for degradation of RNA, accentuated the senescent phenotype [70]. Furthermore, the transient expression of $p16^{INK4a}$ enforced a somewhat reversible G1 arrest, but prolonged exposure to $p16^{INK4a}$ drove the cells into senescence. Interestingly, even after removing the $p16^{INK4a}$ stimulation, a large fraction of the cells that had been exposed to this condition for 6 days maintained many hallmarks of the senescent phenotype [71].

The role of $p21^{Cip}$ is not as clear as that of $p16^{INK4a}$; however, it does seem to have an important influence on the induction of senescence. Human fibroblasts transfected with the papillomavirus type 16 E6 generally have low levels of $p21^{Cip}$, and these cells can only partly acquire the senescent phenotype, even though $p16^{INK4a}$ is normally expressed [72]. This was confirmed using $p21^{Cip-/-}$ mouse embryo fibroblasts [72]. This research strongly suggests an important role for $p21^{Cip}$ in cell cycle arrest and senescence, in addition to $p16^{INK4a}$. Furthermore, $p21^{Cip}$ can directly form complexes with E2F factors in senescent cells [73]. A recent finding is less consistent with the previous results, but still underlines the importance of both CKIs. Using siRNAs, $p21^{Cip}$ and $p16^{INK4a}$ translation was inhibited in senescent human fibroblasts, both independently and concurrently. Strikingly, the majority of senescent cells reentered the cell cycle and no synergistic effect was found when $p21^{Cip}$ and $p16^{INK4a}$ were inhibited concurrently [19]. So, although this is not consistent with the hypothesis that $p21^{Cip}$ is only necessary for the initial cell cycle arrest preceding senescence, it does underline the importance of the continuous expression of CKIs in maintaining the cell in such a state.

Another protein that is indicated to play a role in senescence is the tumor suppressor p53. This protein is well known for its functions in DNA repair and apoptosis [74], and has a multitude of links with the cell cycle. Although its function in arresting cell cycle progression due to DNA damage will not be discussed, its mechanisms in regulating senescence in response to telomere depletion and hypermitogenic arrest might not be that different. p53 involvement in senescence is not surprising, since $p21^{Cip}$ is upregulated with the activation of p53 [75]. Even though telomere depletion is a well-known phenomenon, the concept of hypermitogenic arrest might need some more clarification. The term hypermitogenic arrest was used by Blagosklonny [66, 76], who argued that unlike quiescence, which is induced by a lack of mitogens, senescence is actually invoked by an overstimulation of mitogens, which activate the MAPK pathways. When these pathways are activated transiently, they induce the expression of cyclin D, but also of certain CKIs. Under normal circumstances, CKIs, especially $p21^{Cip}$ and $p27^{Kip}$, can function as activators of cyclin D/CDK4/6 complexes [11]. However, overstimulation of these pathways also increases the CKI levels, which can then reach a threshold to trigger cell cycle arrest. This model also offers an explanation for the metabolically active and relatively large phenotype of a senescent cell, as well as the fairly normal levels of cyclin D/CDK4/6. Because the cells are continuously exposed to mitogens, they are induced to grow and to attain a functional metabolism, although actual cell cycle progression is arrested [66]. This hypothesis explains the observed phenomenon that overactiva-

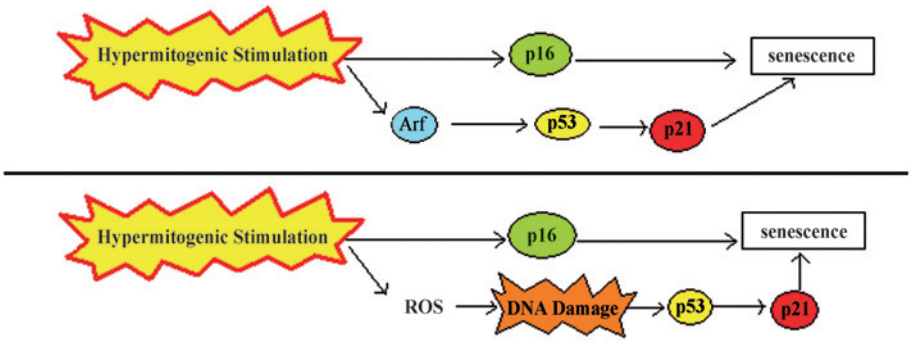

Figure 3. Two possible pathways leading to senescence from hypermitogenic stimulation. One pathway involves the transcription of Arf, leading to p53 activation, and subsequent $p21^{Cip}$ activation. $p16^{INK4a}$ is activated in response to hypermitogenic stimulation in both cases. The second model involves the accumulation of reactive oxygen species (ROS), which damage DNA, leading to upregulation of p53 and possibly $p21^{Cip}$. However, note that $p21^{Cip}$ is also hypothesized to work upstream of ROS accumulation [82].

tion of the MAPK pathways leads to premature senescence [77]. In the literature, two different pathways for senescence have been proposed, one triggered by telomere attrition and one by oncogenic stress (hypermitogenic stimulation). One pathway is the $p16^{INK4a}$/Rb pathway, mainly triggered by oncogenic stress, and the other is the p53/$p21^{Cip}$ pathway, which reacts strongly to telomere erosion [75].

