Ageing as developmental decay: insights from p16^{INK4a}

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ABSTRACT

The $p16^{INK4a}$ cell cycle regulator is one of the best ageing biomarkers, suppressed in early embryogenesis and progressively induced during ageing. $p16^{INK4a}$ plays a crucial role in key cell fate decisions which contribute to ageing, such as cellular senescence and stem cell dynamics. Detailed examination of the pathways regulating $p16^{INK4a}$ expression has revealed an overlap with those regulating early development. Here we present the hypothesis that ageing might be primarily driven by gradual functional decay of developmental pathways. To support this, we summarize the role of $p16^{INK4a}$ in ageing and our current knowledge on $p16^{INK4a}$ regulation. The developmental decay hypothesis implies that the much evidenced damage that is associated with all aspects of ageing might be secondary to such decay.

HIGHLIGHTS

- The $p16^{INK4a}$ cell cycle regulator is a biomarker of ageing.
- $p16^{INK4a}$ plays a key role in cellular senescence and stem cell dynamics.
- $p16^{INK4a}$ is regulated by pathways that drive early development.
- We hypothesize that ageing could result from developmental decay.

p16^{INK4a}: a biomarker of ageing

Ageing (see Glossary) is a gradual deterioration of physiological function leading to reduced fitness, increased susceptibility to pathologies and increased mortality rate. Cancer, diabetes, cardiovascular disorders, immune decay and neurodegenerative diseases are amongst the main ageing-associated pathologies. These organism phenotypes relate to cellular and molecular changes. Nine molecular hallmarks of ageing have been recently proposed, including cellular senescence or stem cell exhaustion [1]. These cell alterations result in impaired tissue homeostasis, regeneration and eventually impact on organ function. Research aimed at understanding the biology of ageing has been hampered by the lack of irrefutable biomarkers for ageing. However, p16^{INK4a}, a cell cycle inhibitor, has emerged in the last ten years as a valuable candidate biomarker [2].

p16^{INK4a} is one of the products of the *INK4/ARF* locus (Box 1; Figure 1), and was molecularly cloned by virtue of its interaction with cyclin dependent kinase 4 (CDK4) [3]. It was soon realised that p16^{INK4a} controls the G1 phase of the cell cycle [3], is a key effector of cell senescence [4] and is frequently inactivated in cancer [5]. More recently, genome-wide association studies (GWAS) have related the *INK4/ARF* locus to many age-related pathologies, including susceptibility to frailty and increased risk of coronary artery disease (CAD), myocardial infarction, type-2 diabetes and late onset Alzheimer's disease. Moreover, a meta-analysis identified the *INK4/ARF* locus as the locus genetically linked to the highest number of age-related pathologies (reviewed in [6]).

Despite its involvement in cell cycle control, p16^{INK4a} is dispensable for early development and is not involved in programmed developmental senescence [7, 8]. Its expression is deeply suppressed in embryogenesis [9] and remains very low in most somatic tissues of young mammals [10]. However, certain stresses, such as acute oncogene expression, efficiently induce p16^{INK4a} both *in vivo* and in tissue culture. As animals age, *CDKN2A* becomes progressively expressed in most tissues and the levels of p16^{INK4a} reflects age in mice and humans [2]. An exponential increase of p16^{INK4a} expression with ageing is also observed by bioluminescence imaging in mouse models that bear a reporter for p16^{INK4a} [10, 11].

How does p16^{INK4a} affect ageing?

Importantly, $p16^{INK4a}$ is a key effector of cellular senescence and controls stem cell dynamics. As both processes are hallmarks of ageing [1], it is not surprising that $p16^{INK4a}$ influences organism ageing and is not simply a passive biomarker.

Contribution of $p16^{INK4a}$ to cellular senescence

Cellular senescence was first described in 1961 by Leonard Hayflick and Paul Moorhead, who reported that human diploid fibroblasts lose their capacity to divide after a number of passages in culture [12]. The maximum number of times primary cells can divide is referred to as the "Hayflick limit", and in human fibroblasts it has been suggested to correlate inversely with donor age. Replicative senescence is observed in the vast majority of primary mammalian cells, with the famous and highly significant exception of embryonic stem cells (ESCs). Senescent cells display a stable cell cycle arrest accompanied with characteristic changes in cell morphology, physiology, chromatin organization, gene expression and their secretome [13]. The latter, or so-called senescence-associated secretory phenotype (SASP), enables senescent cells to influence their microenvironment, and accordingly have a pervasive local effect [14]. Senescent cells accumulate with age in various tissues including liver, kidney, skin, lung, gastrointestinal tract and components of the immune system, including hematopoietic stem cells (HSC) and spleen, and accompany age-associated pathologies such as atherosclerosis or osteoarthritis (reviewed in [15]). Accumulation of senescent cells is in general deleterious for tissue homeostasis, thereby contributing to the disruption of tissue homeostasis observed during ageing [1].

