Trends in Cell Biology

Spatial and Temporal Control of Senescence

--Manuscript Draft--

Manuscript Number:	TCB-D-17-00020R1
Article Type:	Review
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Abstract:	Cellular senescence is an autonomous tumor suppressor mechanism leading to a stable cell cycle arrest. Senescent cells are highly secretory driving a range of different functions through the senescence-associated secretory phenotype (SASP). Recent findings have suggested that the composition of the SASP is dynamically and spatially regulated and that the changing composition of the SASP can determine the beneficial and detrimental aspects of the senescence program, tipping the balance to either an immunosuppressive/pro-fibrotic environment or pro-inflammatory/fibrolytic state. Here we discuss the current understanding of the temporal and spatial regulation of the SASP and the novel finding of NOTCH signaling as a regulator of SASP composition.

Dear Dr. Danielle Loughlin,

Ms number: TCB-D-17-00020

Please find enclosed our revised manuscript with new title, **Spatial and Temporal Control of Senescence.**

Thank you again for your guiding us to revise the manuscript. It has been very helpful. We have incorporate most of your suggestions. Together with our response to reviewers' questions, some point needed further consideration. Particularly we have kept some flow within the text and some sub-titles (in Introduction), which may help readers to follow the now longer introduction. Also we have tried to broaden the content, but the manuscript is still focused on Notch, which was our initial agreement.

We also thank all the reviewers for their careful reading and constructive comments. We have modified the manuscript accordingly. We believe the modifications have substantially improved our manuscript.

Thank you again for your help.

Sincerely,

Masashi

Ms number: TCB-D-17-00020

We thank the reviewers for their careful reading and thoughtful comments, and the editor for her helpful guidance.

The major changes made in this revision are:

- The structure of the main text has been changed as comments/suggestions from the reviewers and the editor.
- The order of (original) Figure 2 and 3 has been changed as the editor's suggestion. Now Figure 2 introduces NOTCH signaling and Figure 3 shows the model of RIS- and RAS-type senescence.
- The changes other than above can be found in 'Point-by-point response'.

Point-by-point response:

Reviewer #1

In this review Ito et al. summarize the state-of-affairs of the senescence-associated phenotype in health and disease. The authors succinctly describe historical and recent developments and interlace them with their own, recently published findings. Overall the review is quite-well balanced but not very accessible to non-specialists. In addition, their own contribution should be toned down.

We agree this is a valid point, which was also raised by the editor. Although, the main focus of the review article is still NOTCH, we have broadened the scope and avoided any overstatements.

1. Globally speaking the phrasing and wording are at times too convoluted and the sense of some sentences is hard to decipher. The abstract as is not very enticing and too narrowly phrased for a wider audience.

We have rephrased the abstract for a wider audience.

1-1 I would not go so far to say that NOTCH is a master regulator. How important it really is needs to be seen. Tone down.

We agree. We have removed the word "master".

1-2 page 1 in abstract: ...leading to a stable cc arrest....

Corrected.

1-3 in abstract: ...leading to complex non-autonomous functionality through the SASP: what is meant to be said here?

This has been rephrased as follows (now page 1, line 17):

"Senescent cells are highly secretory driving a range of different functions through the senescence-associated secretory phenotype (SASP)."

1-4 in Figure 1: telomere (not telomer) dysfunction and developmental cues are missing. Moreover, I think premature senescence is a misnomer because this is only relative to replicative senescence. Physiologically speaking it is not premature but right in time. So, I would replace here with cellular senescence. The distinction is not necessary.
We thank the reviewer for pointing this. We have corrected the typo and added: "developmental cues" as triggers of senescence in fig. 1. We have also used "Cellular senescence" to represent the phenotype in fig. 1 as suggested.

1-5 page 2 line 11 cell autonomous; ...damage from theses stressors...: from which ones; I presume the ones that are mentioned two sentences before.
Yes. This sentence has been moved later in the manuscript and rephrased as follows (now page 2, line 21):

"senescence is an essential autonomous tumor suppressor mechanism preventing accumulation of damaged cells and malignant transformation."

1-6 yes, the commonality is that the mentioned stressors are all potentially oncogenic but not all senescence inducers are. I think the distinction between physiological and patho-physiological senescence should be highlighted better in this paragraph up front.
We have clarified that senescence is involved in both pathological and physiological processes, citing figure 1, and also in the following 'SASP section'.

1-7 cytotoxic drugs also induce senescence (TIS) and it is mostly independent of p53 and Rb status, so here the senescence response is dormant. This should be mentioned and weaved into the text. The importance of the SASP in such a context could be discussed.
We have added the description that TIS can be independent of p53/Rb, which are often defective in cancer. Also, a potential tumorigenic effect of the SASP in this context has been mentioned (now page 3, line 8).

1-8 line 25: Indeed, senescence confers diverse impacts....should read senescence impacts....
This sentence has been moved to the 'SASP section' and rephrased.

1-9 page 3 line 3: ...developing placenta, embryo and....
1-10 line 4 sense: In both situations...but there are more than two here.
We have rephrased to make the statement more accurate and up-to-date as follows (now page 3, line 22):

"Many recent works have clarified the importance of senescence not only as an intrinsic tumor suppressor, but as a highly conserved mechanism underpinning development and homeostasis; physiological senescence has been reported in the developing placenta [18], embryo [19,20] and during wound healing [21,22], driving the appropriate development or restoration of tissue architecture, often through immune surveillance. Moreover, the SASP, and IL6 in particular, has been demonstrated to promote cellular reprogramming [23-26]."

1-11 line 9: How important the SASP is for development is currently only speculative. Thus, I would avoid "critical" here as a judgment or ref the proof.
We agree and have deleted “critical”.

1-12 line 3: cite primary publications here rather than own reviews (Gil and Peeper and Campisi).
These primary papers have been cited as suggested.

1-13 line 16: The SASP includes many pleiotropic factors, driving different, potentially contrasting, impacts on different target cells. Rephrase sentence to read easier. I now what wants to be said here but the construction is too heavy.
This has been rephrased as follows (now page 3, line 6):

“The SASP has been linked to highly context-dependent and sometimes contrasting downstream functional outcomes.”