The $p16^{INK4a}$/Rb pathway is so named because $p16^{INK4a}$ inhibits cyclin D/CDK4/6 complexes, effectively preventing Rb phosphorylation. As described above, this halts the cell cycle. Indeed, it has been shown that $p16^{INK4a}$ exerts its effects through the pocket proteins, especially Rb, and depletion of this protein will result in cells that fail to arrest with $p16^{INK4a}$ expression [78, 79]. In concordance with the hypermitogenic arrest hypothesis, $p16^{INK4a}$ induction has been measured after oncogenic *ras* stimulation [75, 77, 80]. $p16^{INK4a}$ mediated induction of senescence is proposed to occur as follows [80]: $p16^{INK4a}$-inhibits cyclin D/CDK4/6, thus activating Rb by preventing its phosphorylation. This in turn leads to an inhibition of the E2F genes, which are normally responsible for reducing the formation of reactive oxygen species (ROS) formed by mitogenic stimulation. This increase in ROS might in turn induce a senescent state by damaging DNA. This assumption seems plausible considering that ROS have been reported earlier to participate in mitogenic signaling and might even be a second-messenger system [81]. Furthermore, accumulation of ROS has been linked to senescence [82]. It should be noted, however, that ROS accumulation is a consequence of $p21^{Cip}$ rather than of $p16^{INK4a}$ during senescence of human fibroblasts. However, the induction of $p21^{Cip}$ was independent of p53 expression [82]. Even though $p16^{INK4a}$ has been implied to function as a result of oncogenic *ras* stimulation, there is also evidence that the p53/$p21^{Cip}$ pathway plays a role in stimulating this form of senescence, since both $p16^{INK4a}$ and p53 levels increase in response to oncogenic *ras* signaling [77]. Furthermore, in endothelial cells, upregulation of p21 is observed as a result of oncogenic *ras* signaling [83]. In mice, knocking out p53 can be sufficient for reentry of cells into the cell cycle [77]. However, in human fibroblasts, both p53 and $p16^{INK4a}$ need to be inhibited to consistently evade senescence. Interestingly, overexpression of $p21^{Cip}$ or $p16^{INK4a}$ does not result in a senescent phenotype similar to that resulting from oncogenic *ras* signaling [83]. There are two explanations for the apparent activation of the p53/$p21^{Cip}$ pathway in response to oncogenic stress (Fig. 3). First, it might be due to an upregulation of Arf ($p19^{Arf}$ in mice, and $p14^{Arf}$ in humans). Arf is transcribed from the the *INK4A* locus as an alternative reading frame for $p16^{INK4a}$ [84]. Unlike $p16^{INK4a}$, which targets cyclin D/CDK4/6, Arf is an activator of p53. Furthermore, it has been shown that $p19^{Arf}$ is crucial in murine senescence [1, 77] and, in addition, its expression has been detected in response to mitogenic signaling [77] and overexpression of E2F1 [85]. However, the importance of Arf in human cell senescence has been disputed [1] and more emphasis has been placed on $p16^{INK4a}$. Another explanation for clarifying the p53 induction in response to hypermitogenic stimulation is the induction of ROS, as discussed above. These reactive particles might be responsible for DNA damage, which would in turn result in p53 activation [75].

As for hypermitogenic arrest, senescence in response to telomere depletion, which has often been ascribed to the p53/$p21^{Cip}$ pathway, has an overlap with the $p16^{INK4a}$ pathway. Using the acute telomere attrition system, the inhibition of p53 and thus decreased $p21^{Cip}$ levels caused only a partial decrease in the senescent response, comparable to the inhibition of $p16^{INK4a}$ [86]. Inhibiting both p53 and $p16^{INK4a}$ almost completely circumvented the induction of senescence in

response to telomere depletion. Furthermore, telomere-induced p53 senescence was reverted by inactivation of p53 and oncogenic *ras*, but senescent cells that expressed $p16^{INK4a}$ were not easily reversed [62]. All in all, this implies a general mechanism of senescence in which $p16^{INK4a}$ is the mediator of the irreversible physiological arrest program, as proposed by Alcorta et al. [69]. Interestingly, senescence has also been observed in response to cytokines [87], which could imply a physiological means to actively induce cell senescence *in vivo*. It must be noted, however, that there is still controversy surrounding the role of $p16^{INK4a}$ and $p21^{Cip}$. For example, telomere-depletion-induced senescence was independent of $p16^{INK4a}$, and although $p16^{INK4a}$ increased in aging cells, it was independent of the $p53/p21^{Cip}$ pathway [88]. Furthermore, some human senescent cells are either $p21^{Cip}$ or $p16^{INK4a}$ positive [75, 88], instead of both [65, 69]. The different results might be accounted for by cell-specific expression of CKIs.

The findings presented above suggest an important role for the CKIs in inducing a senescent state. One would expect this to have an effect on Rb as well, since CKIs prevent cyclin/CDK activity. However, the activity of the pocket proteins is still not exactly known. As mentioned earlier, cells that lack Rb can still senesce, probably due to redundancy of the other pocket proteins, but cells that lack all pocket proteins fail to senesce [29]. One possibility is that Rb is present in its active hypophosphorylated state, possibly consistent with the lack of downregulation of cyclin D. This in turn might facilitate the expression of metabolic genes, while still repressing the proliferative genes [20] and the passage of R (discussed later). On the other hand, there is also evidence for the presence of p130 in senescent cells [89], as well as unphosphorylated Rb [90], as discussed for quiescence. The latter findings could be explained by the fact that CKI-induced cell cycle arrest and thus Rb hypophosphorylation or even dephosphorylation might be sufficient for the repression of certain proliferative genes, as discussed above. However, more research needs to be done to make either assumption conclusive.

Lastly, it is important to note that senescence is a separate state which seems to exclude quiescence [91]. Chromatin condensation in senescent cells was more similar to cells in late G1/S than to early G1/G0 cells [91]. This is consistent with the observations that cells in a senescent state due to hypermitogenic arrest acquire this phenotype at or after the restriction point [66, 76]. Furthermore, senescent cells are metabolically active and rather large, which is the phenotype of a late G1 cell more than that of a serum-starved cell [76]. The restriction point apparently controls whether the cell has the appropriate growth factors and can only be passed by mitogenic stimulation. Senescence via hypermitogenic stimulation might thus be induced after R is passed. Similarly, telomere depletion is an internal stimulus, thus a restriction point for mitogens might not readily affect such a control point. In the literature on DNA damage, it has indeed been suggested that G1 progression can be stopped closer to G1/S [92]. One can wonder whether hyperphosphorylation of Rb accurately describes the restriction point, since $p16^{INK4a}$ and $p21^{Cip}$ can acquire G1 arrest after R by mechanisms as similar to those that function before R. A possibility might be that mitogenic stimulation results in a certain amount of phosphorylation on Rb, leading to interactions with certain transcription factors to induce growth. However, the hyperphosphorylation and release of E2F transcription factors might occur later in G1, and therefore upregulation of CKIs is still effective to halt G1 progression.

In summary, senescence is an irreversible cell cycle arrest with hallmarks of late G1 cells. CKIs play an essential role in the maintenance of this state. $p16^{INK4a}$, in particular, is frequently implicated in the maintenance of senescene and $p21^{Cip1}$ is implicated sometimes for its initiation, but also for possible maintenance. Senescence results from aging (telomere depletion) and possibly hypermitogenic stimulation. The frequently observed CKI p21 upregulation can result from both p53-dependent as well as p53-independent pathways.