The two products of the *CDKN2A* gene, $p16^{INK4a}$ and ARF, play major roles in the implementation of senescence by controlling the retinoblastoma (Rb; in the case of $p16^{INK4a}$) and p53 (in the case of ARF) tumor suppressor pathways. The relative contribution of these pathways to senescence is species and cell type dependent [13]. Both $p16^{Ink4a}$ and $p19^{Arf}$ (the murine homologue of human $p14^{ARF}$) accumulate during senescence in murine embryonic fibroblasts, but spontaneous escape from senescence occurs preferentially through loss of a functional Arf/p53 pathway [16]. Similarly Arf-null MEFs, but not Ink4a-null MEFs [17, 18] are immortal, suggesting a prominent role for Arf over Ink4a on MEF senescence. However, this property is cell type-dependent: functional data and that derived from frequency of inactivation during the establishment of immortal cell lines, suggest that $p16^{Ink4a}$ is the key regulator of senescence in most human cells [19]. In primary human fibroblasts, replicative senescence is triggered by the combination of telomere shortening and derepression of the *INK4/ARF* locus. In the majority of human

cell types other than fibroblasts, replicative senescence is primarily driven by the progressive and powerful increase in $p16^{INK4a}$ expression upon cell division [20]. Therefore, $p16^{INK4a}$ has a key role in implementing senescence in response to excessive replication and other stresses.

The impact of $p16^{INK4a}$ on stem cell dynamics

Stem cell exhaustion is observed upon ageing in many, but not all, adult stem cell compartments, and causes a decline of the regenerative capacity of these tissues [21]. Loss of the self-renewal potential of adult stem cells or more differentiated progenitors upon ageing is associated with $p16^{INK4a}$ induction. For example, $p16^{INK4a}$ limits the proliferation of human mesenchymal stem cells by inducing senescence [22]. In old muscle stem cells, a decline in Notch activation and induction of TGF-β/pSmad3 causes the upregulation of $p16^{INK4a}$ expression and impairment of their regenerative potential [23]. $p16^{INK4a}$ accumulates in geriatric muscle satellite cells, which switch from quiescence to senescence, thus losing their regenerative and self-renewal functions during ageing [24]. $p16^{INK4a}$ loss also attenuates the age-dependent decline in the self-renewal potential of forebrain progenitors [25]. Interestingly, the accumulation of $p16^{Ink4a}$ and $p19^{Arf}$ occurring during ageing in neural stem cells (NSCs) is linked to reduced expression of HMGA2 [26]. In HSCs $p16^{INK4a}$ accumulates upon ageing and limits their cycling activity and repopulation capacity [27]. Lineage-specific inactivation of $p16^{INK4a}$ in lymphocyte progenitors demonstrated that $p16^{INK4a}$ contributes to T-cell immune ageing in old mice [28]. Inhibition of $p16^{INK4a}$ expression by FGF-7 administration results in partial rejuvenation of early T-cell progenitors [29]. However, the impact of $p16^{INK4a}$ on HSCs is still a matter of debate as other studies only found a limited effect of $p16^{INK4a}$ in serial transplantation experiments, or failed to detect any contribution of $p16^{INK4a}$ in the steady-state ageing of HSC [30]. In the pancreas, $p16^{INK4a}$ overexpression causes a decline in islet regenerative capacity, while $p16^{INK4a}$ deficiency attenuates the age-induced decline in proliferation of pancreatic β cells [31]. Importantly, $p16^{INK4a}$ does not have a universal role in regulating regenerative capacity. For example, despite the key tumour suppressive role of CDKN2a in melanoma, melanocyte ageing (and graying) occurs at normal rate in *Ink4/Arf*$^{-/-}$ mice [32]. However, these studies show that increased $p16^{INK4a}$ expression upon ageing causes loss of self-renewal capacity in multiple tissues.

In addition, $p16^{INK4a}$ also impairs the reprogramming of somatic cells to induced pluripotent stem cells (iPSC). Generation of pluripotent cells from differentiated progenitors is of high

interest for basic biology, drug development and regenerative medicine [33]. However, the low efficiency of the process is a major barrier for translating iPSC production into clinical applications [34]. In 2009 several groups reported that the p53/ARF and the $p16^{INK4a}$/RB pathways limit the efficiency of iPSC generation [34]. Upon expression of pluripotency-associated factors, p53 is activated and $p16^{INK4a}$ expression is quickly induced. The histone demethylase JMJD3, which is upregulated upon reprogramming, removes the repressive H3K27me3 marks on the *INK4/ARF* locus [35, 36]. When $p16^{INK4a}$ is knocked down, somatic cell reprogramming to iPSC occurs faster and more efficiently [9, 35]. Once cells are reprogrammed, the epigenetic status of the *INK4/ARF* locus is reset and its expression suppressed, as in ESCs [9, 35]. In line with $p16^{INK4a}$ upregulation during organismal ageing, iPSC are derived less efficiently from old cells than young ones, however efficiency can be improved by knocking down *INK4a/ARF* expression [9]. Therefore $p16^{INK4a}$ can regulate stem cells, both via limiting stem cell self-renewal and by inhibiting the acquisition of pluripotent properties by somatic cells (Figure 2). In this way, $p16^{INK4a}$ limits tissue regeneration. The critical role of $p16^{INK4a}$ in the implementation of senescence and induction of stem cell exhaustion has favored the hypothesis that $p16^{INK4a}$ promotes ageing, but this linear view has been challenged by analyzing how $p16^{INK4a}$ affects ageing in mouse models.

