Notch and senescence

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Abbreviations: RAS, rat sarcoma virus oncogene; BRAF, BRaf proto-oncogene; CDK, cyclin-dependent kinase; SA β-GAL, senescence-associated beta-galactosidase; ECM, extracellular matrix; SASP, senescence-associated secretory phenotype; IL, interleukin; CCL, C-C motif chemokine ligand; RelA, v-rel reticuloendotheliosis viral oncogene homolog A; C/EBPβ, CCAAT/enhancer binding protein beta; BRD4, bromodomain containing 4; H3K27ac, histone 3 acetylated at lysine 27; CXCR, C-X-C motif chemokine receptor; TGFβ1, transforming growth factor beta 1; EMT, epithelial-mesenchymal transition; NK cells, natural killer cells; HES, hairy and enhancer of split; bHLH transcription factor; HEY, hairy/enhancer-of-split related with YRPW motif; MYC, myelocytomatosis proto-oncogene; RBPJ, recombination signal binding protein for immunoglobulin kappa J region; MAML1, mastermind like transcriptional coactivator 1; FBXW7, F-box and WD repeat domain containing 7; DLL, delta-like ligand; T-ALL, T-cell acute lymphoblastic leukaemia; SHH, sonic hedgehog; MEF, mouse embryonic fibroblast; JAG1, Jagged-1; SMAD, Mothers against decapentaplegic; PTEN, phosphatase and tensin homolog; JAK, Janus kinase; STAT, signal transducer and activator of transcription; CXCL, chemokine (C-X-C motif) ligand; G-CSF, granulocyte colony stimulating factor; PD1, programmed death 1.

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Abstract

Cellular senescence, previously thought of as an autonomous tumour suppressor mechanism, is emerging as a phenotype and effector present throughout the life of an organism from embryogenesis to senile decline. Senescent cells have powerful non-autonomous effects upon multiple players within their microenvironment mainly through their secretory phenotype. How senescent cells co-ordinate numerous, sometimes functionally contrasting outputs through their secretome had previously been unclear. The Notch pathway, originally identified for its involvement in *Drosophila* wing development, has more recently been found to underpin diverse effects in human cancer. Here we discuss recent findings that suggest that Notch is intimately involved in the development of senescence and how it acts to co-ordinate the composition and functional effects of the senescence secretome. We also highlight the complex physical and functional interplay between Notch and p53, critical to both senescence and cancer. Understanding the interplay between Notch, p53 and senescence could allow us develop the therapeutics of the future for cancer and ageing.

1. Cellular senescence

Somatic cells have a variety of tumour suppressor mechanisms to prevent cellular damage leading to transformation into cancer. Amongst there is increasing recognition that cellular senescence not only plays a crucial role in the pathogenesis of cancer and the cancer microenvironment, but also more generally in wound healing and embryological development. When cells enter cellular senescence they undergo a long-term stable exit from the cell cycle, but can remain viable and metabolically active for a prolonged period. Cellular senescence was originally identified from cultured cells demonstrating a finite *in vitro* growth capacity. Subsequent work linked this proliferative arrest to progressive attrition of telomere length and the development of a telomere-derived DNA damage signal [1].

The identification that activation of oncogenic RAS in primary human cells could also lead to the development of senescence linked this process to tumour suppression (Fig. 16.1a) [2]. Senescence was then found to underpin the suppression of human cancers, such as arresting BRAF-expressing cells in senescence and thereby preventing melanoma development [3]. Since this time, many genetic lesions leading to oncogene expression or loss of tumour suppressor activity have been found to drive cellular senescence. Evidence of senescence has been described in a variety of human pre-neoplastic lesions suggesting that the acquisition of tumorigenic mutations is actively repressed *in vivo* by cellular senescence [4, 5].

Senescence has also been shown to underpin the successful response of some tumours to chemotherapy. In the murine Eμ-Myc model of lymphoma, treatment with chemotherapy induces senescence within the tumour and leads to tumour regression. Abrogation of senescence in these mice prevents the response to treatment and leads to a worsening of survival [6]. Further, in established murine liver cancer with inactivation of senescence, re-establishment of senescence can lead to complete resolution of the tumour [7]. Therefore, not only does senescence resist the development of cancer, but may also underpin the successful response to cancer treatment.

Senescent cells accumulate in otherwise healthy organisms with progressive ageing [8, 9]. Utilising genetic labeling from the promoter of p16, a cyclin-dependent kinase (CDK) inhibitor and putative senescence marker, several studies have found differential accumulation of senescent cells within diverse organs. In wild-type mice, the number of senescent cells progressively increases with ageing, but is heterogeneous across otherwise genetically identical litter-mates [10]. Further, the level of senescence within an organ does not predict the development of tumours. Targeted clearance of these senescent cells increases the healthy lifespan of both wild-type and prematurely aged mice through reduction of both tumorigenesis and age-related pathologies [11, 12]. This is, perhaps, paradoxical, but as we shall see later, senescence can have contrasting oncogenic and tumour suppressive effects. Importantly, even when the senescent cells are deleted late in life, when age-related

pathologies have already developed, this prevents the progression of those pathologies, holding the promise of future therapies that arrest or even reverse age-related decline. These findings have reinforced the notion that senescence demonstrates antagonistic pleiotropy: a process that defends the organism and promotes reproductive fitness by repressing tumorigenesis early in life, but is deleterious in later life through declining organ function and age-related illness [13].

More recent studies have broadened our understanding of senescence as a developmental mechanism underpinning both healing and organogenesis. Senescent cells can be found in skin wounds in mice (Fig. 16.1a). These cells are actively involved in the appropriate restoration of homeostasis as deletion of senescence in these mice delays the healing of the wound [14]. Senescence can also be detected during embryogenesis in the developing inner ear and urinary tract of mice where it is crucial to appropriate development of these organs [15, 16]. This form of senescence shares all the features of other models of senescence (see later section on markers of senescence), other than a DNA-damage signal. Importantly, these developmental senescent cells express a typical pro-inflammatory secretome that is crucial in the regulation of the surrounding tissue. Loss of senescence during development leads to developmental abnormalities. Therefore, more than simply a tumour suppressor mechanism, senescence seems to be a highly conserved developmental pathway, intrinsic to a range of cellular behaviours, that can function in a stress-responsive mode to resist transformation.