1-14 page 4 line 6: The transition sentence is odd and should be rephrased.
This has been rephrased (now page 4, line 15).

1-15 line 19: What is meant here? What interaction are we talking about? Functional or direct prot-prot; gene network...Please, clarify.
page 5 line 8: "are" instead of "were also unclear".
We have rephrased this sentence as follows (now page 5, line 13):

“How BRD4 activity integrates with NF-κB and C/EBPβ activity to influence SASP gene expression remains unknown.”

1-16 lines 10-13: This paragraph should be deleted. I think their own contribution should be humbled in a review. Any we etc should be avoided whenever possible.
- Put a transition sentence here.
‘We’ etc have been deleted but we have kept the paragraph to clarify that this review is focused on ‘NOTCH-mediated senescence control’ (this was the initial agreement with the editor).

1-17 page 10 line 16: It should be discussed why dynamic NOTCH activity could be important for OIS.
This is an important and interesting point. In the original version, we extensively discussed the potential significance of dynamic NOTCH regulation in tissue remodeling. However, it is unclear how it is also important for OIS. We speculate that at an early stage of OIS, NOTCH signaling might serve as amplification machinery for pro-senescence TGFb signaling to ensure senescence establishment. And then timely switch to inflammatory SASP would facilitate senescence surveillance. This is highly speculative, thus discussed in the section ‘conclusion remarks’.

1-18 page 24 line 11: This conditioned media..should read These...or This...medium...
As the editor’s suggestion, we have deleted all the glossary terms.

1-19 Trends box: cell-cell contact rather than cell-contact
Corrected.

2. Given the latest findings by Keyes et al in Genes/Dev and Serrano et al in Science this information should be included because it supports the argument of a dynamic SASP function.
This is a good point. We have added the information about the SASP-cell plasticity on page 4, line 2.

We also include a speculative view regarding the Notch-cell plasticity connection at the conclusion section.

3. The conclusion again is quite narrow and should express research opportunities and future directions more vividly.
We have removed notch-specific discussions and, instead, have added some particularly speculative views. At the same time, we have modified 'outstanding questions' to make less Notch-centric.

Reviewer #2
This manuscript is a review about the Notch signalling pathway in the context of senescence. Following their publication on 2016, where they described NOTCH1 as the master controller of the switch between two different secretomes, they here offer an extended discussion of the role NOTCH plays regarding SASP control and the non-cell autonomous spatial propagation of senescence phenotype. Overall, this is a well-written discussion about the novel role of NOTCH1 in senescence (recently described by Narita´s laboratory).

1. Although this review is focused on NOTCH1, as it´s the protein of interest of their recent publication, I would suggest adding more background and discussion about the other three NOTCH receptors and their role in senescence.
Other Notch receptors have also been linked with senescence. Although these studies were cited in the original version, we failed to describe the finer details. We have now extended the background of individual Notch receptors, particularly in the senescence context as follows (now page 7, line 19):

"NOTCH can also drive senescence independently of other stressors as ectopic expression of the ICDs of all of the NOTCH receptors induces senescence [52-54]. Upregulation of some cyclin-dependent kinase (CDK) inhibitors has been demonstrated as the molecular mechanism for NIS. Ectopic NOTCH3 in HMECs leads to upregulation of p21 through direct binding to its promoter [54]. Ectopic N1ICD leads to NIS through transcriptionally upregulating p16, and subsequent activation of Rb [53]."

2. In their current proposed model, an initial wave of NOTCH-TGFB signaling associated to an specific secretome preceded a second NFKB-CEBPB specific "canonical SASP" phase where TGFB signaling is switched off. However, others have shown that some factors of the TGFB family such as INHBA sustained TGB signaling during the late "canonical SASP" phase and are important for some senescence non autonomous effects such as paracrine senescence. authors should discuss how these other TGFB-family factors such as INHBA or BMPs fit into the

proposed model.
The following discussion has been added (now page 12, line 1):

“A recent study of OIS has reported persistent upregulation of other TGF-β family ligands, such as the activin/inhibin subunit, inhibin beta A (INHBA) [13]. Although *INHBA* is upregulated to similar levels in both RIS and NIS, endogenous activin antagonists *follistatin-like 3* and *BAMBI* are both downregulated in RIS and upregulated in NIS (unpublished data), suggesting further potential post-translational switching in TGF-β signaling by NOTCH and RAS. “

3. The bibliography is well chosen and up to date with some exceptions that have been stated in the comments.
In general and after some additions this would be a good review and I recommend its published in your journal.
We have added new references as suggested.

Reviewer #3
Comments on "Notching up and down: temporospatial control of senescence" by Ito et al

Overall, this is a very well written article, very nicely summarizing the recent finding by the group on the role of a Notch-mediated switch in the senescence-associated secretory phenotype, and the novel implications for senescence in general. The introduction and context of senescence and the SASP is well written for non-experts in the field to understand the context and significance. The details of their discovery and the mechanisms are well explained and discussed. In general, this reviewer only has some very minor comments on spelling and typing mistakes, and congratulate the authors on a very nice piece.

Minor comments

1. In the first paragraph on page 8, the sub-heading is "Two types of SASP", whereas in the text, they more so describe different stages in the temporal evolution of the senescence program. This is a little confusing suggesting two separate processes, rather than two stages of the same process. Perhaps the subheading could read "Two stages of the SASP" or something similar.
We thank the reviewer for pointing this out. We have replaced the sub heading with ‘*NOTCH-mediated secretome switch’*.

2. Telomere misspelled in fig 1
Corrected.

3. p3, Line 4: should not use "both" situations:
This has been clarified. Please see our response to reviewer #1-1-10

4. p5, Line 15: evolutionarily misspelled
Corrected.

5. p7, line 4: avoid repeated use of "more"
Corrected.