The effect of $p16^{INK4a}$ on organismal ageing

Monitoring organismal ageing in $p16^{INK4a}$-related mouse models has provided interesting insights. The hypothesis that $p16^{INK4a}$ has a pro-ageing activity is supported by observations made in mice harboring a hypomorphic mutation of *BubR1*, which causes premature ageing due to chromosomal instability [37]. In this model, accumulation of $p16^{INK4a}$ and induction of senescence are observed for example in the skeletal muscle and fat, which show early ageing-associated phenotypes. $p16^{INK4a}$ inactivation attenuates cellular senescence and premature ageing in these tissues, while $p19^{ARF}$ ablation exacerbates senescence and ageing [38]. Through the design of the transgene *INK-ATTAC*, which allows inducible elimination of $p16^{INK4a}$-positive cells, it has been shown that life-long clearance of $p16^{INK4a}$-positive cells in this BubR1 progeroid model delays the onset of ageing-associated phenotypes such as cataract and sarcopenia [39]. Moreover, late-life removal of $p16^{INK4a}$-positive cells attenuates progression of already established age-associated disorders [39]. In addition, unbiased genetic studies have shown that $p16^{INK4a}$ gain-of-function alleles have a protective role in some age-related diseases such as atherosclerosis [40, 41].

Other models suggest a more complex scenario. A mouse strain carrying a transgenic copy of the *INK4/ARF* locus exhibits normal ageing and lifespan [42]. Increasing *INK4/ARF* dosage with two additional copies of the locus resulted in lower scores of ageing markers and extended longevity [43]. A recent report also showed that deletion of $p16^{INK4a}$ in mice deficient for Pot1B (protection of telomeres 1B) does not rescue the ageing phenotype, but rather accelerates organ impairment and shortens organismal lifespan [44]. These models thus argue for a protective role of $p16^{INK4a}$ in ageing. Altogether, this mixed picture might suggest that $p16^{INK4a}$ is beneficial for suppressing the propagation of damaged cells, but detrimental for the regenerative capacity of tissues. Overall, however, $p16^{INK4a}$ is intimately implicated in ageing and its regulation is crucial.

Regulation of $p16^{INK4a}$ expression by developmental pathways

Given the prominent role of $p16^{INK4a}$ in ageing, cancer and other diseases (and by extension the *INK4/ARF* locus), it is extremely important to keep this locus faithfully repressed. Study of $p16^{INK4a}$ regulation has revealed a common theme; namely, that multiple developmental pathways converge to repress $p16^{INK4a}$. These include Polycomb, Wnt/β-catenin, Homeobox, T-box proteins, Fox and the HH/GLI pathways (Figure 3). Given that $p16^{INK4a}$ is induced in a wide range of tissues during ageing, these findings may imply that the progressive molecular and cellular defects contributing to organismal ageing could be secondary to the deterioration in developmental pathways.

Polycomb repressive complexes

A key layer controlling the *INK4/ARF* locus is epigenetic repression by Polycomb repressive complexes (PRC1 and PRC2; Box 2). The role of PRC in repressing *INK4/ARF* has been extensively investigated using knockout mice for PRC components [45]. For example, mice lacking the PRC1 component Bmi1 suffer skeletal transformations and severe neurological and hematopoietic defects [46]. While the skeletal alterations are linked to the deregulation of Homeobox genes, the other phenotypes are mostly reversed by crossing *Bmi1*$^{-/-}$ with *Ink4a/Arf*$^{-/-}$ mice [47]. *Ink4a/Arf* deregulation in *Bm1*$^{-/-}$ mice, affects the homeostasis of adult stem cells, including HSCs and NSCs [48]. In contrast, increasing the function of PRC1 complexes, achieved by overexpression of individual components such as Cbx7 or Cbx8, bypasses senescence due to the repression of *INK4/ARF* [47]. PRCs bind directly to the *INK4/ARF* locus [49]. Factors that mediate PRC recruitment to the *INK4/ARF* locus include the non-coding RNA ANRIL [50], the transcription factor Zfp277 [51], Twist1 [52], HIC1 [53] and Homeobox proteins [54].

Homeobox proteins

Homeobox proteins form a large family of transcription factors sharing a conserved 60 amino acid DNA binding domain called the homeodomain. *Drosophila* homeotic genes, which control the pattern of body formation during embryonic development, are the proteotypic family members. Many Homeobox genes are represses by PRCs. In mammals, ~260 Homeobox genes exist that are important in development patterning and regulation of proliferation, apoptosis, differentiation and epithelial-to-mesenchymal transition [55]. Homeobox proteins such as MEOX2, HOXA9 and HLX1 have each been shown to regulate *INK4a* expression. While MEOX2 was characterized as an *INK4a* inducer in a functional screen [56], HOXA9 represses *INK4/ARF* and overcomes replicative senescence or senescence induced by RAS and AML-ETO1 [57 , 58]. HLX1 was identified by its ability to bypass replicative senescence in human primary fibroblasts. HLX1 binds to the *INK4a* promoter through its homeodomain and repress *INK4a* expression by recruiting PRC2. Other homeobox proteins including DLX3, HOXB13, HOXC13, HOXD3, HOXD8 and HOXA9 also behave similarly [58].