Critical to the development of senescence are two major pathways (Fig. 16.1b) frequently mutated in human cancer: the p53-p21 [2, 17] and p16-Rb pathways [18]. Viral oncoproteins, that can drive the development of human cancers, are known to inactivate these pathways: the SV40 large T antigen is able to bind both of these factors leading to their inactivation and subsequent senescence bypass; similarly, the adenoviral E1A protein inhibits their function and promotes tumour formation [19]. Activation of p53 and Rb in senescence seems to rely, in large part, on the activity of two proteins, $p16^{INK4A}$ and $p14^{ARF}$, expressed from the *CDKN2A* locus. p16 inhibits the CDK4/6-dependent inactivation of Rb, whereas p14 (p19 in mice) interferes with the ability of MDM2 to inhibit p53. Ectopic expression of p16 can induce a senescent phenotype in cancer cell lines [20] and this relationship has more recently become a potential therapeutic target with the development of CDK4 inhibitors, such as palbociclib. In fact, these drugs mimic the effect of p16 by preventing CDK4-dependent Rb phosphorylation and thereby repressing E2F-target genes, crucial for cell cycle progression. Chronic CDK4 inhibitor treatment is able to drive senescence in cancer cells that have lost both p53 and p16, but only when Rb remains intact [21]. This raises the possibility of using such drugs to restore an appropriate senescence response in cancer, even when some endogenous tumour suppressors have been lost [22].

p53, on the other hand has a multitude of effects in senescence in a range of cellular pathways [23]. This seems to be in part related to a distinct set of chromatin

binding sites and therefore distinct transcriptional programme compared to those seen in the acute activation of p53 in acute cellular stress [24]. In the autonomous aspects of senescence, p53 promotes growth arrest by upregulating p21 expression that acts in turn to inhibit CDK2-dependent Rb phosphorylation. Significantly, p53 seems to repress some of the non-autonomous activities of senescence [25] that, as we shall discuss later, underpin much of the functionality of the senescent cell.

1.1 Markers of senescence

A number of putative defining characteristics of senescence have been identified (Fig. 16.1c). However, none are truly sensitive or specific, which has hampered efforts to understand the role of senescence in human disease. Due to their lack of specificity, the presence of senescence is normally inferred by the simultaneous presence of several of these markers. In fact, senescent cells are defined by a combination of several of the following features: a lack of proliferation; activation of p53-p21 and p16-Rb pathways [18]; formation of senescence-associated heterochromatic foci (SAHF), at least in oncogene-induced senescence [26]; a persistent DNA-damage response (DDR) [27]; expression of a lysosomal enzyme termed senescence-associated beta-galactosidase (SA β-GAL) [28]; and the secretion of a range of cytokines, chemokines and extracellular matrix (ECM) modifying factors termed the senescence-associated secretory phenotype (SASP) (Fig. 16.1c) [29, 30].

1.2 The senescence-associated secretory phenotype

It is through the SASP that senescent cells exert significant effects upon their surrounding environment. Most previous studies have focused on the secretome of cells undergoing oncogene-induced senescence (OIS) or DNA-damage-induced senescence (DDIS) and demonstrated that the typical SASP consists of proinflammatory cytokines such as interleukin-1α (IL1α) and IL6, and chemokines such as IL8 and C-C motif chemokine ligand 2 (CCL2) [29]. Transcriptionally the SASP is positively regulated by the transcription factors v-rel reticuloendotheliosis viral oncogene homolog A (RelA / p65 (an NF-κB family member)) [31], CCAAT/enhancer binding protein beta (C/EBPβ) [32] (possibly in a tight positive feedback loop with IL1α [33]) and the chromatin binding factor bromodomain protein 4 (BRD4) which dynamically binds to super-enhancers, related to many SASP genes [34]. Chien et al identified RelA through an unbiased proteomic screen of RAS-senescent chromatin. Subsequent functional investigation found that loss of RelA, during *in vitro* senescence, failed to bypass senescence but did prevent the senescence-associated upregulation of IL1α, IL6 and IL8 [31]. *In vivo*, loss of RelA

leads to a failure to develop treatment-induced senescence and relapse after chemotherapy [31].

C/EBPβ was identified as a SASP regulator through a search for putative transcriptional regulators of the prototypic SASP component IL6 in BRAF-induced senescence [32]. Endogenous C/EBPβ binds to the core promoter of IL6 during OIS and ectopic C/EBPβ could drive IL6 expression; loss of either IL6 or C/EBPβ can bypass BRAF-induced-senescence in primary human cells. Ectopic expression of C/EBPβ can drive senescence in both primary human cells [32] and transformed breast cancer cell lines [35]. However, crucial to the activation of C/EBPβ in response to the RAS/MAPK pathway is the activity of the cell-cycle inhibitor p19 (p14 in humans). RAS/MAPK activation in transformed cells lacking p19 fails to drive C/EBPβ expression and stimulates proliferation rather than senescence. Restoration of p19, leading to an upregulation of C/EBPβ, or ectopic C/EBPβ expression re-establishes the senescence response to RAS/MAPK activation [36]. Therefore, C/EBPβ sits downstream of p19 in the development of RAS-senescence.

The identification of BRD4 as a major regulator of the SASP has emerged from analysis of the changing epigenetic landscape of RAS-senescent cells that must underpin the simultaneous repression of cell-cycle-related genes and the activation of secretory-related genes [34]. In other biological contexts where significant functional reprogramming occurs, this is underpinned by changes at genetic regulatory elements termed enhancers, marked by acetylation of histone 3 at lysine 17 (H3K27ac). Amongst these enhancer elements, those marked by long stretches of H3K27ac are termed super-enhancers. Analysis found significant remodeling of super-enhancers in the context of senescence adjacent to genes encoding SASP components. As a putative H3K27ac binding partner, increased chromatin binding of BRD4 was confirmed at these loci and its inhibition, either genetically or pharmacologically, leads to abrogation of the proinflammatory SASP and reduction in SASP signalling to immunocytes both *in vitro* and *in vivo* [34].

The secretome is also significantly modulated at the post-translational level through the inflammasome [37], p38 MAPK [38], mTOR [39, 40] and autophagy pathways [41, 42]. The SASP has been found is nearly all forms of senescence thus-far described, other than senescence induced by overexpression of p16 [43] and mostly relies on a persistent DNA-damage signal [25].

Functionally the SASP is important due to the diverse downstream effects that senescent cells can exert on multiple players within the microenvironment. Firstly the secretome can act in an autocrine manner to reinforce the senescent phenotype. Here signalling from C/EBPβ or through C-X-C motif chemokine receptor 2 (CXCR2) leads to senescence, whereas loss of these factors results in senescence bypass [32, 44]. Secondly the SASP can enforce a paracrine senescence upon surrounding normal cells, through the secretion of transforming growth factor beta 1 (TGFβ1) and IL1β, potentially providing a means of controlling transformation in the context of a cancerisation field effect [37, 45]. Thirdly, the SASP from senescent cells has been

demonstrated to have significantly pro-oncogenic effects upon certain cell types. In *Drosophila* Ras-induced imaginal epithelial senescence drives proliferation of neighbouring epithelial cells through the SASP [46]. Similarly, senescent human fibroblasts are able to drive the growth of co-cultured pre-malignant and fully transformed human cell lines, in addition to promoting their growth in xenografts [47]. This effect is, at least partially, dependent upon NF-κB, as metformin-induced loss of NF-κB signalling prevents the senescence-driven growth of adjacent prostatic cancer cell lines [48]. Furthermore, senescent cells in co-culture can promote the epithelial-mesenchymal transition (EMT), with enhanced invasiveness, in the target cell population [49]. Lastly the SASP has been shown to have significant effects upon components of the immune system.