Differentiation

Unlike quiescence and senescence, which are resting states for cells in response to certain stimuli, differentiation is a process which allows a cell to irreversibly acquire a specialization. As with quiescence and senescence, CKIs also play an important role in regulating differentiation. The major two CKIs that are encountered in differentiation are $p21^{Cip}$ and $p27^{Kip}$. First, a substantial amount of the literature indicates a progressive accumulation of $p27^{Kip}$ in cells that are about to differentiate. $P27^{Kip}$ accumulation in oligodendrocyte progenitors was related to differentiation and this led to the suggestion that $p27^{Kip}$ might function as an intrinsic timer [93]. A subsequent knockout mouse study demonstrated that oligodendrocyte $p27^{Kip-/-}$ progenitors initiated differentiation in the presence of mitogens later than $p27^{Kip+/-}$ and $p27^{Kip+/+}$ mice [94]. This indeed does indicate some sort of timer function for $p27^{Kip}$ in these cells. On the other hand, it should be mentioned that $p27^{Kip-/-}$ cells deprived of mitogens did differentiate normally and it was concluded that although $p27^{Kip}$ might be a

counting mechanism, it is not necessary for the serum deprivation cell cycle arrest preceding differentiation [94]. More support for a progressive accumulation of $p27^{Kip}$ comes from research on erythroid differentiation. However, unlike the oligodendrocytes, in these cells, $p27^{Kip}$ is sequestered by CDK4 in a stabilizing mechanism, but as it reaches higher levels, it starts inhibiting CDK2 action and induces cell cycle arrest. Furthermore, $p21^{Cip}$ is also highly expressed in these cells, but its function appears to be independent of cell cycle arrest and more focused on inducing an actual differentiation program [95]. Similar conclusions were obtained with respect to the importance of $p27^{Kip}$ for cell cycle arrest and $p21^{Cip}$ for actual differentiation, using mouse oligodendrocytes [96]. $p21^{Cip-/-}$ progenitor cells retreat from the cell cycle like normal progenitors, but these mice do display a lack of cerebral myelination, apparently due to a lack of oligodendrocyte progenitor differentiation. The importance of $p27^{Kip}$ in cell cycle arrest for erythroid progenitors is supported by studies that indicate inhibitory binding of CDK2 by $p27^{Kip}$ [97].

The notion that $p21^{Cip}$ is an active inducer of differentiation finds more support in the neuronal differentiation literature. Upregulation of $p21^{Cip}$ was observed during neuroblastoma differentiation, along with also sustained $p21^{Cip}$ levels after the initiation of the process [98]. However, in many non-neural cells, a contradictory mechanism is observed. $P21^{Cip}$ will often be transiently expressed at the initiation of differentiation, while the $p27^{Kip}$ levels accumulate progressively afterwards [99]. In murine keratinocytes, for example, the $p21^{Cip}$ levels initially increase, but increased levels of $p21^{Cip}$ in later stages of differentiation will actually have an antidifferentiation effect [100]. Forcing $p21^{Cip}$ expression in these cells resulted in a lack of expression of differentiation markers for keratinocytes. Similar findings were obtained using human fetal intestinal epithelial cells [101]. Expression of $p21^{Cip}$ was high with the induction of differentiation, but dropped rapidly afterwards, while the expression of $p27^{Kip}$ again accumulated. Interestingly, $p27^{Kip}$ did not bind or inhibit cyclin/CDK complexes, which were already bound to $p21^{Cip}$. A possible direct involvement of this CKI with transcription factors seems attractive, because of the observation that a significant lag exists between Rb activation and the expression of differentiation proteins in cells with low $p27^{Kip}$ levels [101]. Even though this assumption remains to be demonstrated, there is some evidence for the role of Cip/Kip CKIs as transcriptional cofactors [102].

Even though involvement of the Cip/Kip CKIs is undisputed in differentiation, the role of INK4 CKIs is less clear. It has sometimes been reported that $p16^{INK4a}$ does not participate in differentiation. As a matter of fact, if $p16^{INK4a}$ is expressed in intestinal epithelial cells, it does cause cell cycle arrest, but it also inhibits the expression of differentiation markers [101]. In hematopoetic progenitors also, $p16^{INK4a}$ inhibits differentiation [103]. On the other hand, it should be noted that forced expression of $p16^{INK4a}$ in $p21^{Cip-/-}$ murine keratinocytes can restore normal differentiation properties [100]. Furthermore, in a comprehensive review by Muñoz-Alonso and Léon [99], $p16^{INK4a}$ and other INK4 proteins, such as $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{Arf}$, are even mentioned to be upregulated in certain cell types during differentiation. This makes it rather complicated to draw conclusions about the role of INK4 CKIs in differentiation, and their participation is likely to be cell specific.

Although the exact patterns and roles of $p21^{Cip}$ and $p27^{Kip}$ in differentiation are still unclear due to many contradictory findings, the two proteins are upregulated in most cell types during differentiation [99]. This raises the next question, whether forced expression of these proteins under normal conditions is sufficient to induce differentiation. Although there are again several exceptions, simply overexpressing CKIs is not sufficient for differentiation. For example, whereas differentiation of human keratinocytes is related to the CKIs $p27^{Kip}$, $p21^{Cip}$, and $p16^{INK4a}$, overexpression of these proteins will not result in differentiation [104]. Similarly, ectopic expression of $p27^{Kip}$ in rat oligodendrocyte progenitors was not sufficient in facilitating differentiation, although cell cycle arrest was achieved [105]. In erythroid differentiation also, cell cycle arrest was not adequate [97], and the same applies to intestinal epithelial cells [101]. However, before concluding that in general for all cells, cell cycle arrest is not sufficient to induce differentiation, several studies show increased expression of differentiation markers after CKI overexpression [106, 107].

As briefly mentioned above, in many cell types, differentiation occurs paradoxically under conditions that trigger quiescence, namely serum deprivation. An illustrative example might be that myoblasts do not differentiate in the presence of the mitogen fibroblast growth factor (FGF) [108]. Furthermore, the same CKIs as were discussed for quiescence ($p21^{Cip}$ and $p27^{Kip}$) appear to be crucial in establishing a differentiation process. Indeed, the similarities even lead to the assumption that cells start differentiation from the G0 state [109, 110]. However, a considerable body of evidence has accumulated that suggests that cell differentiation and quiescence actually exclude each other. First, the recent research by Coller et al. [16] discussed above for quiescence is partly devoted to this specific question. They used human dermal

fibroblasts and transfected these with the *MyoD* gene, a transcription factor for muscle differentiation. Activation of MyoD by β-estradiol led to expression of muscle-specific proteins and myogenic differentiation. Interestingly, cells that were arrested by forced expression of $p21^{Cip}$ started differentiation, while quiescent cells that were serum starved and initiated the quiescent program described earlier did not exhibit differentiation [16].