T-Box proteins

T-box (TBX) proteins are defined by a common domain (T-box) binding to the DNA consensus sequence TCACACCT and control early embryonic cell fate decisions, embryonic patterning and organogenesis. TBX proteins were first characterized through a mutation, Brachyury, causing truncated tails in mice [59]. The ability of TBX2 and TBX3 to regulate the *INK4/ARF* locus was unveiled in independent genetic screens [60, 61]. TBX3 is mutated in Ulnar-Mammary syndrome, a genetic developmental pathology accompanied by hypoproliferation of cells in a number of tissues, including the breast. Point mutations of TBX3 found in this syndrome impair its capacity to repress ARF [61].

WNT/β-catenin pathway

WNT signalling regulates primary embryonic axis formation, segmentation, organogenesis and stem cell proliferation. In the WNT/β-catenin, or canonical WNT pathway, extracellular WNT proteins bind to transmembrane receptors of the Frizzled family. This disrupts a cytoplasmic complex formed by the GSK3β kinase and its substrate β-catenin. β-catenin then accumulates and translocates to the nucleus where it regulates gene expression cooperating with TCF and LEF transcription factors [62]. There is a β-catenin/LEF/TCF binding site present in the *INK4a* promoter [63]. While β-catenin induces Arf in mouse cells

[64], in human colorectal cancer it decreases proliferation by inducing $p16^{INK4a}$ [63]. However, other reports indicate that activated β-catenin immortalizes melanocytes by repressing, rather than activating, *INK4a* [65], so a context-dependent role might exist. In agreement with this repressive role, WNT3A was identified as a repressor of $p16^{INK4a}$ in a genome-wide siRNA screen for $p16^{INK4a}$ modulators [66].

Hedgehog pathway

Hedgehog (HH) controls embryonic cuticle patterning in *Drosophila*. The HH pathway regulates a wide variety of processes in embryonic development and adult stem cell maintenance [67]. Upon recognition of the HH ligand by its receptor Patched, GLI transcription factors are activated, regulating transcription of target genes. A siRNA screen for $p16^{INK4a}$ modulators identified SUFU, a negative regulator of the HH pathway, as an activator of $p16^{INK4a}$ [66]. GLI2 binds to and represses *INK4a* [68]. Conversely, $p16^{INK4a}$ knockdown rescues the loss of cell expansion caused by progressive inhibition of the HH pathway cell senescence, and increases the formation of primary cilia, microtubule-containing cell-surface protrusions where some components of HH localize [66]. The HH pathway intersects with other developmental pathways that also repress $p16^{INK4a}$. Indeed, TBX2 is swiftly activated by HH signalling [69]. Moreover, several intersections exist between the HH and WNT signalling cascades. For example, SUFU interacts with β-catenin, decreasing β-catenin nuclear levels and TCF-dependent transcription [70].

FOX proteins

In mammals more than 40 Forkhead box (FOX) family proteins exist and members of this family share a DNA-binding domain termed a Forkhead box. Forkhead, the first gene identified of the Forkhead box (FOX) family, promotes terminal development in the *Drosophila* embryo. FOXA, the orthologues of Forkhead in mammals, control the development of the embryonic node, lung and gut [71]. PHA-4 is the orthologue of FOXA in *Caenorhabditis elegans*, and PHA-4 mutants remain short-lived under diet restriction [45]. FOXA1 induces senescence in primary fibroblasts through the activation of $p16^{INK4a}$ [72]. Mutations in DAF-16, the ortholog of mammalian FOXO proteins in *C. elegans,* also affect longevity. Indeed, DAF-16 mutations suppress the increase in lifespan caused by DAF-2 mutations [45]. In response to insulin, activation of the PI3K/AKT pathway leads to FOXO phosphorylation, its cytoplasmic retention, and deactivation of FOXO target genes [71]. In mammals, Myc signaling induces the accumulation of FOXO3a in the nucleus and promotes its binding to the *INK4/ARF* locus [73]. FOXO1a and FOXO3 proteins directly

activate *ARF* and *INK4b* in an AKT-dependent manner [74]. In contrast, another report suggests that FOXO3 represses *INK4a* in HSCs [75]. Interestingly, FOXO and β-catenin interact functionally. In *C. elegans*, mutants of β-catenin BAR-1 are as short-lived as DAF-16 mutants [76]. β-catenin binds to FOXO proteins and enhances FOXO transcriptional activity, particularly in the response to oxidative stress [77]. Altogether, multiple developmental pathways intersect for keeping $p16^{INK4a}$ expression at bay (Figure 3). It is thus interesting to ask how these pathways are affected upon ageing and how these affect $p16^{INK4a}$ expression.