One of the critical findings of senescence in most model systems is that senescent cells are able to trigger their own immune-mediated destruction. Through the pro-inflammatory SASP, senescent cells recruit diverse members of the immune system leading to targeted killing and subsequent clearance, in a process termed senescence surveillance. In mouse models of NRAS-induced hepatocyte senescence, the NRAS-expressing cells secrete pro-inflammatory cytokines and are progressively removed from the liver [50]. $CD4^+$ T-lymphocytes are necessary for this process, as their deletion allows persistence of senescent cells and subsequent liver cancer development. Other groups have demonstrated that differing immunocytes are responsible for senescence surveillance in different contexts, such as macrophages and NK cells [51, 52]. Not only are senescent cells responsible for immune cell recruitment, but also for re-programming and controlling the downstream function of the recruited immunocytes [53]. Senescent hepatic stellate cells secrete a SASP that is able to modulate the polarisation, secretome and function of recruited macrophages [52]. Curiously, this SASP-dependent modulation of monocyte maturation and function can be antagonised *in vivo* by fully transformed cells within the same environment, through mechanisms that are not fully understood at present [53].

Therefore, there is a range of different downstream functions of the SASP, some with clearly contrasting effects on different target cell populations: senescent cells are able to direct development, control wound healing, resist transformation and control the composition and function of parts of the immune system. Until recently it remained unclear how a senescent cell could coordinate these different effects through a SASP of singular or static composition. However, recent data point to a role for the Notch pathway in the dynamic control of both SASP composition and its net functional output.

2. Notch

2.1 Notch signalling pathway

In order to respond to cues from neighbouring cells or the microenvironment, a variety of different signalling pathways have evolved to sense and direct cellular behaviour. Among these, Notch has emerged as a critical pathway in a variety of different cellular contexts. The role of Notch in development was originally identified from spontaneous mutations in *Drosophila*, where haploinsufficiency leads to an obvious notch in the wing edge [54]. Since this fortuitous discovery, comparative genomics has demonstrated that components of the Notch-signalling pathway are highly conserved in bilateria through evolution, suggesting that this signalling pathway arose around 550 million years ago. Subsequent work has demonstrated the significant role that Notch plays not only in directing cell-fate decisions during development, where loss of function of Notch receptors or ligands leads to dysgenesis of the vasculature, biliary tree and nervous system, but also in the development and progression of cancer.

In all organisms where Notch is described, the pathway is notable for the simplicity of the components involved in the core signalling pathway and downstream transduction. Indeed, given the simplicity of the core components, lack of enzymatic amplification steps and the multiplicity of downstream functional outcomes that Notch has been linked with, there must be a significant role for the diverse set of non-core components that have been described to modulate Notch signalling. In its simplest form the Notch pathway consists of a single-pass transmembrane Notch receptor which, when bound to a canonical ligand on an adjacent cell, undergoes a conformational change and subsequent proteolytic cleavage by the transmembrane metalloproteinase ADAM17 [55]. This results in a membrane-tethered intermediate form that is susceptible to further cleavage by γ-secretase, a multi-molecular complex responsible for cleavage of a range of membrane-bound substrates including Notch receptors. This second cleavage releases the Notch intracellular domain (NICD) from the inner envelope of the plasma membrane to traverse the cytoplasm and enter the nucleus. Within the nucleus the NICD binds to the highly conserved DNA binding protein recombination signal binding protein for immunoglobulin kappa J region (RBPJ [CBF1 / LAG-1 / Su(H)]) displacing transcriptional repressors and recruiting transcriptional activators, such as mastermind-like 1 (MAML1). Upon binding to NICD, RBPJ is converted to a transcriptional activator, recruiting the acetyl-transferase p300 and initiating the downstream Notch transcriptional program [55]. Notch is able to regulate a range of genes, including the hairy and enhancer of split (HES) and hairy/enhancer-of-split related with YRPW motif (HEY) family of transcription factors, MYC and Cyclin D3. In addition to being a critical transcriptional co-activator, MAML1 controls the half-life of the NICD through regulating its phosphorylation by CDK8 [56, 57]. This phosphorylation renders the NICD susceptible to ubiquitination by F-box and WD repeat domain containing 7 (FBXW7) [58] and subsequent degradation, thereby limiting the duration of signalling.

Within mammals there are four separate Notch genes, all of which are able to

liberate a distinct intracellular domain and drive distinct downstream signalling events, despite all binding to RBPJ. Similarly, in *Drosophila* there are two (Delta and Serrate) but in mammals at least five (Delta-like ligand (DLL) 1; DLL3; DLL4; Jagged1 and Jagged 2), canonical Notch ligands. These ligands have differing binding affinities for the different Notch receptors and drive distinct downstream functions [59]. Control of the affinity of the different ligands for the Notch receptors is in part controlled by the Fringe-mediated post-translational glycosylation of the receptors prior to their trafficking to the plasma membrane [59]. However, the basis for distinct functional outcomes from a pathway involving multiple receptors and ligands, but a single DNA binding protein remains unclear.

2.2 Notch in cancer

Alterations in the Notch-signalling pathway have been linked to the development and progression of cancer. The earliest suggestion that Notch could be oncogenic came with the identification of a rare chromosomal translocation between chromosomes 7 and 9 leading to the constitutive expression of the NOTCH1 intracellular domain (N1ICD) in lymphocytes in human T-cell acute lymphoblastic leukaemia (T-ALL) [60]. Subsequent studies revealed that the majority of patients with T-ALL had activating mutations due to indels of the NOTCH1 gene rather than major structural rearrangements [61]. These mutations either led to ligand-independent receptor cleavage or enhanced the stability of the NICD once liberated from the membrane [62]. The same studies found that a significant minority of patients without NOTCH1 mutations had mutations of FBXW7, leading to increased stability of the N1ICD [58].

With increasing knowledge of the genetic structure of diverse cancer types it has become apparent that NOTCH is frequently mutated or that the Notch signalling pathway is activated in several human cancers, other than T-ALL. Activating mutations or increased downstream signalling have been described in many solid organ malignancies such as breast cancer, hepatocellular carcinoma, cholangiocarcinoma, colorectal carcinoma and melanoma [62, 63]. The precise pathways that Notch regulates to drive cancer remain unclear as, in addition to driving its own transcriptional program, Notch also exerts significant cross-talk to diverse other cellular pathways such as Wnt-β–catenin, RAS-MAPK and others. Candidate pathway approaches suggest that Notch exerts its actions through transcriptional regulation of both cell-cycle and apoptosis-related genes; in particular, Cyclin D1 and D3 are direct transcriptional targets of N1ICD and drive cell cycle progression [64].