Research undertaken more than 25 years ago is consistent with this notion that quiescent cells are resistant to differentiation. In two papers, the temporal profiles and kinetics of differentiating cells were compared to quiescent states [111, 112]. Two quiescent states were identified, namely G_S (in response to serum starvation) and G_N (in response to nutrient starvation). Interestingly, although G_S and G_D (the differentiation state) could not be well separated temporally, G_D cells could still become G_S cells, while the opposite was not true. The authors concluded that "prior to the expression of a differentiated phenotype, proadipocytes must arrest their growth at a distinct state in the G1 phase of the cell cycle, G_D" [112]. The latter research was accomplished before the knowledge of cyclins, CDKs, and CKIs emerged, but it might be consistent with results mentioned above that first a cell cycle arrest occurs, possibly mediated by $p21^{Cip}$ and $p27^{Kip}$ [16], and then a decision point emerges between differentiation and quiescence, and if the latter path is taken, there is no way back. Examining the role of the pocket proteins during differentiation might shed light on the molecular mechanisms that could underline the decision point between quiescence and differentiation.

A pocket protein that has often been implicated in differentiation is Rb. There is quite some evidence that Rb can act as a direct activator of differentiation genes and as a repressor of differentiation repressors (Fig. 4). In myogenic differentiation, hypophosphorylated Rb can directly bind MyoD, a myogenic transcription factor, in an activating way [113]. Rb has also been shown to interact with other differentiation-inducing transcription factors, such as C/EBP, NF-IL6, MEF2, GRα, and others [114–116]. In the formation of osteoblasts also, Rb associates with the osteoblast transcription factor CBFA1, thereby promoting differentiation. Of course, a second way in which Rb promotes differentiation is by enforcing the required cell cycle arrest [115]. Similarly, Rb has been shown to bind and inhibit differentiation repressors. A well-researched example is the case of HBP1, which stimulates cell cycle exit in muscle cells [4], maintains a quiescent state [117], and represses differentiation when forcibly expressed [4]. The latter effect may be due to the ability of HBP1 to bind and inhibit MyoD [4]. Paradoxically though, at the beginning of differentiation, HBP1 levels actually increase. However, as the ratio Rb/HBP1 changes, the potential for differentiation changes as well. Shih *et al.* [4] report that a high Rb/HBP1 ratio facilitates differentiation, while a low Rb/HBP1 ratio seems to inhibit it. This led to the assumption of a possible differentiation checkpoint that needs to be overcome, with Rb as a possible positive regulator and HBP1 as a negative regulator. Furthermore, not only do the HBP1 levels increase, but p130 does as well. This pocket protein was discussed for the role it plays in quiescence, and in differentiation it plays a repressor role. In osteogenic differentiation, for example, knocking out Rb has detrimental effects on differentiation, while knocking out p107 and p130 does not affect the process [118]. This is supported by knockout adipocytes as well [117]. As a matter of fact, the latter authors also report decreased differentiation when expressing p130 in these cells. It should also be mentioned that like Rb, p130 does bind to HBP1 [4, 117]. So what is observed in muscle cells is an increased level of both p130 and HBP1 which can inhibit MyoD at the start of differentiation. Then, underphosphorylated Rb starts to accumulate more slowly, eventually raising the Rb/HBP1 ratio and thereby triggering activation of the MyoD cascade leading to differentiation [117]. Interestingly, if a sufficient ratio of Rb/HBP1 is not achieved, the quiescence maintainers p130 and HBP1 will prevent differentiation and perhaps possibly start a quiescence program as discussed above.

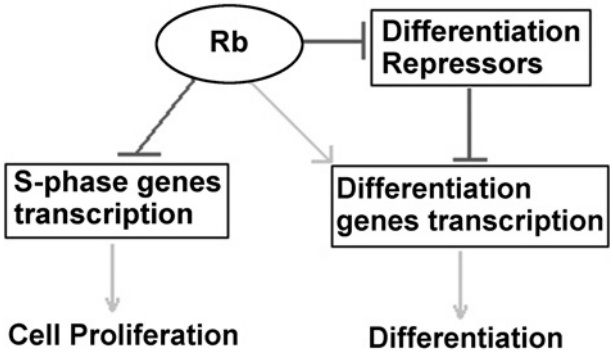

Figure 4. Mechanisms by which Rb can facilitate differentiation. First, it inhibits the transcription of S phase genes by inhibiting E2F. Furthermore, it can directly stimulate the expression of differentiation genes by binding to transcription factors. In addition, Rb is implicated in inhibiting differentiation inhibitors.

Muscle cells are not the only cells that support this notion of a kind of differentiation checkpoint. In adipocytes, for example, a protein similar to HBP1 named CHOP has a similar inhibitory effect on the differentiation transcription factor c/EBP, and anoth-

er such protein, p202, was discovered for myogenic differentiation [117]. So far, the possible internal mechanisms for differentiation have been discussed. As mentioned above, serum starvation conditions are often used to stimulate differentiation. However, as discussed in the section on quiescence, it remains a question whether this condition accurately portrays physiological circumstances. Perhaps it is more plausible that differentiation is triggered directly by 'differentiation factors' rather than serum starvation. Interesting research considering this assumption comes from differentiation as a result of NGF stimulation. NGF can act both as a mitogen and differentiation inducer [119, 120] depending on the cell type. Its functions as differentiation factor are mostly limited to sympathetic neurons [119]. In chick embryos, for example, exposure to exogenous NGF will increase the number of sympathetic neurons [121]. Insight into the molecular mechanism behind NGF-induced differentiation comes from research using PC12 pheochromocytoma cells, cells that have both EGF and NGF receptors [122, 123]. NGF binds to the receptor TrkA, which results in the eventual phosphorylation of MAPK. As discussed above, the activation of MAPK is a common pathway for mitogenic stimulation. However, in PC12 cells, MAPK activation occurs while the cells exit the cell cycle and differentiate. Interestingly, the signal transduction pathway of EGF, a mitogen that leads to proliferation of PC12 cells, seems to be rather similar, since rapid MAPK activation occurs as a result of both mitogens [124]. However, the activation of MAPK was transient for EGF, while NGF stimulation results in a sustained MAPK activation in PC12 cells [124]. Furthermore, no nuclear translocation of MAPK after EGF stimulation was detected, while NGF stimulation did result in MAPK translocation to the nucleus [124]. The temporal activation of MAPK might influence cell fate decision.