Spatial and Temporal Control of Senescence

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Key words:

Senescence, senescence-associated secretory phenotype (SASP), inflammation, C/EBPβ, NOTCH, lateral induction

Abstract

Cellular senescence is an autonomous tumor suppressor mechanism leading to a stable cell cycle arrest. Senescent cells are highly secretory driving a range of different functions through the senescence-associated secretory phenotype (SASP). Recent findings have suggested that the composition of the SASP is dynamically and spatially regulated and that the changing composition of the SASP can determine the beneficial and detrimental aspects of the senescence program, tipping the balance to either an immunosuppressive/pro-fibrotic environment or pro-inflammatory/fibrolytic state. Here we discuss the current understanding of the temporal and spatial regulation of the SASP and the novel finding of NOTCH signaling as a regulator of SASP composition.

Cellular senescence and its secretory phenotype

Cellular senescence was originally identified as a loss of proliferative capacity after prolonged culture of human diploid fibroblasts (HDFs) [1,2]. This form of senescence, induced by 'replicative exhaustion' and thus called replicative senescence, was later shown to be due to telomere attrition. Although replicative senescence was the prototypic form of senescence, similar phenotypes can be induced by a wide-range of stresses, including oxidative and genotoxic stress, cytokines, chromatin perturbation and unrestricted activation of oncogenes or mitogens [3,4]. Thus, senescence is a collective phenotype found in a range of cell and tissue types with diverse triggers, both pathological and physiological (Figure 1). An essential feature of senescence is persistent cell cycle arrest, which is unresponsive to extrinsic growth factor signals [1]. This arrest is most obviously demonstrated in oncogene-induced senescence (OIS), where cells stop proliferating even in the presence of continuous activation of the RAS/MAPK pathway, underscoring the tumor suppressive role of senescence. Critical to the senescent phenotype is activation of the p53-p21 and p16-retinoblastoma protein (Rb) pathways; loss of these pathways, as occurs in many human cancers, permits senescence-bypass and tumorigenesis. Importantly this phenotype can be vital in the response to some anti-cancer treatments, termed therapy induced senescence (TIS) [5,6]. Appropriate development of tumoral senescence after chemotherapy underpins tumor regression and improved prognosis [7]. Thus, senescence is an essential autonomous tumor suppressor mechanism preventing accumulation of damaged cells and malignant transformation.

Senescent cells also have significant non-cell-autonomous activities, which are crucial for many facets of senescence *in vivo*, including tumorigenesis, tissue repair, and embryological development. Soluble proteins secreted by senescent cells include inflammatory cytokines, chemokines, growth factors, and matrix-modifying enzymes, contributing to the senescence-associated secretory phenotype (SASP) [8-10]. The SASP has been linked to highly context-dependent and sometimes contrasting downstream functional outcomes. The SASP can be tumorigenic and promote the growth of neighboring transformed cells [10-12]. This is particularly significant in the *in vivo* TIS context, where the SASP (either from TIS or stromal senescent cells) could facilitate tumor recurrence from residual non-TIS or 'incomplete' TIS cancer cells, which are often p53/Rb defective [5,6]. However, in other contexts the SASP can be tumor suppressive, with induction (e.g. TGF-β [13,14]) or reinforcement (e.g. IL6, IL8 [8-10]) of a senescent phenotype in neighboring 'normal' cells (Figure 1). In addition, secretion of inflammatory cytokines by senescent cells has profound effects upon the immune system. The SASP can drive recruitment and activation of immune cells with subsequent removal of the senescent cell, termed senescence surveillance [15-17]. This senescence surveillance is important for the tumor suppressive role of senescence as inhibition or loss of immune mediators allows chronic persistence of senescent cells and subsequent tumorigenesis [17].

Many recent works have clarified the importance of senescence not only as an intrinsic tumor suppressor, but as a highly conserved mechanism underpinning development and homeostasis; physiological senescence has been reported in the developing placenta [18], embryo [19,20] and during wound healing [21,22], driving

the appropriate development or restoration of tissue architecture, often through immune surveillance. Moreover, the SASP, and IL6 in particular, has been demonstrated to promote cellular reprogramming [23-26].

Senescence is a fundamental intrinsic pathway arising in a variety of situations, whose appropriate function depends on its secretome. The SASP drives a wide range of potentially contrasting outcomes, requiring complex regulatory mechanisms. Understanding these mechanisms could permit therapeutic targeting of the SASP to promote an anti-tumorigenic response [27]. This review will focus on the temporal and spatial regulation of SASP composition and function, as well as novel contact-dependent cell signaling pathways in senescence, including NOTCH signaling (Figure 2).

SASP regulation

The realization that the SASP can have contrasting physiological functions has sparked interest in understanding its regulation. However, how the net functional output of such a complex entity is regulated has been unclear. Regulation of the SASP has been linked to signaling pathways, such as the DNA damage response (DDR) [28], p38MAPK [29,30], JAK/STAT [27], the inflammasome [13] and autophagy/mTOR [31-35]. Although some SASP effectors seem to act post-transcriptionally [31-33], most SASP regulators converge on two transcription factors, NF-κB and C/EBPβ, which co-operatively regulate many SASP components and inflammatory cytokines [8,9,34,36,37]. Originally, IL6 was proposed to be a central regulator of the inflammatory SASP; depletion of IL6 in the context of OIS results in collapse of the network, where a positive feedback loop between IL6 and

the transcription factors, C/EBPβ and NF-κB is engaged [9]. Later it was shown that IL1α, which can activate C/EBPβ and NF-κB, is upstream of IL6 in SASP control [38]. Indeed, ectopic expression of IL1α is able to phenocopy many aspects of RAS-induced senescence (RIS), including expression of IL6, IL8 and CCL2 [13]. Thus, proximal cytokines and inflammatory transcription factors act in a positive feedback loop to amplify the signaling of the SASP (Figure 3A).

More recent work has expanded our understanding of this signaling network: GATA4 has been recognized as an upstream regulator of NF-κB signaling in senescence. Activation of the DDR leads to GATA4-dependent activation of NF-κB and hence SASP expression [34]. The chromatin reader protein BRD4 is another newly identified regulator that dynamically binds to super-enhancer elements adjacent to inflammatory SASP genes [39]. How BRD4 activity integrates with NF-κB and C/EBPβ activity to influence SASP gene expression remains unknown.