Conversely it has become apparent that Notch and downstream signalling can be tumour suppressive in some circumstances. Sequencing has demonstrated that a significant number of patients with bladder cancer [65] and head and neck squamous cell cancer [66] skin cancer [67] and small-cell lung carcinoma [68] have inactivating mutations of NOTCH1 suggesting a tumour suppressive role in these cancers [69].

Notch has also been demonstrated to be tumour suppressive in mouse models. Inducible knock-out of Notch1 leads to the spontaneous development of basal-cell carcinoma of the skin and accelerated tumour development after chemical carcinogenesis, partly due to loss of Notch1-mediated repression of the sonic hedgehog (Shh) pathway, previously implicated in basal cell carcinoma of the skin [67, 70]. Similarly, mesenchymal-specific loss of RBPJ, the DNA-binding protein for Notch, leads to the development of skin inflammation and subsequent tumour generation [71].

In some tissue types modulation of different Notch receptors has been demonstrated to have opposing effects upon tumorigenesis. Human pancreatic carcinoma is ubiquitously associated with the expression of oncogenic KRAS, in addition to other genetic lesions such as loss of the p53 encoding gene TP53. Mouse models with pancreas-specific expression of $KRas^{G12D}$ lead to the development of the pre-neoplastic lesion pancreatic intraepithelial neoplasia (PanIN). In mice with combined pancreas-specific expression of $KRas^{G12D}$ and loss of Notch1, there is an increased number and more advanced grade of PanIN compared to mice with $KRas^{G12D}$ alone [72, 73]. However, in similar mice with pancreas-specific loss of Notch2, survival is longer with reduced development of PanIN, but the mice develop late, highly anaplastic pancreatic carcinoma [73]. Therefore, in the same tissue, different Notch receptors can have complex and potentially opposing effects upon tissue differentiation and tumorigenesis.

It is clear that Notch receptors can be either oncogenic or tumour suppressive in different tissues, depending on context. The molecular basis for this duality of function in different cancer types is currently unknown but one possibility is the emerging role for Notch and downstream signalling in the autonomous and non-autonomous functions of senescence.

2.3 Notch in senescence

The association of Notch signalling with cellular senescence is a relatively recent finding and several studies have identified different NOTCH receptors in different model senescence systems. Replicative senescence is associated with the upregulation of NOTCH1 in both normal human prostatic cells and oesophageal keratinocytes [74, 75]. Similarly, all of the NOTCH receptors are up-regulated during *in vitro* culture of human endothelial cells [76] and upregulated in murine endothelium overlying atherosclerosis, thought to have features of senescence [77]. Manipulation of downstream Notch function is also able to modulate these senescent phenotypes. Pharmacological inhibition of Notch signalling by the γ-secretase inhibitor DAPT is able to increase *in vitro* replicative lifespan and reduce features of replicative senescence in oesophageal keratinocytes, such as SA β-GAL and p16 expression [75]. Several Notch receptors are upregulated in other forms of senescence beyond

replicative senescence. There is increased NOTCH3 expression in several forms of stress-induced senescence including replicative senescence, DDIS and oxidative stress-induced senescence in human fibroblasts [78]. In this context, knockdown of NOTCH3 is able to delay the onset of proliferation arrest and reduces features of senescence, such as SA β-GAL and p21 expression [78].

2.4 Multiple Notch receptors can drive a senescent phenotype

In addition to modulating the senescent phenotype induced by diverse other stressors, several Notch receptors are able to drive senescent phenotypes independently of other stimuli. Over-expression of NOTCH3 induces both a proliferative arrest by up-regulating the cyclin-dependent kinase inhibitor p21 through direct binding to its core promoter and a senescent phenotype [78]. Interruption of downstream NOTCH3 signalling, through expression of a dominant negative MAML1 (dnMAML1), or knockdown of p21 are able to partially rescue this NOTCH3-induced senescent phenotype. Cancers may bypass this NOTCH-induced senescence (NIS) through reduction of NOTCH receptor expression. Expression of NOTCH3 is significantly down-regulated in human breast cancer compared to normal breast tissue and is correlated with the level of p21 expression in the same tumour. Ectopic NOTCH3 expression, in breast cancer cell lines with low endogenous NOTCH3 expression, is able to drive the cells into NIS, suggesting that some degree of senescence-bypass could be associated with reduction of NOTCH3 signalling or that selection for clones with low Notch signalling could occur in human breast cancer [78].

Similarly to NOTCH3, several studies have shown that ectopic NOTCH1 also drives a senescent phenotype with reduced proliferation, increased SA β-GAL expression and upregulation of the CDK4/6 inhibitor p16, with subsequent loss of Rb phosphorylation. This NIS also requires NOTCH-mediated transcription, as it can be rescued by inhibition through co-expression of dnMAML1 [75, 79]. Importantly, the cells remain arrested in NOTCH-induced senescence, even after removal of ectopic N1ICD, a cardinal feature of senescence [79]. This confirms that this phenotype is true senescence and not simply quiescence, that can be induced through expression of HES1 [80]. In this context NOTCH1-induced senescence seems to be dependent upon the p16-Rb signalling axis as knockdown of p16, but not p14, is able to rescue this NIS phenotype [75]. Whether there is genuine specificity of NIS for the p16-Rb or p53-p21 pathways induced by signalling from the different Notch receptors or whether these different reports are describing a common, conserved NIS phenotype remains unclear.

The role of RBPJ in NIS is more controversial. In one context, N1ICD-mediated senescence can be rescued by concurrent shRNA-mediated knockdown of RBPJ, with reduced p16 expression and continued proliferation [75]. However, other studies have suggested that loss of RBPJ, in the absence of ectopic N1ICD expression

can also lead to the development of senescence [81]. Murine dermal fibroblasts with loss of Rbpj or human fibroblasts with shRNA-mediated knockdown of RBPJ have increased expression of p15, p16, p21 and SA β-GAL [81]. Further, RBPJ can directly bind to DNA at enhancer elements upstream of both p16 and p21 genes [81]. This apparent discrepancy can be explained by the dual role that RBPJ plays, dependent upon the presence of the NICD. In the Notch-inactive state RBPJ acts as a transcriptional repressor of multiple genes through constitutive DNA binding [82]. Upon Notch-activation, binding of the NICD leads to conversion of RBPJ to a transcriptional activator. Therefore, loss of RBPJ in the study by Procopio and colleagues, in the absence of NICD, removes the transcriptional repression on these genes and drives a similar senescent phenotype to NICD-mediated conversion to a transcriptional activator. Consistent with this, ectopic expression of N1ICD in their models leads to a similar phenotype to RBPJ loss [81].