Besides MAPK activation, several other changes take place in PC12 cells stimulated by NGF. First, an upregulation of $p21^{Cip}$ is observed, which might account for the withdrawal from the cell cycle for differentiation [125, 126]. Furthermore, an accumulation of hypophosphorylated Rb was observed in PC12 cells treated with NGF [127]. This could be consistent with the previously discussed notion of Rb as an inducer of differentiation by activating differentiation transcription factors and inhibiting differentiation repressors. Interestingly, during the development of chick embryos, adrenal medullary cells of different ages seem to respond differently to NGF. When cultured with NGF, the percentage of neural differentiating cells has an optimum at 10–12 days of embryonic age, with the response declining thereafter [128]. This is an example of the possible growth factor receptor regulation discussed for quiescence. In addition, in PC12 cells NGF stimulation results in a decreased number of EGF receptors [122], thus acting as a mitogen for these cells. This supports the notion discussed for quiescence that a serum-starvation-like state might be achieved *in vivo* by growth factor receptor modification/regulation.

A model that differentiation occurs from a cell cycle arrest state, referred to as the G0 state, has been proposed [117]. However, the evidence discussed above that quiescence actually excludes differentiation, seems to contradict this suggestion. A possibility could be that cells that will become quiescent and cells that differentiate exit the cell cycle in a similar point in time, consistent with Scott et al. [112], who show little temporal difference between the G_D and G_S state. The mediators of this initial cell cycle arrest could be $p21^{Cip}$ and/or $p27^{Kip}$ in response to mitogen withdrawal, differentiation factors, or perhaps even as a result of CKI accumulation as an intrinsic timer. During this arrest, the upregulation of quiescence maintainers such as p130 and HBP1/CHOP prevent cell cycle reentry as well as differentiation. If Rb is sufficiently upregulated during this time, perhaps as a result of extracellular signaling, the Rb/repressor ratio can be increased, leading to inhibition of differentiation repressors and thus the expression of differentiation genes. If Rb does not reach the critical level required to sufficiently raise the Rb/HBP1 ratio, the differentiation checkpoint will not be passed and the cell will initiate a quiescence program which would exclude differentiation [16] (Fig. 5).

Apoptosis

Unlike the previously discussed cell fates, apoptosis does not allow for cell survival and is actually often referred to as 'programmed cell death.' This is due to the fact that apoptosis is a highly structured and regulated form of cell death. The functions of apoptosis are mainly to maintain tissue homeostasis, to help in development, e.g., the formation of separate digits [2] and the development of the nervous system [129], and as a reaction to oncogenic stress to prevent tumor formation. Considering these functions, it is not surprising that apoptosis is related to the cell cycle machinery, but although the pathways to apoptosis are rather well known, its exact relation to the cell cycle is still unclear. Before elaborating on the relation between the cell cycle and apoptosis, it should be noted that the scope of the discussion here is focused primarily on apoptosis under physiological conditions, which excludes much of the research on apoptosis as a

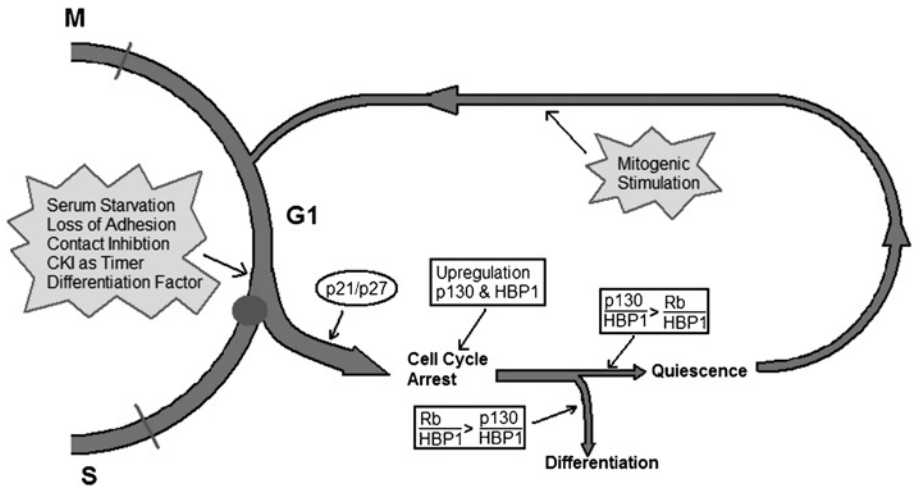

Figure 5. A stimulus triggers a cell cycle exit before R and the subsequent cell cycle arrest is mediated by $p21^{Cip}$ or $p27^{Kip}$. Subsequently, the arrest is reinforced by quiescence maintainers such as p130 and differentiation repressors such as HBP1. When Rb/repressor ratios exceed the threshold, the cell will differentiate if it is able to. When the p130/repressor ratios are higher than the Rb/repressor ratios, the cell will become quiescent.

result of invoked DNA damage or pharmacological intervention.