Ageing-associated decay of developmental pathways

Several studies have described a drift of developmental pathways during senescence and ageing. For example, expression of PRC2 components, such as EZH2, are downregulated during senescence in human and mouse fibroblasts [49]. This decrease, and the upregulation of the H3K27 histone demethylase JMJD3, induces displacement of PRC1 and transcriptional activation of the *INK4/ARF* locus during oncogene-induced senescence but also during ageing [45]. Decline in EZH2 expression also accompanies the increase of INK4a and ARF expression in ageing pancreatic β-cells *in vivo* [78]. Age-dependent attenuation of PDGFR-α expression limits EZH2 expression in these cells through a decline in ERK and RB phosphorylation. With age, association of the transcriptional activator E2F1 on the EZH2 promoter decreases in favor of the transcriptional repressor E2F4 [79]. TBX2 is also downregulated upon replicative senescence in primary human fibroblasts [80]. A more complicated picture has been described for the WNT pathway. Activation of the canonical WNT pathway occurs in aged myogenic progenitors, as shown by a decrease in the amount of active GSK3β and an increase in the amount of active β-catenin, and in the transcription of the *Axin2* WNT target gene [81]. Increased WNT signalling has also been reported in Klotho-deficient mice, a model of accelerated ageing [82]. However, expression of the WNT2 ligand and downstream signals of the canonical WNT pathway are repressed in replicative senescence in human fibroblasts [83]. Elevation of WNT5a expression during HSC ageing induces a switch from canonical to non-canonical WNT signalling that causes stem cell ageing [84]. Downregulation of HH signalling is also associated with ageing [85]. As for the $p16^{INK4a}$-activating FOXA1, a robust induction of its expression is observed in fibroblasts upon replicative senescence [72]. Altogether this fits with the theory presented here: that ageing can result from developmental decay. In summary, the trend is for the activity of key developmental pathways to be altered upon ageing. This suggests that the postnatal derepression of

p16^{INK4a} could stem from this progressive drift, or functional dissociation, of developmental pathways during ageing.

Concluding remarks and future perspectives

Understanding the genetic pathways and molecular mechanisms underlying ageing is a major challenge both for basic and translational research (Box 3). The *INK4/ARF* locus has well-documented links with ageing and ageing-related disorders, however how *INK4/ARF* alterations impact on ageing-related diseases is not straight-forward. Despite the fact that GWAS studies have shown that most of the disease-associated SNPs linked to 9p21 map to ANRIL, the significance of this observation is unclear. These SNPs could either affect regulatory regions in the *INK4/ARF* locus, or ANRIL. Moreover, ANRIL could function in *cis* (affecting *INK4/ARF*) or in *trans*, affecting other loci. Although this requires further investigation, studies in mice have shown that deletion of the 9p21 non-coding coronary artery disease (CAD) risk interval resulted in decreased expression of the *INK4/ARF* locus and linked CAD with diminished senescence [86]. However, there is also evidence that senescence in vascular cells protects from atherosclerosis [87]. These apparently conflicting observations reflect that p16^{INK4a} accumulation, and senescence, can be either beneficial or detrimental depending on the specific organ, genetic context, time or disease analysed [15]. The functional relevance of cells accumulating high p16^{INK4a} levels on ageing is further highlighted in work with the *INK-ATTAC* mice [39], where elimination of p16^{INK4a}-positive cells in a progeroid mouse model improved different age-related pathologies.

p16^{INK4a} is currently considered as one of the best biomarkers of ageing, being induced during age and functionally linked with key cell fate decisions which contribute to ageing, such as cellular senescence and stem cell dynamics. Interestingly, senescence plays a key role in early development by promoting tissue remodelling [7, 8]. However p16^{Ink4a} is not an effector of developmental senescence [7, 8]. Senescence is as a potential example of antagonistic pleiotropy, beneficial at an early stage for coordinated development but detrimental later in life by contributing to ageing. Similarly, the decay of developmental pathways that control programmed senescence during development could be also causally involved in p16-mediated ageing.

Recent work has shown that p16^{INK4a} is regulated by pathways that drive early developmental decisions and remain active in the adult, but are altered upon ageing. Postnatal derepression of p16^{INK4a} could thus be one of many consequences of the drift of

these developmental pathways upon ageing. A better understanding of $p16^{INK4a}$ regulation, how these pathways intersect and are affected upon ageing, and which signals induce their alteration, as well as $p16^{INK4a}$ derepression, will help exploration of this idea. Prevalent theories attribute ageing to the progressive accumulation of molecular and cellular damage throughout the life of organisms. In light of recent data on $p16^{INK4a}$ regulation, we propose that ageing could be due primarily to age-associated defects in developmental pathways, and that damage accumulation might be secondary, but display a self-catalytic property, eventually driving the end-stages of ageing and age-associated disease.

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GLOSSARY

ARF ($p14^{ARF}/p19^{Arf}$)^{RF}: small protein of 14 kDa in human or 19 kDa in mouse, encoded by *ARF* (*Alternative Reading Frame*) in the *INK4/ARF* locus. By binding to MDM2 and inhibiting its ubiquitin ligase activity, $p14/p19^{ARF}$ARF stabilizes p53.

Ageing: time-dependent functional decline affecting living organisms.

Forkhead box (FOX) proteins: family of transcription factors sharing a highly conserved ~100 residue Forkhead DNA binding domain. FOX proteins play key roles in embryonic development as well as in metabolism and longevity.

Hedgehog (HH) pathway: signalling pathway termed after the *Hedgehog* gene, identified as a critical regulator of body segmentation in *Drosophila*, and also involved in the maintenance of stem cell populations in the adult.

Homeobox proteins: family of transcription factors characterized by a conserved 60 amino acid DNA binding domain (termed the homeodomain). The first genes identified in this family were *Drosophila* homeotic genes, which control the pattern of body formation during embryonic development.