At present there is no evidence that NIS is specific for NOTCH1 or 3. Indeed, in cultured endothelial cells, ectopic expression of the NICDs from NOTCH1, 2 or 4, but not the NOTCH-target genes HEY1 or HEY2 are able to drive a similar senescent phenotype with reduced proliferation, increased expression of SA β-GAL and upregulation of both p16 and p21 [76, 77]. Functionally this is associated with increased endothelial permeability [76] and increased endothelial pro-inflammatory cytokine expression [77] *in vitro*. Therefore, seemingly all of the NOTCH receptors can trigger a senescence response in certain contexts.

This NOTCH-induced senescent phenotype does not involve the HEY family of transcription factors [76]. Indeed, other studies have found that HES1, a canonical NOTCH-target gene, is important in resisting irreversible cell-cycle exit associated with prolonged expression of p21 in fibroblasts and thereby controlling the cell-fate decision between quiescence and senescence. In this context, 4 days of expression of p21 are sufficient to lead to irreversible senescence. However, restoration of HES1 expression permitted cell cycle entry and proliferation even after long periods of proliferative arrest [80]. This function of HES1 was also found in the context of OIS, where HES1 was able to resist the entry into RAS-induced senescence and prolong cellular proliferation.

It is interesting to speculate that cMyc could represent a plausible intermediary underpinning NIS. Myc is known to be a direct transcriptional target of NOTCH1 [83, 84]. Chronic activation of cMyc has also been shown to drive a form of senescence, when the cyclin-dependent kinase CDK2 is lost or inactivated [85]. In the Eu-Myc mouse model of lymphoma, cellular senescence has been demonstrated to underpin the response to chemotherapy and is critical to an improvement in survival [6]; Eu-Myc mice with biallelic loss of CDK2 have spontaneous development of senescence within lymphoid tissue and significantly improved prognosis compared to CDK2 heterozygotes [85]. Notch is also known to repress the expression of CDK2 [86], suggesting a model where NOTCH expression could simultaneously up and down-regulate cMyc and CDK2 respectively, driving senescence; this remains to be tested.

In addition to senescence driven by dysregulated signalling from the activated forms of the Notch receptors, abrogation of FBXW7 and interruption of normal degradation of the NICD has also been linked to senescence. Disruption of FBXW7 promotes endogenous Notch signalling and is able to lead to a proliferative arrest and senescence-like phenotype [87]. Loss of FBXW7 in mouse embryonic fibroblasts (MEFs) leads to a specific retention of N1ICD, amongst other FBXW7 targets, and significant up-regulation of a range of Notch-target genes. The growth arrest could be rescued by inhibition of Notch signalling by DAPT or subsequent loss of p53 function. This suggests that loss of FBXW7 leads to prolonged and upregulated Notch1 signalling that is able to drive a senescent phenotype.

These effects of Notch signalling upon senescence can be recapitulated in mouse model systems. Specific expression of N1ICD in murine endothelial cells is associated with reduced angiogenesis and increased *ex vivo* SA β-GAL expression in cultured aortic tissue, compared to tissue from control mice [76]. Ectopic expression of N1ICD specifically in the renal tubules after renal injury, not only prolongs the resolution of injury, but also leads to increased markers of tubular senescence with upregulation of both p16 and p21 [88]. In these mice, treatment with the Notch inhibitor DAPT leads to a significant reduction in the level of both p16 and p21, suggesting either enhanced clearance or reduced development of senescence in the kidneys.

Therefore, there is abundant evidence that sustained Notch activation from increased activity or impaired degradation of several Notch family members is able to drive a senescent phenotype, including *in vivo* senescence. However, the basis for Notch acting as a tumour suppressor to drive senescence or as an oncogene leading to malignancy, such as T-ALL remains elusive.

2.5 Notch regulates the SASP

There has been indirect evidence of a link between NOTCH and non-autonomous signaling previously; loss of Notch1 in mouse skin is associated with increased influx of immune cells, suggesting a role for Notch in suppression of inflammatory signaling [70]. Mechanistically Notch signalling has a complex relationship with the secretome and TGFβ signalling in particular. There seems to be a strong positive feedback loop between NOTCH and TGFβ1. Treatment of mesenchymal cells with TGFβ1 leads to the upregulation of the Notch ligand JAG1, through the canonical TGFβ1 targets Mothers against decapentaplegic 3 (SMAD3) [89] and SMAD4 [90], whereas in epithelial cells the TGFβ1-JAG1 pathway can drive an EMT through Notch [91]. Indeed, there is some evidence that the NICD is able to physically bind to both SMAD9 [92] and SMAD3; the latter interaction has been demonstrated to enhance downstream Notch signalling [93] Certainly this signalling axis seems to underpin the proliferative arrest [94] and Notch-induced senescence of cells in response to TGFβ1

treatment [75]. Co-operative signalling through these two pathways seems to be critical for induction of p21 [94]. Both TGFβ1-mediated growth arrest and SA β-GAL expression are rescued through concurrent treatment with DAPT or knockdown of NOTCH1. Therefore, NOTCH signalling seems to be a downstream effector of non-autonomous signalling through TGFβ1. Indeed, in addition to blindly augmenting TGFβ-signalling, activation of Notch seems to be able to shape the response to TGFβ. Notch is able to modulate the relative expression levels of the different SMAD proteins, promoting SMAD3 in particular and altering downstream signalling networks from TGFβ [95].

From our own work we have established that N1ICD is sufficient to induce expression of both TGFβ1 and TGFβ3, cell surface expression of the latency-associated peptide, cleaved from the TGFβ1 propeptide and to induce downstream TGFβ-signalling with increased chromatin-bound SMAD3. In addition, blockade of TGFβ1 signalling through antagonists of TGFβR1 or expression of a dominant negative form of SMAD4 is able to partially rescue the NIS phenotype [79].

However, the relationship between Notch and a more widespread role in control of the composition of the secretome and thereby net functional non-autonomous output of a cell was much less clear. NOTCH1 was identified as significantly upregulated in an unbiased plasma membrane proteomic screen looking for senescence-associated cell surface proteins. Validation confirmed that NOTCH1 was upregulated in several forms of senescence and in different cell types. Despite being progressively upregulated through the transition to RAS-induced senescence (RIS), NOTCH1 is only functionally active during the transition to senescence, with loss of downstream signalling when cells are fully senescent. Through pharmacological and genetic pathway manipulation during senescence it was possible to identify that NOTCH1 is able to drive expression of several TGFβ-family members at the same time as repressing the typical pro-inflammatory SASP of RIS cells. Transcriptional profiling confirmed that RAS and NOTCH1 co-regulated the secretome towards two polar opposite secretory phenotypes. In the case of RAS, the secretome consisted of proinflammatory cytokines such as IL1α, IL1β and ECM-degrading matrix metalloproteases (MMP), whereas the NOTCH-driven secretome consisted of several TGFβ-family members, collagens and extracellular matrix components such as fibronectin. Importantly, when co-expressed, N1ICD is dominant over RAS in determining the secretome composition. Therefore, the level of NOTCH signalling acts as a rheostat upon the secretome composition and net functional output of cells undergoing senescence. The burst of NOTCH1 signalling during the transition to senescence is able to direct a pro-fibrotic and immunosuppressive SASP, prior to subsequent loss of Notch signalling and secretome switch to an anti-fibrotic and proinflammatory SASP [79]. We found that Notch was functionally active during *in vivo* RAS-induced hepatocyte senescence, where autonomous expression of Notch1 was increased. Utilising hydrodynamic tail-vein delivery of an oncogenic NRAS-containing transposon, we were able to induce RAS-senescence of hepatocytes [50,