The principal determinant for apoptosis is the activation of so-called caspases [130]. These proteins inhabit the cytosol in an inactive form (procaspases) and upon activation start actively cleaving and activating effector caspases [131] that, among other properties, will have proteolytic and DNase activity, ultimately leading to cell death [130]. The activation of caspases occurs through two main pathways. Firstly, cytochrome c is released from the mitochondria and stabilizes an Apaf-1/caspase 9 complex, which can then activate effector caspases. Secondly, mammalian cells have so-called death receptors. Activation of these (i.e. by tumor necrosis factor or CD95 receptors) activates FADD, which in turn activates caspase 8, again leading to downstream effector activation [131]. When examining the instigators of apoptosis, one of the well-researched ones is p53 which was already discussed for having a large role in senescence. p53 acts by stimulating release of cytochrome c from the mitochondria and thereby directly stimulating apoptosis due to caspase activation [132]. P53 is often activated in response to stress factors, such as DNA damage [133] and telomere erosion [132]. Another potent activator of p53 is overexpression of c-Myc. Under normal circumstances c-Myc is an important mediator of G1 progression and G0–G1 transition [134]. However, it also plays a role in apoptosis and both overexpression and underexpression can lead to such a fate, as well as c-Myc expression under serum starvation conditions [134]. The increased presence of the myc protein induces Arf expression, which in turn stabilizes p53 by binding to Mdm-2, a protein responsible for the sequestration and inactivation of p53 [135]. A similar effect is found when overexpressing E2F, inhibiting Rb [136], and upregulating cyclin D/CDK4/6 [137]. Both p53-dependent and -independent apoptosis occurs as a result, as well as upregulation of crucial apoptosis proteins, such as Apaf-1 and effector caspases [138]. Interestingly, when Rb is coexpressed, apoptosis does not occur in E2F-overexpressing cells [134]. Similarly, p53 overexpression in $p53^{-/-}$ and $Rb^{-/-}$ HeLa cells leads to apoptosis, while p53 coexpressed with Rb induces cell cycle arrest [139, 140]. So, an inappropriate activation of proliferation transcription factors, such as c-Myc and E2F, can lead to apoptosis by p53 activation. This could be a defense mechanism to protect the cell against inappropriate oncogenic stress. It should be noted, however, that apoptosis is not solely dependent on p53, and several p53-independent mechanisms exist to achieve this goal. For example, erythroid progenitors deprived of Epo will undergo apoptosis without p53 [141] and corticosteroid-invoked apoptosis also takes a p53-independent pathway [142].

It is rather salient that apoptosis and senescence, both of which are implicated in aging, can use similar mechanisms. However, what decides whether a cell will senesce or commit suicide? This seems to be largely due to extracellular signals. p53 itself is known as a transcription factor for both proapoptotic and antiapoptotic genes. Depending on extracellular signals, a route is chosen [143]. For example, β-catenin, a protein important in cell-cell adhesion, can function as a messenger to the nucleus that stimulates $p19^{Arf}$ and in turn activates p53. However, a senescent phenotype rather than apoptosis results in this case [143]. This may be due to stimulation of the cell cycle arrest functions of p53, such as the induction of $p21^{Cip1}$, while inhibiting the transcription of proapoptotic genes. Even though this seems plausible, p53 is still a direct stimulant of apoptosis by increasing cytochrome c release from the mitochondria. A possibility for cell survival of senescent cells might be an upregulation of chaperones that inhibit cytochrome c release and

caspase activation [144]. These findings indicate the possibility of a decision point between apoptosis and senescence after stress-induced p53 activation. More support for such a notion comes from research that used the apoptosis inducer doxorubicin in human neuroblastoma cells; subsequent inhibition of caspase-3 shifted the predominant cell fate toward senescence [145].

As previously discussed, senescence appears to be induced after R, since R is a checkpoint merely for the confirmation of sufficient growth factors. Consistent with the assumption that telomere attrition and hypermitogenic stress should not trigger a hypomitogenic cell cycle arrest, a possible decision point between senescence and apoptosis could be situated behind R. This could be consistent with findings discussed earlier that p53-overexpressing fibroblasts will induce apoptosis, while coexpression of Rb inhibited this effect. It could be that the enforced expression of Rb creates a cell cycle arrest at R, which will consequently prevent the cells from traversing the restriction point and arriving at the apoptosis/senescence decision point.

Although the previous section mostly covered apoptosis as a response to cellular stress, there are also good indications of a separate apoptosis point that appears to reflect more physiological parameters, i.e. development. In neuronal development, for example over 50 % of newly developed oligodendrocytes go into apoptosis shortly after mitosis [146]. This assumption is supported in rat cerebral cortex development [147]. More structural research using synchronized mitotic CHO cells that were exposed to serum starvation conditions for 24 h showed that these cells exit the cell cycle shortly after mitosis [5]. These cells subsequently display a quiescence-like morphology, $p27^{Kip}$ upregulation, and cyclin D downregulation. However, upon serum addition, which would drive normal G0 cells back into the cell cycle, these cells remained in this state, which is referred to as a G_0^- state [5]. Not only were these cells irreversibly arrested, they also showed hallmarks of apoptosis (i.e. caspase activation) and 40 % of cells were apoptosed after 3 days [5], leading to the hypothesis that there is a checkpoint early in G1 where cells decide to continue G1 and proliferation or to go into apoptosis. The mechanism behind this checkpoint might involve PI 3-kinase, an important proliferative protein for cell cycle progression, since the inhibition of this protein resulted in early G1 cell cycle arrest and increased caspase activation [148]. The notion of an early G1 checkpoint is consistent with the findings presented before of early apoptosis in neuronal and oligodendrocyte development, and this point could represent the more physiological apoptosis checkpoint that is important in development and tissue homeostasis.

One could hypothesize that, in line with senescence, p53 mediates the stress-related apoptosis discussed in the first part and that developmental apoptosis might take alternative routes. This is supported in mouse mammary gland involution. After lactation, the mammary gland will change back into a non-active form and apoptosis is an essential process for achieving this. During this developmental apoptosis, p53 is not required, since the mammary glands of p53-deficient mice undergo normal involution [149]. However, it should be noted that contradictory findings have been reported. A delayed involution was observed in p53-deficient mice [150], and although p53-independent pathways also appear to function, p53 does partake in normal mouse mammary gland involution.

As mentioned above, developmental apoptosis seems to be induced from a separate and irreversible quiescence-like state [5]. Indeed, after induction of a quiescence program resulting from mitogen withdrawal, loss of adhesion, and loss of cell contact, antiapoptotic proteins are often upregulated [16]. So, apoptosis does not seem to occur after quiescence is initiated; instead, apoptosis occurs early in G1, and possibly late in G1 after R in response to stress (Fig. 6).