***INK4/ARF* locus:** locus comprising the *INK4a*, *INK4b* and *ARF* genes and exons for the antisense non-coding RNA ANRIL. The *INK4/ARF* locus is the locus genetically linked to the highest number of ageing-associated diseases.

$p15^{INK4b}$: small ankyrin-repeat protein of 15 kDa encoded by *INK4b* in the *INK4/ARF* locus. $p15^{INK4b}$ binds to, and inhibits, the cyclin-dependent kinases CDK4 and CDK6, thus inducing a cell cycle arrest in G1.

$p16^{INK4a}$: small ankyrin-repeat protein of 16 kDa encoded by *INK4a* in the *INK4/ARF* locus. $p16^{INK4a}$ binds to and inhibits the cyclin-dependent kinases CDK4 and CDK6, thus inducing a cell cycle arrest in G1.

Pluripotency: ability of cells to differentiate into several cell types.

Polycomb group proteins (PcG): large group of repressors of gene expression, first identified in *Drosophila* as regulators of axial body patterning. In mammals, PcG proteins are part of two large multi-protein complexes, Polycomb repressive complexes 1 (PRC1) and 2 (PRC2), which induce gene silencing through histone modification.

Progenitor cell: early descendant of a stem cell, but with limited differentiation capacity.

Reprogramming: generation of induced pluripotent stem cells (iPSC) from differentiated somatic cells.

Self-renewal: ability of cells to divide and form more cells with identical properties, allowing indefinite regeneration of the cell population.

Senescence: State of stable cell cycle arrest accompanied with specific changes in cell morphology, physiology, chromatin organization, gene expression and changes in the secretome. Cellular senescence is triggered by telomere shortening or signals such as oncogene activation or overexpression of pluripotency factors.

Somatic cells: all the cells of a living organism except germline cells (egg and sperm).

Stem cells (SC): cells that have both the potential to self-renew and to differentiate into diverse cell types. Embryonic stem cells (ESC) are derived from the inner cell mass of the blastocyst and can give rise to any cell or tissue type found in an organism. Adult stem cells (ASC) are found in different tissues of the adult organism and can differentiate into any cell types of their tissue of origin.

T-box (TBX) proteins: family of transcriptions factors characterized by a highly conserved domain (T-box) binding to the DNA consensus sequence TCACACCT. T-box proteins are required for early cell fate decisions, differentiation and organogenesis.

WNT pathways: signalling pathways termed as a contraction of the *Wingless* and *Int1* genes. The canonical WNT pathway triggers β-catenin accumulation in the cytoplasm and translocation to the nucleus where β-catenin regulates gene expression. This pathway controls embryonic development processes such as body axis patterning and cell fate specification as well as cell proliferation and migration.

BOXES

Box 1. The *INK4/ARF* locus

$p16^{INK4a}$ is encoded by the *INK4/ARF* locus, which spans around 35kb on human chromosome 9p21.3 [45]. This complex genetic locus comprises the three intimately linked tumor suppressor genes. *INK4b* (also known as *CDKN2B*), *INK4a* and *ARF* (these two jointly are encoded by the *CDKN2a* gene), which respectively encode the two cyclin-dependent kinase inhibitors (CDKI) $p15^{INK4b}$ and $p16^{INK4a}$ and the unrelated $p14/p19^{ARF}$. In addition, a large antisense non-coding RNA termed ANRIL (also referred as CDKN2B antisense or CDKN2BAS) is transcribed from this locus [88] (Figure 1). $p16^{INK4a}$ was functionally characterized as a small ankyrin repeat protein acting as a cyclin-dependent kinase inhibitor . By specifically binding to CDK4 and CDK6 and blocking their activity, $p16^{INK4a}$ prevents the phosphorylation of RB and the subsequent release of E2F. As a result $p16^{INK4a}$ induces a cell cycle arrest in G1. In addition to its function on regulating senescence and cell cycle progression in a manner dependent on inhibition of CDK4/6, other functions have been proposed for $p16^{INK4a}$. For example, $p16^{INK4a}$ has been linked with repression of the *hTERT* gene, which encodes the catalytic subunit of telomerase. It has been shown in human mammary epithelial cells that $p16^{INK4a}$ expression induces the silencing of the *hTERT* promoter through histone 3 lysine 27 trimethylation [89]. Although this observation suggests an involvement of the Polycomb Repressive Complexes (PRC), the mechanism underlying this regulation, whether direct or indirect, remains to be clarified. Other CDKI such as p21 and p27 have been shown to regulate transcription via direct chromatin binding [90, 91]. Such a role for $p16^{INK4a}$ has not been shown yet, but might be worth exploring.

Box 2. Polycomb repressive complexes.

Polycomb group proteins (PcG) were first characterized in *Drosophila* as regulators of axial body patterning. They form a large group of proteins involved in transcriptional repression through histone modification [92]. In mammals, they assemble into two canonical large macromolecular complexes, termed Polycomb repressive complex 1 (PRC1) and 2 (PRC2). PRC1 comprises BMI1, Ph1/2, Pc/chromobox (CBX) and the ubiquitin E3 ligase RING1A/B. In addition, PRC1 components have in non-canonical functions that involve alternative PRC1 complexes incorporating RYBP instead of CBXs

[48, 92]. The core of PRC2 contains EED, SUZ12 and the histone methyltransferase EZH1/2 which catalyzes histone H3 lysine 27 trimethylation. PRC2 initiates repression of target genes with this epigenetic mark recognized by CBX proteins (CBX2, CBX4, CBX6, CBX7 or CBX8) present in canonical PRC1 complexes. In turn PRC1 then catalyzes monoubiquitination of lysine 119 of histone H2A and implements gene silencing [93].