79]. Co-delivery of dnMAML1 with RAS promoted recruitment of T-lymphocytes to the liver, associated with enhanced clearance of the RAS-senescent hepatocytes, presumably related to an enhanced pro-inflammatory SASP. A role for Notch in suppressing inflammation would be consistent with previous studies of Notch in other contexts. In mice with mesenchymal-specific loss of Notch signalling spontaneous inflammation of the skin was observed, with upregulation of a diverse array of inflammatory mediators and MMPs, ultimately driving the development of tumours [71].

Mechanistically NOTCH1 inhibits the pro-inflammatory SASP through repression of both expression and chromatin binding of the transcription factor C/EBPβ. C/EBPβ is thought to act in concert with the NF-κB component RelA in transcriptionally regulating the SASP [31, 32]. C/EBPβ has been demonstrated to be a critical factor in the generation of the SASP, with loss of C/EBPβ causing loss of pro-inflammatory cytokine expression and senescence bypass [32]. Importantly, we could not find any significant effect of NOTCH1 activation upon expression or chromatin binding of any NF-κB family member in the context of senescence, despite previous evidence of a link between NOTCH and NF-κB [96]. N1ICD was able to reduce the binding of C/EBPβ to enhancer elements upstream of the *IL1A* locus, as well as previously identified binding sites in the core promoters of *IL6* and *IL8* [79]. Therefore, our data place NOTCH1 amongst the master regulators of the senescence secretome. In particular, NOTCH1 appears to be upstream of IL1α, critically important for the regulation of various inflammatory cytokines including IL6 and IL8. The precise mechanism by which NOTCH1 is able to repress C/EBPβ remains unclear, including whether this repression is direct. Previous studies have identified that the canonical NOTCH1-target HES1 is able to transcriptionally repress C/EBPα [97], but whether a similar mechanism operates for C/EBPβ is unknown.

Some evidence for a SASP with an evolving composition and downstream functionality has been found before. In skin wound healing senescent myofibroblasts are important to normal wound repair and their loss prolongs the time to restoration of the wound [14]. In this context the SASP directs the operation of the healing microenvironment, before ultimately directing the immune-mediated destruction of the senescent cell, necessitating a time-dependent switch between non-autonomous signalling modules with contrasting downstream functionalities. Indeed, many studies have demonstrated that the onset of senescence is associated with a reduction in tissue fibrosis, potentially relating to a switch from a pro-fibrotic to pro-inflammatory secretome [51, 98].

Therapeutically, there is much interest in the concept of manipulating the composition of the SASP to enhance passage into senescence or clearance of senescent cells to prevent the development of cancer. In the context of PTEN-loss associated senescence in the prostate, the typical SASP is immunosuppressive, leading to recruitment of myeloid-derived suppressor cells and preventing immune-mediated clearance of the senescent cells [99]. These cells also show evidence of

signalling through the Jak2/Stat3 pathway leading to expression of chemokine (C-X-C motif) ligand 2 (Cxcl2) and granulocyte colony-stimulating factor (G-CSF). Upon genetic deletion of Stat3 or pharmacological inhibition of Jak2, the SASP of the senescent prostatic epithelium shows significant reductions in Cxcl2 and G-CSF, associated with enhanced immune-cell infiltration and reduction in subsequent tumour development [99]. In the case of Notch, genetic inhibition through dnMAML1 is able to enhance the clearance of RAS-senescent hepatocytes from the mouse liver, associated with enhanced recruitment of $CD3^+$ T-lymphocytes. Therefore, modulation of SASP composition, in order to promote chemotaxis and senescence surveillance, can be demonstrated through two different pathways in two distinct models of senescence. Therefore, the combination of SASP modulation to enhance recruitment and immune-checkpoint blockade, such as anti-programmed death 1 (PD1) therapy, to enhance immune activation could be a rational combination in the treatment of neoplastic and pre-neoplastic lesions.

2.6 Notch and p53

TP53 is one of the most commonly mutated genes in human cancers. It also plays a critical role in both senescence and the SASP. There is increasing evidence that Notch and p53 have a complex relationship with regulation of both factors by the other (Fig. 16.2) [100]. In *Drosophila*, Notch is a direct target of p53 activity and mediates p53-dependent cell behaviour between apoptosis and proliferation [101]. Similarly, in both mammalian keratinocytes and epithelial cells NOTCH has been demonstrated to be a direct p53 transcriptional target, where p53 up-regulates NOTCH1 expression [102, 103]. UV irradiation of the skin leads to upregulation of both Notch1 expression and activity, in a p53-dependent manner [104], where Notch acts to repress UV-damage induced apoptosis.

Previous studies of Notch-mediated regulation of p53 have again revealed a duality of Notch function between activation and repression of p53. In the context of haematological malignancy, Notch is known to repress p53 function, potentially through regulation of the p53-regulatory protein MDM2 [105] or through direct physical interaction with p53 itself [106]. Indeed p53 has been demonstrated to bind to both RBPJ [81] and MAML1 [107, 108] in different contexts. Through this direct interaction, the N1ICD is able to repress the expression of p53-target genes such as p21, in a dose-dependent manner [106]. In some tumour cell lines, NOTCH1 activity is responsible for repression of p53-dependent apoptosis, through reducing the stability of the p53 protein [109].

However, other studies have shown that activated Notch-signalling can positively regulate p53 function. The canonical Notch targets HES1 and HEY1 have been shown to positively regulate p53 activity, through negative regulation of MDM2 [110]. The Notch-dependent cell-cycle arrest attendant with FBXW7 loss can be rescued by knockout of p53 [87].

Despite their complex reciprocal regulation, in the context of senescence, Notch and p53 signalling seem to drive coordinated endpoints with autonomous cell cycle arrest and cellular senescence. Similarly, loss of the constitutive repressive RBPJ activity is also able to drive this phenotype [81]. Physically RBPJ binds both to the p53 promoter [111] and to p53 itself and reduces its transcriptional activity [81]; bait oligonucleotides containing the promoter sequence of the canonical p53-target p21 were able to pull down both p53 and RBPJ, suggesting that these two factors are physically and functionally linked in the same gene space, related to the senescence program. Indeed increasing levels of ectopic RBPJ lead to a dose-dependent reduction of p53 transcriptional activity [81], suggesting a functional interaction between these factors. Paradoxically, increasing levels of the NOTCH co-activator MAML1 are able to increase p53-directed gene transcription [107, 108], potentially though enhancing its stability and activation [108].