Therefore, whilst our understanding of the regulation of the inflammatory SASP has improved, understanding of other SASP modules and whether they are regulated through other pathways is less explored. However, it has become clear that the composition of the SASP can be modulated to achieve anti-tumorigenic effects [27].

Senescence is a progressive phenotype and there is some evidence that global SASP functionality is also temporally regulated. Senescent cells accumulate in skin wounds where they act to promote wound healing. Senescent cell secretion of PDGF-AA promotes wound closure and deletion of these senescent cells delays wound healing. However, senescent cells also subsequently drive their own immune-

mediated clearance, suggesting a temporal switch from orchestration of wound repair to inflammatory recruitment of immune cells [22]. Consistently, a recent study has begun to unpick the mechanistic underpinnings of the temporal regulation of the SASP, suggesting that there are at least two distinct types of SASP: a 'NOTCH-induced senescence (NIS) type' (or 'TGF-β type'); and a 'RAS-induced senescence (RIS) type' (or 'inflammatory type') [40] (Figure 3A).

NOTCH in senescence

NOTCH is an evolutionarily conserved family of four cell surface receptors that play an essential role in embryonic development controlling cell fate determination and tissue homeostasis in many adult tissues [41,42]. The functions of NOTCH signaling are highly tissue-type specific, either promoting or inhibiting cell proliferation, viability and differentiation. In addition, since NOTCH ligands, such as the DLL and JAG family of proteins, are also plasma membrane proteins, NOTCH signaling is mediated through cell-cell contact, contributing to complex bio-patterning that is dependent on 'specification' of both signal-sending (ligand expressing) and signal-receiving (receptor expressing) cells [43] (Figure 2, Box 1).

Upon ligand-receptor interaction, NOTCH receptors undergo a series of proteolytic cleavages liberating the active NOTCH intracellular domain (NICD). NICD translocates to the nucleus, where it forms a complex with the DNA-binding transcription factor RBP-J and the co-activator Mastermind-like 1 (MAML1) proteins. RBP-J is a transcriptional repressor, which is converted to a transcriptional activator when bound to the NICD driving expression of NOTCH-target genes such as the HES and HEY family of transcription factors [41,43] (Figure 2).

NOTCH can be oncogenic or tumor suppressive in different cancer types. Indeed, constitutively active NOTCH is oncogenic in T-cell acute lymphoblastic leukaemia (T-ALL) [44,45], while NOTCH can be tumor suppressive in both bladder and head and neck squamous cell cancer [46,47]. The basis for this context-dependent duality is unclear, but one hypothesis is the complex interaction between cancer and tumor stroma as NOTCH signaling in stromal cells has been shown to modulate the tumor microenvironment [48].

Hyperactive Notch1 signaling can induce cell cycle arrest and apoptosis in early neural progenitor cells [49] and mouse embryonic fibroblasts [50], but its role in senescence was unclear until recently. Several NOTCH receptors or components of NOTCH signaling are upregulated during different forms of stress-induced senescence in various cell types, including human and mouse fibroblasts, human endothelial cells, human umbilical vein endothelial cells (HUVEC), human esophageal keratinocytes, human mammary epithelial cell (HMEC), and mouse primary renal tubular epithelial cells (PTEC) [51-55]. Inhibition of Notch, usually through γ-secretase inhibition (Figure 2), prevents or delays senescence in most models. NOTCH can also drive senescence independently of other stressors as ectopic expression of the ICDs of all of the NOTCH receptors induces senescence [52-54]. Upregulation of some cyclin-dependent kinase (CDK) inhibitors has been demonstrated as the molecular mechanism for NIS. Ectopic NOTCH3 in HMECs leads to upregulation of p21 through direct binding to its promoter [54]. Ectopic N1ICD leads to NIS through transcriptionally upregulating p16, and subsequent activation of Rb [53]. Whilst different Notch receptors are upregulated or activated in

various senescence models, the basis of determination between NOTCH-mediated apoptosis and senescence is unclear. In addition, the functional relevance of NOTCH signaling in the downstream functions of senescence has been largely unexplored.

NOTCH-mediated secretome switch

NOTCH1 was found to be significantly upregulated in RIS through plasma membrane proteomics [40]. Whilst NOTCH1 receptor expression is continuously upregulated during RIS, downstream signaling from NOTCH1 occurs transiently at the early phase of RIS or DNA damage-induced senescence (DDIS) (Figure 3A). NOTCH1 activity closely correlates with the expression of both TGF-β1 and TGF-β3 and inversely correlates with typical inflammatory cytokines, such as IL1α, IL6 and IL8. Indeed, NOTCH1 signaling is necessary and sufficient for TGF-β1 upregulation in RIS cells, whereas it negatively regulates expression of inflammatory cytokines. This suggested that there might be at least two distinct phases of SASP during senescence. Transcriptional profiling found that this regulation was not restricted to a few canonical SASP cytokines, but a generalized NOTCH-mediated reprogramming of the secretory output of the senescent cell. While the RIS-type SASP included many well-known inflammatory factors, shown by other groups [10,56,57], NIS was accompanied by expression of a distinct set of secreted factors. These two distinct secretomes exhibit a strikingly reciprocal pattern: RIS factors are mostly downregulated in NIS cells whereas NIS factors tend to be downregulated in RIS cells [40]. Furthermore, sustained activation of NOTCH1 in late RIS cells results in a SASP more similar to the NIS-type SASP, indicating a dominant role for NOTCH in determining SASP composition: the higher the NOTCH activity, the stronger the

TGF-β type components. This model suggests that the dynamic nature of NOTCH1 activity contributes to a switch between these two SASPs during senescence.

One of the key unanswered questions is how the level of NOTCH signaling is regulated during senescence. There are a number of potential candidates, including activity of the gamma-secretase complex and endogenous NOTCH inhibitors such as NUMB, IKAROS, ITCH and FBXW7 [58-60]. However, further investigations will be required to understand this mechanism and whether it could be targeted.