Box 3. Outstanding questions.

- Which molecular pathways control ageing?
- What determines whether $p16^{INK4a}$ is beneficial or detrimental *in vivo*?
- Does $p16^{INK4a}$ have E2F-independent functions?
- Which signals and pathways induce $p16^{INK4a}$ postnatal derepression *in vivo*?
- How are developmental pathways regulated upon ageing?
- Does the drifting of developmental pathways affect other ageing-related phenotypes?
- What is the relationship between developmental decay and damage during ageing?

FIGURE LEGENDS

Figure 1. p16^{INK4a} identity card: gene and function. (**A)**. The *INK4/ARF* locus. The genetic structure of the locus is shown, with coloured boxes representing coding exons of *ARF*, *INK4a* and *INK4b* and non-coding exons for ANRIL. Single nucleotide polymorphisms associated with age-related disorders (frailty, coronary artery disease, myocardial infarction, type-2 diabetes and late onset Alzheimer's disease) are indicated by arrows. Map is not drawn to scale and positions are approximate. (**B)**. p16^{INK4a} function as a cyclin-dependent kinase inhibitor. Cell cycle progression from G1 to S phase requires the activation of the cyclin-dependent kinases CDK4 and CDK6, which inactivate RB through phosphorylation. RB phosphorylation releases E2F transcription factors which activate the expression of genes promoting S-phase entry. By directly binding to the cyclin-dependent kinases CDK4 and CDK4, p16^{INK4a} blocks their assembly with cyclin D and thus their activation, leading to a cell cycle arrest in G1.

Figure 2. Impact of p16^{INK4a} on stem cell dynamics. (A). Stem cell self-renewal and pluripotency. Accumulation of p16^{INK4a} upon ageing causes stem cell exhaustion and impairs tissue formation and regeneration **(B).** Somatic cell reprogramming to induced pluripotent stem cell. Upon expression of pluripotency-associated factors, p16^{INK4a} expression is induced and acts a barrier for reprogramming by triggering senescence.

Figure 3. Members of developmental pathways regulating the *INK4/ARF* locus. Members of developmental pathways reported to repress or activate the expression of the genes of the *INK4/ARF* locus are indicated. These factors act as transcriptional or epigenetic regulators.

Figure 1
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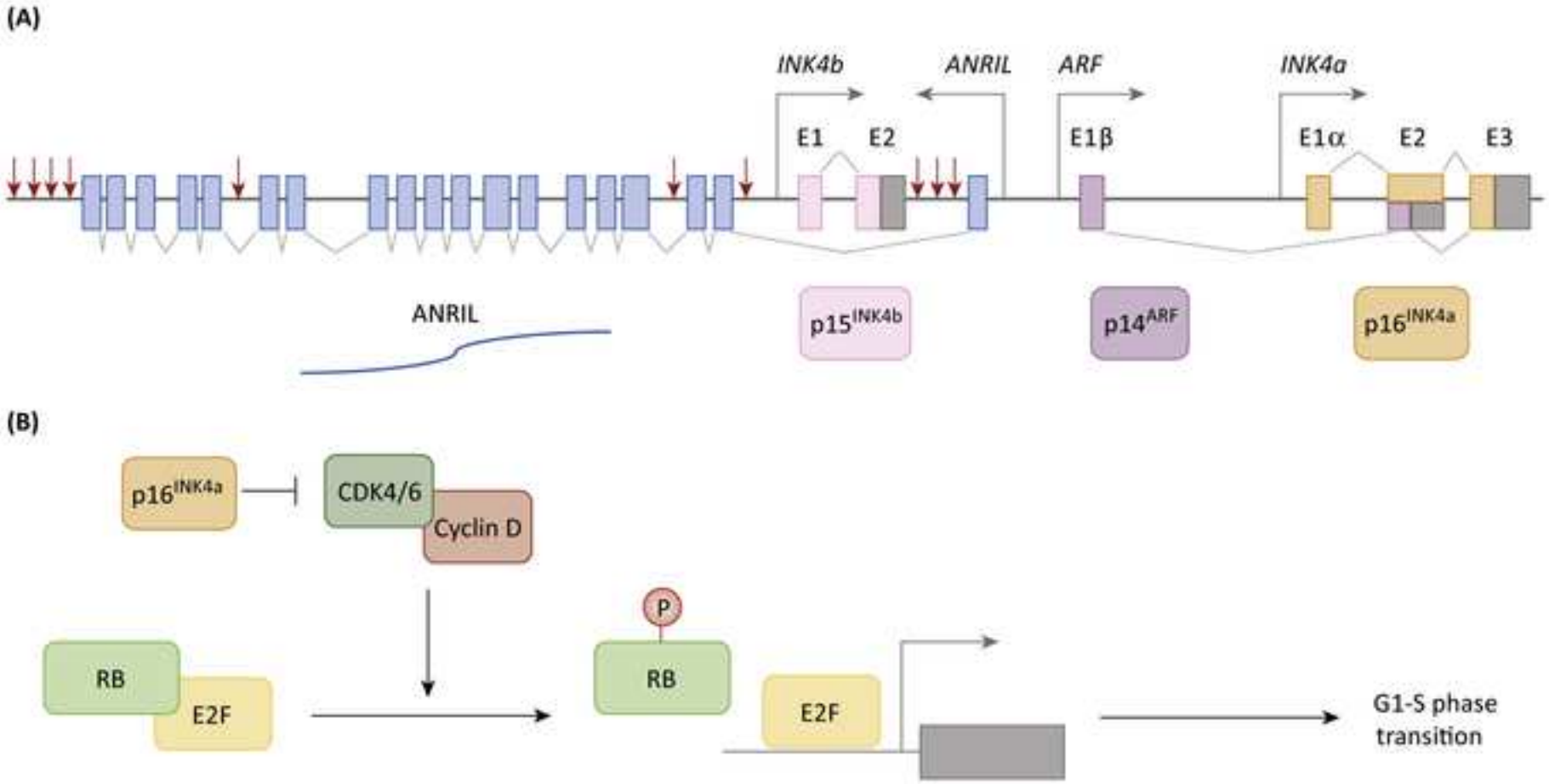