Integrative model

From the findings discussed above, the following model for G1 phase progression and cell fate decision during this phase is proposed (Fig. 7). After mitosis is completed, the cells enter G1 phase. Shortly thereafter the first checkpoint will be encountered that decides on continuation of the cell cycle or apoptotic cell death. Crucial in this checkpoint appears to be PI 3-kinase, which is under extracellular control. The inhibition of PI 3-kinase leads to exit from the cell cycle in early G1 and entry into a quiescence-like albeit irreversible state, which will lead to apoptosis [5]. A role for p53 in the early apoptosis checkpoint remains to be confirmed, although extracellular signaling leading to p53 induction might also occur here. With sufficient PI 3-kinase or other antiapoptotic signaling, the cell passes this checkpoint and continues G1 progression, characterized by growth. Cyclin D is upregulated at the beginning of G1 and forms complexes with CDK4/6 that phosphorylate Rb. When Rb is sufficiently phosphorylated it will become inactive and E2F transcription factors can in turn upregulate S phase genes. Keeping Rb in a hypophosphorylated state prevents E2F activation and progres-

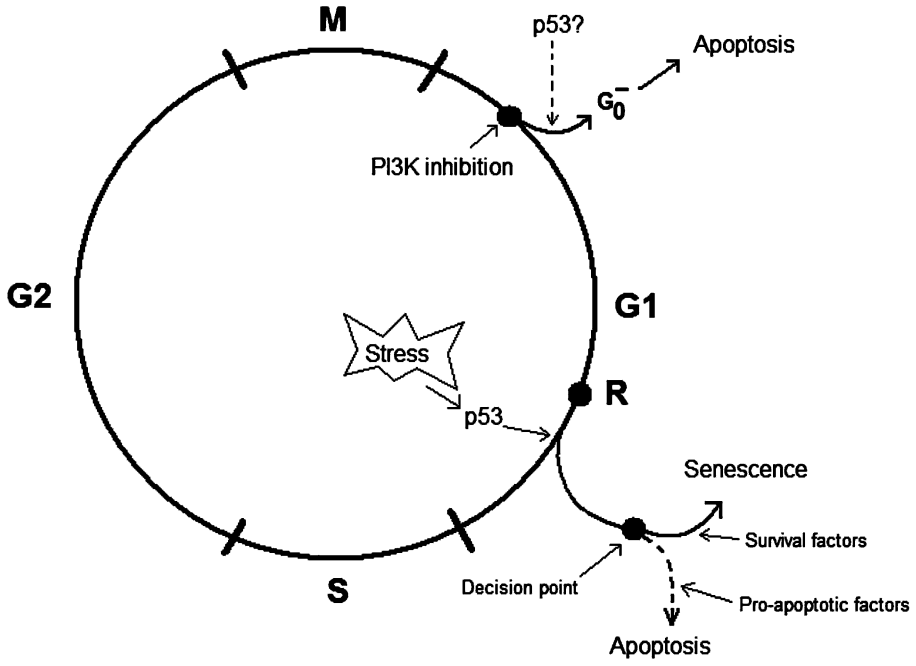

Figure 6. Possible model for the induction of apoptosis during G1 phase progression. Early after entry into G1 the cells pass a point during which mitogenic stimulation is crucial. PI 3-kinase (PI_{3K}) inhibition leads to cell cycle arrest and possible apoptosis. Later in G1, after R, cells can exit the cell cycle in response to oncogenic or genotoxic stress. The decision between apoptosis and senescence at the decision point is influenced by the presence of survival factors or proapototic factors.

sion past the restriction point R [9]. Upon conditions of serum starvation, loss of adhesion, or contact inhibition, and possibly upon induction by differentiating factors, CKIs are upregulated, which prevents Rb phosphorylation by cyclin D/E/CDK complexes and results in a cell cycle arrest at R. Note that this cell cycle arrest, mostly induced by $p21^{Cip}$ and/or $p27^{Kip}$, is not synonymous with quiescence or G0 and precedes this state.

The cell cycle arrest is reinforced by the expression of p130 and cell-type-specific differentiation repressors, such as HBP1 and p202 for myocytes and CHOP of hepatocytes [117]. The upregulation of Rb, an inducer of differentiation, possibly by extracellular differentiation-promoting factors, shifts the Rb/repressor ratio. If Rb reaches sufficient levels, it will surpass the cell cycle arrest maintainers HBP1/p202/CHOP and p130 and act as a differentiation activator by stimulating and facilitating the transcription of cell-type-specific proteins [117]. In the absence of sufficient Rb levels, cell cycle arrest is reinforced and a quiescence program is initiated, while differentiation and apoptosis are excluded as fates [16]. The quiescent state is reversible and the cell can be induced to reenter the cell cycle by mitogenic stimulation. As discussed earlier, several different quiescent programs seem to exist, depending on the cause of the cell cycle arrest (i.e. growth factor deprivation, contact inhibition, or loss of cell adhesion). More research could elucidate the role of these three extracellular stimuli in the three different quiescence programs.

Even though the processes of differentiation and quiescence appear to exclude each other, there might still be a link between them. When considering terminal differentiation, the process of differentiation into a non-proliferative specialized cell, these cells seem to rest in a G0 phase. Furthermore, terminally differentiated myotubes can partly reenter the cell cycle after serum addition [151], which is also a hallmark of quiescence. Therefore, it is possible that after differentiation, cells reenter a quiescent state. Once the cell reenters the cell cycle or if it has not stopped at the restriction point, it encounters a possible cell stress checkpoint. Hypermitogenic stress, telomere attrition, and other stress factors can induce p53 and possibly $p16^{INK4a}$. Both p53, via $p21^{Cip}$, and $p16^{INK4a}$ can induce a withdrawal from the cell cycle. The cell now encounters another checkpoint leading it to senescence or committing it to apoptosis. In the presence of survival factors, such as β-catenin, or possibly Akt-activating pathways [152], the cell can go into irreversible senescence. In the presence of proapoptotic factors, such as activators of the 'death receptors,' the cell can go into apoptosis as a result of the stress.