Modulation of senescence surveillance by NOTCH

The inflammatory SASP is critical for senescence surveillance where immune cells are recruited to eliminate senescent cells, thereby preventing tumorigenesis [17,27,61,62]. Therapeutics can reprogram the SASP to promote senescence surveillance [27]; therefore, de-repression of the inflammatory SASP through NOTCH-inhibition could promote this process. Utilizing a *NRAS*^{G12V}-driven murine hepatocyte senescence model, where RIS hepatocytes are progressively cleared by a CD4+ T-cell-dependent immune reaction [17], autonomous inhibition of Notch activity accelerates clearance of senescent cells, with increased T-cell recruitment [40]. NOTCH modulation has been considered in cancer therapy [63,64], and this study implies that NOTCH could be considered a target for preneoplastic lesions in early therapy for the prevention of malignancy, at least in some tissue types.

NOTCH negatively regulates C/EBPβ

Whilst NOTCH activity tunes the SASP composition during senescence, this does not exclude the hierarchical model of inflammatory-type SASP regulation, which

involves proximal cytokines (IL1α in particular) and the transcription factors, NF-κB and C/EBPβ. NOTCH1 negatively regulates C/EBPβ in RIS (Figure 3A), while NF-κB activity is largely unaffected [40], despite previous studies suggesting significant crosstalk between these two pathways [65].

Notably, although *IL1B/6/8* are well known targets of C/EBPβ, how *IL1A* is regulated is unclear. Similarly to IL1β/6/8, IL1α activates C/EBPβ (and NF-κB) [38], and C/EBPβ directly induces *IL1A,* which can be disrupted by NOTCH through depletion of C/EBPβ from an *IL1A* enhancer. Indeed, chromatin-binding profiles demonstrate that C/EBPβ binds to prominent upstream enhancer regions of *IL1A* although it also seems to bind the core promoter more weakly. The latter might be a reason for the apparent lack of evidence for the direct regulation of *IL1A* by C/EBPβ. These upstream enhancers directly associate with the *IL1A* promoter as shown by high-throughput 3D chromatin interactome mapping in HDFs, suggesting that they are *IL1A* enhancers [66]. Similarly C/EBPβ was found to predominantly bind one of the enhancer regions rather than the core promoter of *IL1A* during RIS, and this interaction was reduced by ectopic N1ICD expression [40] (Figure 3B).

Considering the central role for IL1α in the induction and subsequent amplification of the inflammatory SASP [13,38], NOTCH could provide a highly sensitive and robust mechanism of global SASP regulation. This view is corroborated with the near mutually exclusive NIS- and RIS-type secretome profiles (Figure 3A). Moreover, the pleiotropic activities of both factors suggest that such a regulatory relationship between C/EBPβ and NOTCH may have wider implications in development, differentiation, and cancer biology.

NOTCH and TGF-β

TGF-β is an important SASP factor that has major roles in several contexts. Firstly, it underpins paracrine senescence through p21-mediated cell cycle arrest. Co-culture of normal target cells with cells undergoing various types of senescence, or culture of normal cells in conditioned medium leads to target cell senescence, which is dependent on TGF-β and IL1β. [13,14]. Secondly, TGF-β seems to be crucial for the downstream functionality of the recently described developmental senescence [19].

Whilst often cytostatic, the effect of TGF-β on cell proliferation is cell-type dependent [67]. TGF-β alone fails to induce senescence in HDFs, but NIS is, in part, dependent on TGF-β [40]. Thus, TGF-β must cooperatively activate the NIS phenotype with other factors. How NOTCH activates TGF-β during senescence is still unclear. The relationship between NOTCH and TGF-β is complex and they crosstalk at various levels. Functionally, they often cooperate to induce target genes of either or both signaling pathways [68-70] although they can be mutually antagonistic in some contexts, such as muscle aging [71]. For their regulatory relationship, most studies have focused on TGF-β-driven induction of the NOTCH ligand JAG1 [69,72,73]. A positive feedback between these signals could be observed in renal podocytes and prostate epithelial cells [74,75]. Indeed, TGF-β-induced senescence in human esophageal keratinocytes is mediated through JAG1/NOTCH1 activation [53]. In contrast, another study found that JAG1 induction is TGF-β-independent, despite NOTCH-dependent upregulation of both factors. Therefore, NOTCH1 activation can be an upstream event of TGF-β activation during senescence, reinforcing the idea that NOTCH1 is a master modulator of SASP composition [40].

A recent study of OIS has reported persistent upregulation of other TGF-β family ligands, such as the activin/inhibin subunit, inhibin beta A (INHBA) [13]. Although *INHBA* is upregulated to similar levels in both RIS and NIS, endogenous activin antagonists *follistatin-like 3* and *BAMBI* are both downregulated in RIS and upregulated in NIS (unpublished data), suggesting further potential post-translational switching in TGF-β signaling by NOTCH and RAS.

Spatial regulation of senescence

Previous studies have demonstrated that non-cell-autonomous signaling from senescent cells is spatially restricted. RIS HDFs co-cultured with normal HDFs are able to non-cell-autonomously transmit a senescent phenotype to adjacent normal cells, but the effect wanes with distance from the RIS cells [13]. Similarly, this 'paracrine' senescence is incomplete as onward transmission declines with distance from the primary senescent cells. This could prevent runaway senescence and inflammation taking hold throughout a tissue, but its underlying mechanisms are ill-understood. One plausible mechanism is a cell-cell contact-dependent form of signaling rather than solely through the SASP; the non-cell-autonomous function of senescent cells was thought to lie predominantly in their secretome and cell-cell contact-dependent mechanisms have not previously been described in senescence. NOTCH is known to drive signaling through cell-cell contact-dependent feedback loops that involve the induction or repression of NOTCH ligands on adjacent cells. During lateral induction NOTCH-mediated upregulation of NOTCH ligands leads to the transmission of NOTCH signals to neighboring cells; this mode of signaling is responsible for coordinated cell behavior across a tissue [76,77] (Box 1). An

analogous process has been shown to occur during senescence: the NOTCH ligand JAG1 is selectively upregulated by N1ICD, as well as during the early phase of RIS. Co-culture experiments show that the NIS phenotype is transmitted to neighboring 'normal' cells, where JAG1-high NIS cells induce NOTCH signaling in the neighboring cells. This positive feedback loop leads to spatial relay of NIS. Therefore, senescence-associated NOTCH-dependent upregulation of JAG1 is linked to a spatial expansion of the NOTCH-driven phenotype of senescence and secretome control. This lateral induction of NOTCH signaling can be observed *in vivo* during RIS in hepatocytes [40].