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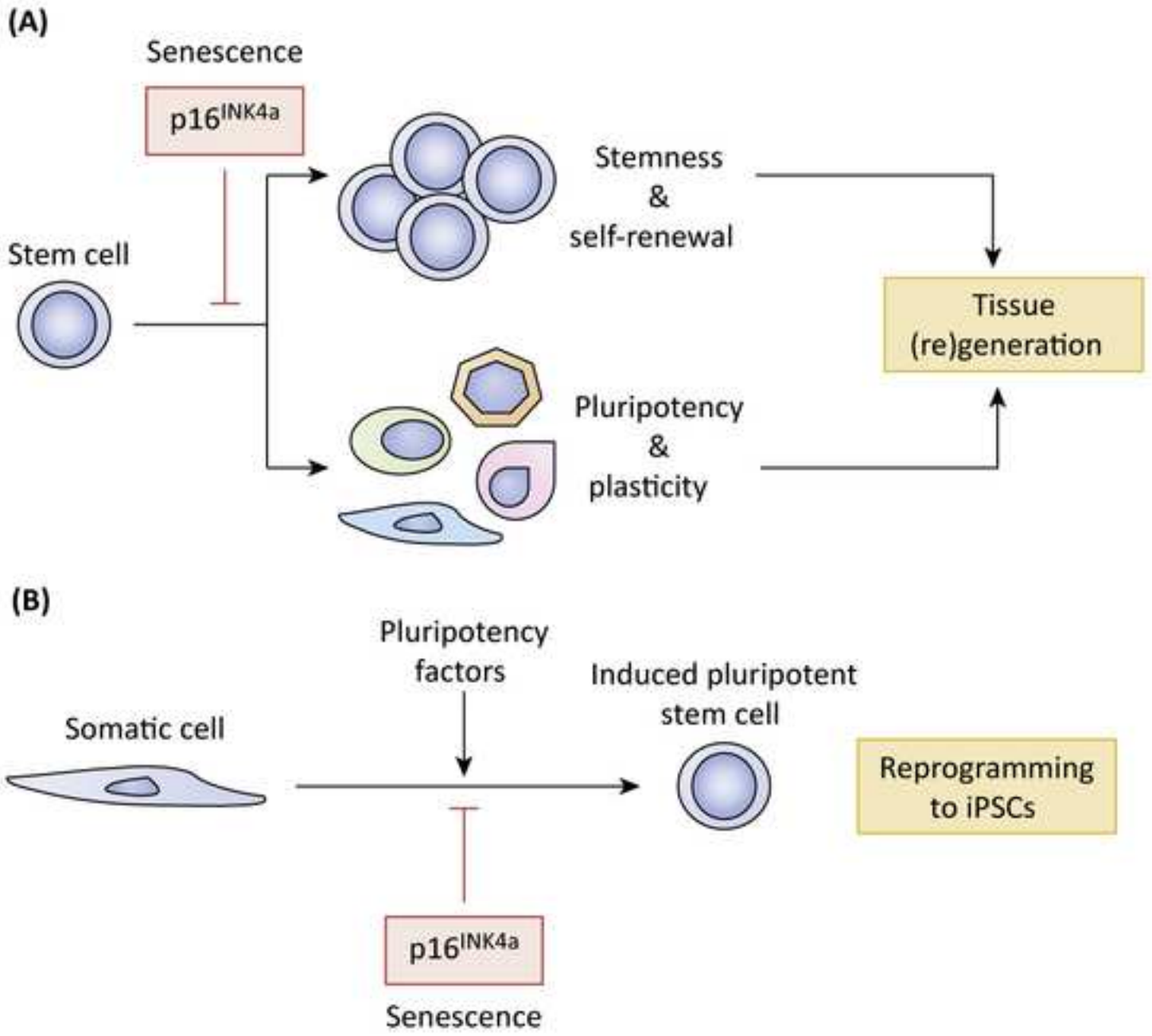

Figure 3
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