However, whereas NOTCH, RBPJ and p53 coordinately regulate the autonomous features of senescence, their roles in the regulation of non-autonomous functionality, and the SASP in particular, is sometimes contrasting. NOTCH1 [75, 79] and p53 [49] both function to promote cellular senescence, whilst repressing the pro-inflammatory SASP. Constitutive RBPJ functions to repress both senescence and the SASP [81], suggesting that NOTCH converts RBPJ to an activated state at senescence-associated genes, but not at genes regulating the SASP or potentially that NOTCH1-mediated regulation of SASP or C/EBPβ could be independent of RBPJ. This remains to be directly tested, but intriguing evidence suggests that this RBPJ-independent, non-canonical function of NOTCH1 could occur in the regulation of IL6, when p53 is lost [112].

Basal-type breast cancer is associated with increased Notch signalling and expression of IL6. Ectopic expression of N1ICD or activation of endogenous NOTCH through JAG1 leads to upregulation of IL6, but only in basal-type breast cancer cell lines that express mutated p53 [112]. Further, expression of a dominant negative RBPJ reduces the expression of canonical Notch-target genes, but has no effect upon N1ICD-regulated IL6 expression. Conversely, N1ICD lacking the RBPJ-binding domain or cytoplasmically-retained N1ICD are both able to up-regulate IL6, but had no effect upon expression of HES and HEY proteins. This effect on IL6 expression could be reversed by co-expression of wild-type p53. The precise mechanism of this interaction remains elusive and whether this occurs with other secreted factors or in contexts outside of breast cancer remains to be evaluated. However, the findings reinforce that various components of the Notch-signalling pathway could have divergent effects upon autonomous senescence and the senescence-secretome and that some of this functionality may not require nuclear localisation or the apparatus of the canonical Notch pathway.

2.7 Notch-mediated juxtacrine signalling

In addition to regulating the non-autonomous behaviour of senescent cells through the secretome, Notch also regulates signalling to the microenvironment through cell-contact dependent pathways. Studies of embryological development have identified two modes of Notch-dependent signalling through a tissue: lateral inhibition and lateral induction [113]. In the former, activated Notch signalling represses the expression of Notch ligands within the same cell leading to a reduction in signal transmitted to neighbouring cells. Thereby, there is a differentiation between Notch active and neighbouring Notch-inactive cells. This mode of signalling has been commonly described as a mode of differentiating cell fate decisions on the level of cells and boundary formation at the level of tissues [114-116].

The second mode, termed lateral induction, describes how Notch-signalling drives autonomous expression of Notch ligands leading to increased transmission of a Notch-signal to neighbouring cells. In this situation both signal sending and receiving cells will be Notch-active. This mode allows for co-ordination of cell fate and a spatial expansion of coordinated Notch-signalling across a tissue [117, 118]. The cellular decision to induce or repress Notch ligand expression seems to involve the strength of the Notch signal and therefore likely the balance and post-translational modification of Notch ligands on neighbouring cells [118].

We identified that ectopic N1ICD was able to specifically induce the expression of JAG1 amongst the other Notch ligands. This up-regulation of JAG1 transmits a Notch signal to surrounding cells leading to non-autonomous transmission of Notch-induced senescence with upregulation of p16 and reduced proliferation in the signal-receiving cells [79]. This form of senescence could be rescued through knockdown of JAG1 expression in the sending cell, inhibiting Notch signalling with dnMAML1 or with DAPT in the receiving cell. Therefore, this represented clear evidence of *in vitro* N1ICD-mediated lateral induction of NOTCH signalling and NOTCH-induced senescence through JAG1. In the mouse liver there was also evidence of both lateral induction of Hes1 and p21 expression from RAS-senescent hepatocytes, suggesting that RAS-induced senescence is associated with the transmission of a cell-contact dependent lateral induction of Notch signalling.

Previously non-autonomous signalling in senescence was thought to involve paracrine, secreted factors alone. The finding of Notch-mediated cell-contact dependent pathways adds complexity to senescence signalling to other players in the microenvironment. It will be interesting to see, not only the effects of this Notch-mediated signalling pathway upon surrounding parenchymal cells, but also upon members of the immune system, where Notch is known to play a profound role in regulating cellular differentiation [119].

3. Conclusions

It is becoming clear that senescence, far from a simple tumour suppressor mechanism, is a highly conserved pathway that is utilised in a variety of physiological and pathophysiological contexts throughout the life-cycle from embryogenesis to age-related decline. Fundamental to our understanding of the role of senescence will be to understand how its non-autonomous functionality is regulated and the net output or signal to the various players within the microenvironment is delivered. This output must be dynamically regulated to deliver behaviours as diverse as inner ear development and co-ordination of skin wound healing. We are only just beginning to understand some of the players that control this process. Notch activity is able to modulate both the net secretory output of the senescent cell as well as a cell-contact dependent form of lateral induction, previously thought of as a developmental patterning program.

We do not understand the many contradictions and dualities that have been described to occur with Notch signalling: how is activation of this pathway oncogenic in one context but tumour suppressive in another?

The ultimate prizes for understanding how senescent cells arise, function and then are cleared will be therapies that may target preneoplastic lesions before they develop into cancer and also treatments for non-cancerous age-related pathologies where senescent cells underpin the decline in function with age.