A recent study has also highlighted other cell-cell contact-dependent signaling in senescence. Senescent cells, both *in vitro* and *in vivo*, are able to transfer proteins to neighboring cells through cytoplasmic bridges; in this model juxtacrine protein transfer from senescent cells to immunocytes was able to facilitate immune-mediated surveillance of senescent cells and could be demonstrated *in vivo* from $Kras^{G12D}$-expressing pancreatic cells to immunocytes [78]. Therefore, more than simply reliant on the SASP, senescent cells are able to demonstrate complex cell-cell contact-dependent signaling patterns that necessarily result in spatially-restricted patterns of behavior preventing widespread changes throughout tissues. It will be fascinating to see the physiological and pathophysiological relevance of this in the future.

Senescence and tissue homeostasis

The NOTCH-mediated temporal regulation of the SASP is probably not limited to RIS, given the similarity between the dynamic pattern of NOTCH1 activity and TGF-β

expression in RIS and DDIS [40]. The two secretomes also appear to represent distinct functionalities. TGF-β signaling is immunosuppressive [79], and also plays a key role in fibrosis upon tissue damage; type 3, 4 collagens and Fibronectin1 (FN1), constituents of fibrotic tissues, are also upregulated in NIS cells. In addition to pro-inflammatory cytokines, the RIS-type SASP components include extracellular matrix-degrading metalloproteases, such as *MMP1/3/10* [16,40]. Therefore, the NOTCH/TGF-β-high and the NOTCH/TGF-β-low phases of senescence might modulate the extracellular matrix through pro-fibrotic and fibrolytic functions, respectively.

This is highly reminiscent of the dynamic alteration of the secretome of hepatic stellate cells (HSCs). This liver-resident cell responds to organ damage through activation and secretion of a pro-fibrotic secretome leading to hepatic fibrosis, potentially underpinned by NOTCH [80]. Post-activation, HSCs undergo senescence with a pro-inflammatory, fibrolytic SASP, thereby both contributing to the resolution of fibrosis and their own immune-mediated clearance [16]. Similar to RIS HDFs [40], matrix components such as type 3, 4 collagens and FN1 are downregulated in senescent HSCs [16]. Thus, the timely shift of secretory profile to the inflammatory-type SASP during senescence is critical for proper resolution of tissue damage and preventing excessive fibrosis.

The dynamic nature of the SASP was also suggested in murine skin wound healing [22]. Accumulation of senescent stromal cells is an early event during healing, where PDGF-A, an early SASP factor, plays a critical role for proper wound healing.

Interestingly, *PDFGA* is highly upregulated in NIS, but downregulated in RIS or DDIS HDFs [40].

Whether NOTCH is involved in these tissue damage/repair models remains to be elucidated, but these studies underscore that timely regulation of the secretome composition during senescence plays an important role in restoration of tissue homeostasis after damage. Consistently, in the kidney, whilst acute activation of tubular epithelial Notch might be beneficial for tissue repair after ischemia-reperfusion injury, chronic activation of Notch triggers senescence and renal fibrosis [55,81,82]. The timely downregulation of NOTCH activity and, hence, the TGF-β type SASP during senescence might contribute to inhibiting 'over-healing' of damaged tissues. Some patients undergo excessive fibrosis of skin wounds termed keloids, associated with exuberant fibrosis production; NOTCH signaling seems to be upregulated in this context [83].

During embryonic development, senescence functions as a mechanism for developmental patterning, and these senescent cells demonstrate upregulation of TGF-β signaling and are subsequently eliminated by immune cells [19,20]. Considering the well-established role for NOTCH signaling in embryonic development, it would be interesting to investigate the involvement of NOTCH signaling and its spatial regulation in developmental senescence.

Concluding Remarks

It has become evident that senescence is far more than an autonomous tumor suppressor mechanism, but plays diverse and dynamic roles in tissue homeostasis

under both physiological and pathological conditions through its non-cell-autonomous activities [35]. Similarly far from having a fixed composition, the SASP is a temporally-evolving entity involving a dynamic shift of its functionality during the transition to senescence. We are only just beginning to understand some of the non-cell-autonomous activities of senescence apart from the secretome, with spatially restricted signaling. In certain contexts NOTCH signaling regulates a dynamic shift of SASP composition and a spatial propagation of senescence phenotype, adding new layers to the control of the non-cell-autonomous activities of senescence in space and time. Its potential significance in tissue damage/repair is discussed above. Senescent cells, through the inflammatory SASP, also play a key role for promoting cellular plasticity of adjacent cells *in vivo* [23-26]. Interestingly, it was shown that Notch inhibition can improve reprograming of mouse and human keratinocytes [84]. Whether or not the Notch-mediated secretory switch and/or lateral induction of Notch signaling contribute to cellular plasticity in keratinocytes and other contexts needs further study. While the significance of such temporospatial regulation of the SASP in tissue homeostasis is conceivable, its functional importance in OIS is not immediately clear. One speculative view would be that, at an early stage of OIS, NOTCH signaling amplifies the senescence-reinforcing TGF-β signaling through the lateral induction to ensure establishment of senescence, which then is followed by timely activation of inflammatory SASP and senescence surveillance.

Senescence is a highly heterogeneous phenotype, in both autonomous and non-autonomous contexts. Better understanding of the temporospatial regulation of the SASP in broader contexts will provide greater opportunities for development of senescence modulatory therapies.

Acknowledgments

We thank Aled Parry for helpful discussion. Y.I. and M.N. are supported by a Cancer Research UK Cambridge Centre Core Grant (C14303/A17197). M.H. is supported by a CRUK Clinician Scientist Fellowship (C52489/A19924).

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Figure 1. Cellular senescence is an autonomous tumor suppressor mechanism providing diverse non-autonomous effects through the senescence associated secretory phenotype (SASP).