Although this review has focused more on physiological rather than artificial conditions, there is no clear distinction between the two, which is important to consider especially for senescence and apoptosis. Although telomere attrition and perhaps a hypermitogenic arrest can reflect physiological events, they do seem to trigger an alarm mechanism that is also induced in response to DNA damage. As a matter of

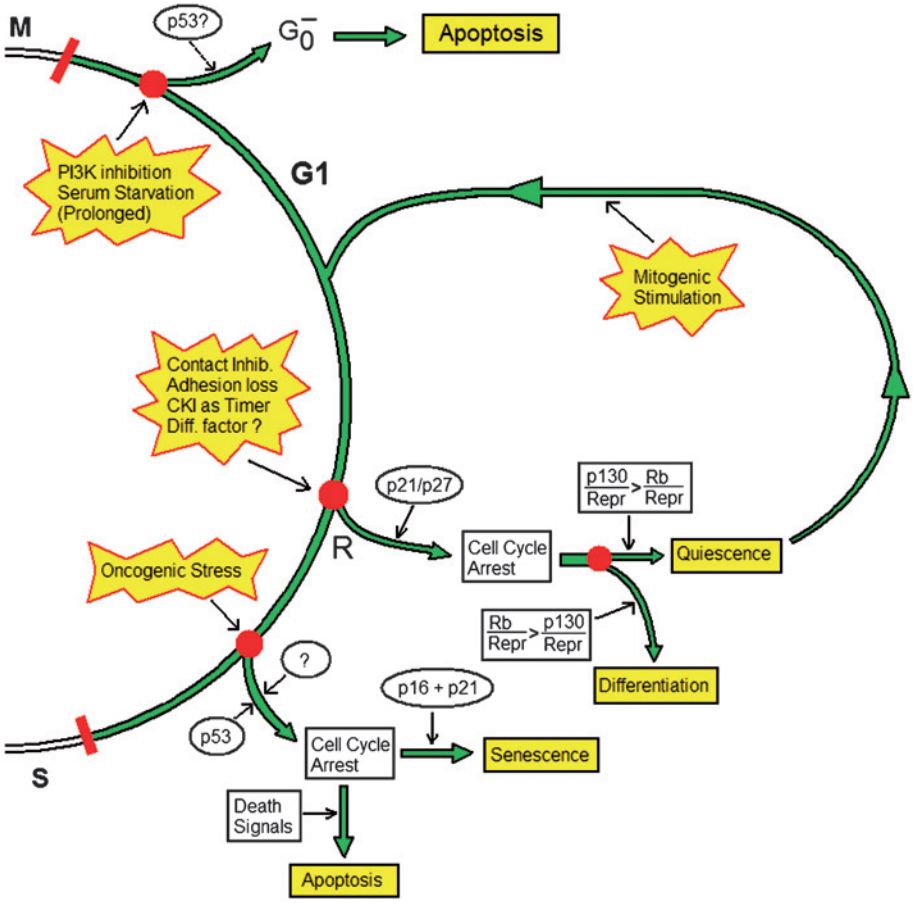

Figure 7. Integrative model. As a cell enters G1, the first checkpoint is reached. Serum starvation or PI3K inhibition at this point will lead to an irreversible quiescent state with subsequent apoptosis. The cells will then progress toward R. Quiescence or differentiation-inducing conditions will result in cell cycle arrest. If the Rb/repressor ratio is sufficiently high, the cells will differentiate if they are able to. If the repressor ratio is high, the cells will become quiescent. If the cells pass R, there is a possibility of arrest near the G1/S border in response to oncogenic or genotoxic stress. Here, cells will senesce or go into apotosis.

fact, it has even been hypothesized that telomere attrition mediates senescence and apoptosis by DNA damage induced when telomeres become too short [153]. Similarly, (hyper)mitogenic stimulation can induce ROS [154] which in turn might damage DNA and induce p53. DNA damage in response to radiation or chemical mutagenesis may thus induce similar pathways to those we have discussed.

It is important to note that much of the research is done under laboratory conditions with cell cultures. This means that not every finding can be generalized to the *in vivo* situation. One problem is encountered in quiescence. Even though loss of adhesion or an abnormal cell density might occur under physiological conditions, the features of serum starvation might not be present under physiological conditions, since the body provides cells with continuous growth factors. Even though most of our cells are in a quiescent state, it remains a question whether this is invoked by growth factor starvation. A possible explanation for the phenomenon might be cellular desensitization or internalization of growth factor receptors which could perhaps mimic serum deprivation conditions. As discussed, in developing chick embryos, NGF sensitivity varies during neuronal development [128, 155], possibly due to receptor modification. Furthermore, one could speculate on the presence of quiescence-inducing factors. More *in vivo* research on the different quiescence programs as well as *in vitro* research on the possibilities of inducing differentiation using cell density and adhesion as parameters might help to clarify this. It is interesting to note that, although the Cip/Kip and the INK4 CKIs play pivotal roles in arresting the cells before the induction of a cell fate, ectopic expression is often not enough for the induction. However, ablating the responsible CKIs is often enough to reverse the cell fate, such as senescence and quiescence, and reintroduce the cells into the cell cycle. So even though they are crucial tools for the maintenance of the discussed cell fates, especially quiescence, differentiation, and senescence, they do not seem to induce the fates themselves, and these seem highly regulated by extracellular signals.

Lastly, the ability to generalize the model is likely to depend heavily on and vary greatly among cell types. For example, as discussed earlier, $p21^{Cip}$ has been mentioned as an initiator of differentiation in some cells and an inhibitor in others. Some researchers report induction of both $p16^{INK4a}$ and p53 in response to hypermitogenic stress, and others state only a role

for the former. There are a multitude of examples and pathways for which contradictory literature can be found, which is partly due to the different mechanisms in different cell types.

Conclusion

In summary, the regulatory mechanisms of four cell fates, namely quiescence, differentiation, senescence, and apoptosis, that can be induced in the G1 phase of the cell cycle have been discussed, leading to the formation of a temporal model. The earliest checkpoint manifests itself early in G1, and cell cycle exit from this point leads to the induction of apoptosis. As the cell progresses through G1 it will encounter the restriction point R. Several factors, such as serum starvation and loss of adhesion, induce cell cycle arrest which is reinforced by antidifferentiation proteins such as HBP1 and p130. Increased ratios of Rb/repressor sensitize the cell for differentiation, while low ratios of Rb/repressor and high rations of p130/repressor invoke a quiescent state. The precise quiescence program is determined by the stimulus that induced the preceding cell cycle arrest. After traversing the restriction point, cellular stress, such as telomere erosion and hypermitogenic stress, can invoke p53 and possibly $p16^{INK4a}$ for a cell cycle arrest which, depending on the presence of either extracellular survival or death signals, can lead to senescence or apoptosis. The latter mechanisms prevent the formation of tumor formation and are often mutated in cancer. Although laboratory research is required to test the proposed temporal model of cell fate determinations in the G1 phase, it might be a small step toward a comprehensive understanding of the cell cycle and how the determination of cell fate is related to this important cellular regulatory mechanism.

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