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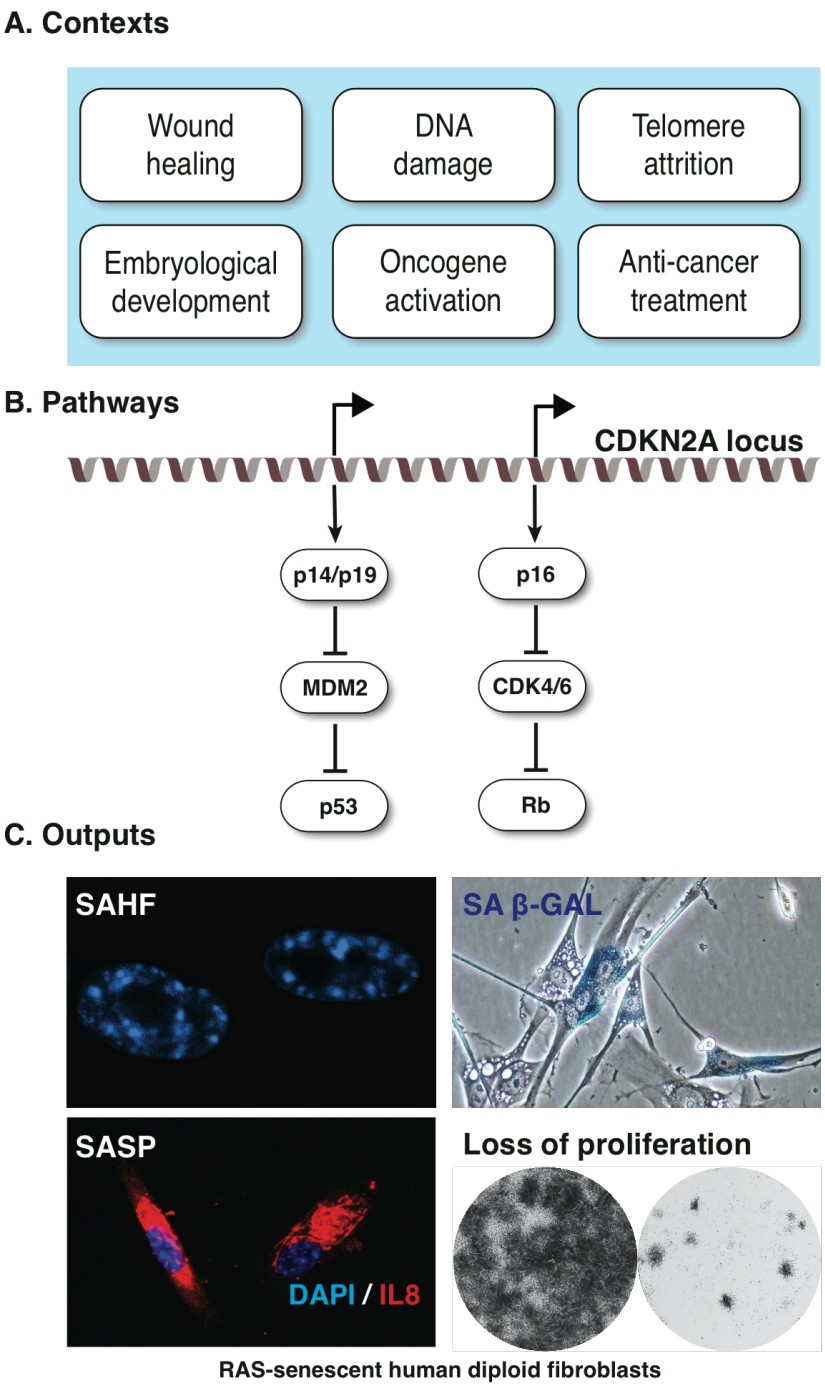
Figure legends

Fig. 1. Cellular senescence is a highly conserved cellular pathway involved in diverse biological contexts. (A) Whilst originally identified in the context of telomere attrition and then suppression of oncogene-induced transformation, cellular senescence is now recognized to occur in contexts as diverse as embryological development, wound healing and the response to anti-cancer therapies. (B) Cellular senescence is underpinned by two cellular pathways driven by gene-products of the CDKN2A locus. Both $p14^{ARF}$ (p19 in mice) and $p16^{INK4A}$ are expressed from this locus and result in p53 and Rb-dependent cellular responses, respectively. There is enormous interest in the recently developed CDK4/6 inhibitors, such as palbociclib, which can restore a tumour suppressive senescence response in cancer cells that have intact Rb signalling. (C) The presence of senescence within a cell is inferred by a panel of markers and downstream effector functions, none of which are truly specific for senescence. The chromatin of RAS-senescent IMR90 human diploid fibroblasts undergoes a significant architectural change to form DAPI-dense foci of heterochromatin called senescence-associated heterochromatic foci (SAHF). Senescent cells have an expansion of their lysosomal compartment and express galactosidase activity at a non-optimal pH, termed senescence-associated beta-galactosidase (SA β-GAL). Senescent cells are highly secretory and produce a range

of cytokines, growth factors and matrix-modifying enzymes termed the senescence-associated secretory phenotype (SASP); shown here are RAS-senescent IMR90s expressing the chemokine IL8. The most fundamental characteristic of senescent cells is their lack of proliferation, even upon growth factor or oncogenic stimulation, demonstrated here by lack of colony forming ability compared to control cells.

Fig. 2. NOTCH and p53 are involved in complex reciprocal regulation, but drive coordinated outputs in senescence. NOTCH and p53 have been demonstrated to reciprocally regulate each other, including through physical binding, leading to stimulation or inhibition in a context-dependent manner. However, in the context of senescence, both drive common outputs with an autonomous cell-cycle arrest and subsequent senescence, in addition to inhibiting the pro-inflammatory senescence secretome and therefore coordinately modulating the non-autonomous functionality of senescent cells.

Hoare - Figure 16.1 Chapter 16 Notch & senescence



RAS-senescent human diploid fibroblasts

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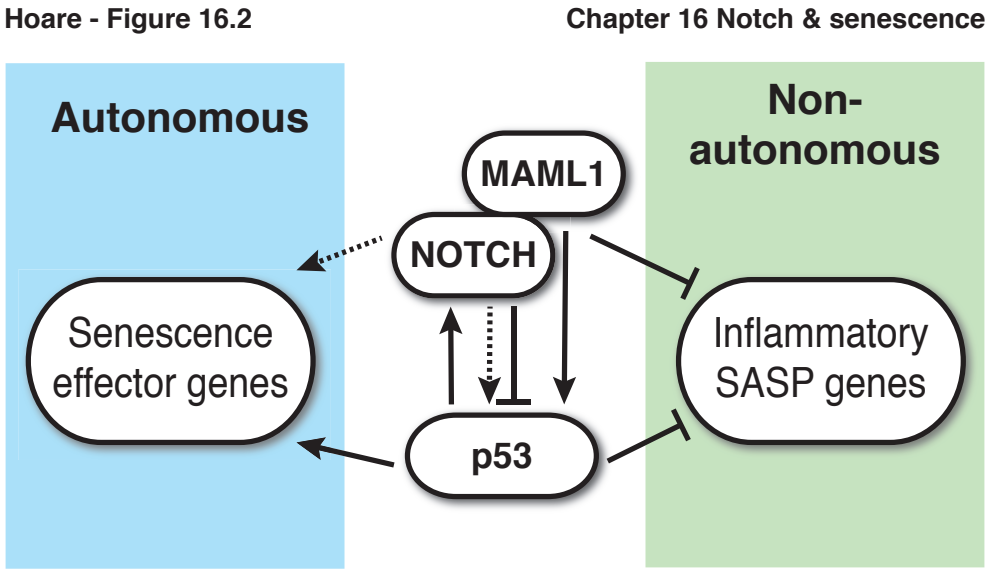
Hoare - Figure 16.2
Chapter 16 Notch & senescence
Autonomous
Non-autonomous
MAML1
NOTCH
Senescence effector genes
Inflammatory SASP genes
p53