Cellular senescence is a state of a stable cell cycle arrest mediated by the p53-p21 and p16-Rb pathways. It can be induced by a wide-range of cellular stresses. In particular, senescence triggered by telomere dysfunction during repetitive cell division is termed replicative senescence, whereas other forms of stress-induced senescence can also develop (premature senescence). Senescent cells are highly secretory and have diverse impacts on the neighboring cells and the tissue microenvironment, co-ordinating the behavior of surrounding normal, senescent and transformed cells. Functionally it has been demonstrated to be critically important not only during tumorigenesis, but also wound healing and embryonic development.

Figure 2. The canonical NOTCH signaling pathway.

NOTCH signaling is transmitted from a 'signal-sending' cell to a 'signal-receiving' cell through ligand–receptor binding in a cell-cell contact dependent manner. In humans there are five DSL-family ligands, Jagged (JAG) 1 and 2, and Delta-like (DLL) 1, 3 and 4, and four single-pass transmembrane-protein receptors, NOTCH 1, 2, 3 and 4. Upon ligand-receptor binding, the NOTCH receptor undergoes a series of proteolytic cleavage steps: metalloproteases of the ADAM/TACE family cleave the receptor ectodomain, allowing a second cleavage step by the γ-secretase complex releasing the NOTCH intracellular domain (NICD). The NICD can then translocate to the nucleus and bind to the DNA-binding protein RBP-J on chromatin, displacing co-repressors from the complex and driving recruitment of transcriptional co-activators such as MAML1, p300 and others to form active transcription complexes on NOTCH-

target genes, such as the HES and HEY family of transcription factors. Pharmacological or genetic disruption at each critical point in the pathway can inhibit NOTCH signaling. γ-secretase inhibitors (GSIs) or expression of the dominant-negative form of MAML1 (DN-MAML) are examples.

Figure 3. NOTCH signaling regulates a dynamic transition between two distinct SASPs through control of C/EBPβ during RAS-induced senescence.

(A) At the early phase of RAS–induced senescence (RIS), upregulation of the active form of NOTCH1, the NOTCH1 intracellular domain (N1ICD) represses the expression and chromatin binding of the pro-inflammatory transcription factor C/EBPβ. This transient N1ICD upregulation is closely correlated with upregulation of TGF-β expression, thus resulting in an immunosuppressive and pro-fibrotic state resembling the secretory phenotype of N1ICD-induced senescence (NIS-type SASP). At the later phase downregulation of N1ICD permits de-repression of C/EBPβ. In cooperation with NF-κB this gives rise to a pro-inflammatory and fibrolytic secretome (RIS-type SASP) through expression of inflammatory cytokines such as IL6 and IL8. (B) *IL1A* is a direct target of C/EBPβ. The promoter and upstream enhancers of *IL1A* associate as a higher order chromatin structure where C/EBPβ binds and activates *ILA* transcription. N1ICD controls an IL1α - C/EBPβ-mediated inflammatory loop during RIS by regulating C/EBPβ activity, confirming NOTCH1 signaling as a master regulator in switching-over between the NIS- and RIS-type SASP (see also A).

Box 1. Lateral inhibition and induction by juxtacrine signaling

NOTCH-induced lateral inhibition or induction are modes of juxtacrine signaling described in embryonic development, where these processes determine cell fate and tissue domain specification by cell-cell contact dependent communication.

Lateral inhibition of NOTCH signaling creates a divergence in NOTCH signal and therefore cell fate between sending and receiving cells through a negative feedback loop. (Figure I. A) A certain cell (light blue circle) among an otherwise homogenous population starts exhibiting higher NOTCH-ligand expression (L) than surrounding cells, which leads to upregulation of NOTCH activity (N) in the signal-receiving cells (light yellow circle). This elevated NOTCH activity represses ligand expression through transcriptional regulation of the effector gene (*p*), resulting in reduced reciprocal signaling from receiving to sending cell, further de-repressing ligand expression. This negative feedback loop amplifies the difference in the ligand expression and NOTCH activity of each cell and eventually the 'ligand-high' signal-sending cell (blue circle) is surrounded by 'NOTCH-high' signal-receiving cells (red circle(s)). The most well known example of this behavior operates when embryonic proneural clusters divide into sensory organ precursors (SOP) (future neurons) and supporting cells at the late stage of inner ear development. Cells differentiating into SOP express higher levels of ligand, repressing ligand expression in the surrounding cells and inhibiting further neuronal generation [76]. Forced expression of Notch-ligand Dl1 in the chick retina leads all cells to become supporting cells due to mutual lateral inhibition, whereas interruption of this pathway causes all cells to become neurons, emphasizing the importance of this system in differentiating cell fate [85].

Although less well described, lateral induction of NOTCH signaling is observed in early progenitor domain specification and is mediated by a positive feedback loop.

(Figure I. B) The ‘NOTCH-high’ signal-receiving cell upregulates NOTCH-ligand expression (dark-red circle); as a consequence, this efficiently returns NOTCH signaling back to the neighboring cells (light-yellow circle). The positive feedback loop delivers trans-activation of NOTCH signaling, delivering co-ordinate cellular behavior across a tissue. This mode of signaling is found in the early stage of developing mouse otic epithelium. Ectopic expression of N1ICD (Notch1 intracellular domain) leads to the ectopic development of sensory-type epithelium throughout the inner ear due to a Jag1-dependent lateral spreading of Notch signaling [77].

The molecular basis of the decision to induce or repress NOTCH-ligand is not known, but may involve the strength of the NOTCH signal transmitted by different members of the NOTCH ligands [86].

- NOTCH signaling is dynamically regulated during senescence

- NOTCH1 signaling reciprocally regulates inflammatory cytokines and TGF-β during senescence

- NOTCH1 signaling suppresses IL1α through downregulation of the transcription factor C/EBPβ

- The NOTCH1-JAG1 pathway mediates cell-cell-contact dependent lateral induction of senescence

- How is NOTCH activation and signaling regulated during senescence?

- How general is the involvement of NOTCH in senescence?

- What is a functional significance of temporospatial regulation of senescence in broader contexts?

- What is the mechanism behind the NOTCH-mediated regulation of TGF-β and C/EBPβ during senescence?

- Does dysregulation of NOTCH-mediated SASP switching contribute to aging and age related phenotypes?

Figure 1

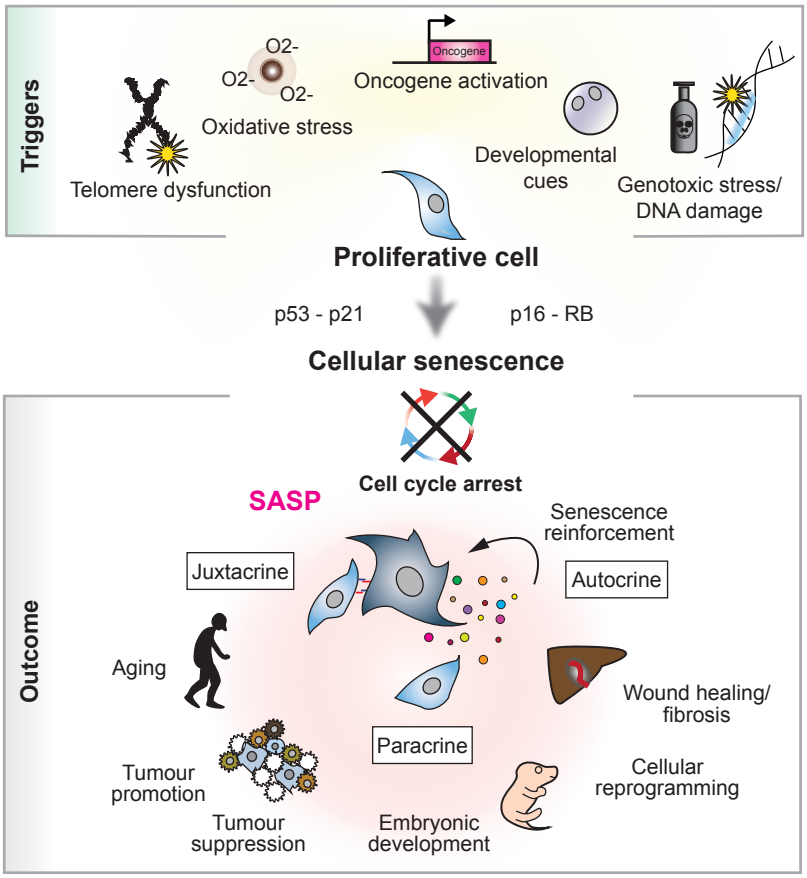
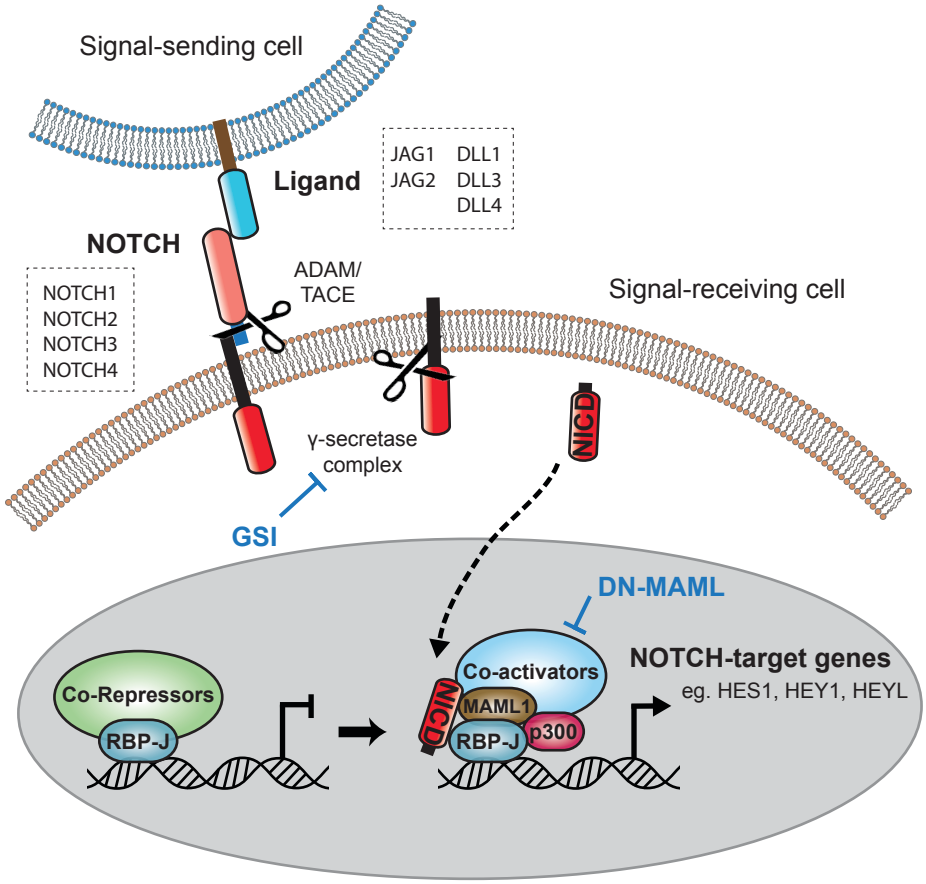

Triggers
O2-
O2-
O2-
Oxidative stress
Oncogene
Oncogene activation
Telomere dysfunction
Developmental cues
Genotoxic stress/ DNA damage
Proliferative cell
p53 - p21
p16 - RB
Cellular senescence
Cell cycle arrest
SASP
Senescence reinforcement
Juxtacrine
Autocrine
Outcome
Aging
Wound healing/ fibrosis
Paracrine
Tumour promotion
Cellular reprogramming
Tumour suppression
Embryonic development

Figure 2


Signal-sending cell
Ligand
JAG1
JAG2
DLL1
DLL3
DLL4
NOTCH
NOTCH1
NOTCH2
NOTCH3
NOTCH4
ADAM/
TACE
Signal-receiving cell
γ-secretase
complex
NICD
GSI
DN-MAML
Co-activators
NOTCH-target genes
eg. HES1, HEY1, HEYL
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RBP-J
NICD
MAML1
RBP-J
p300

Figure 3

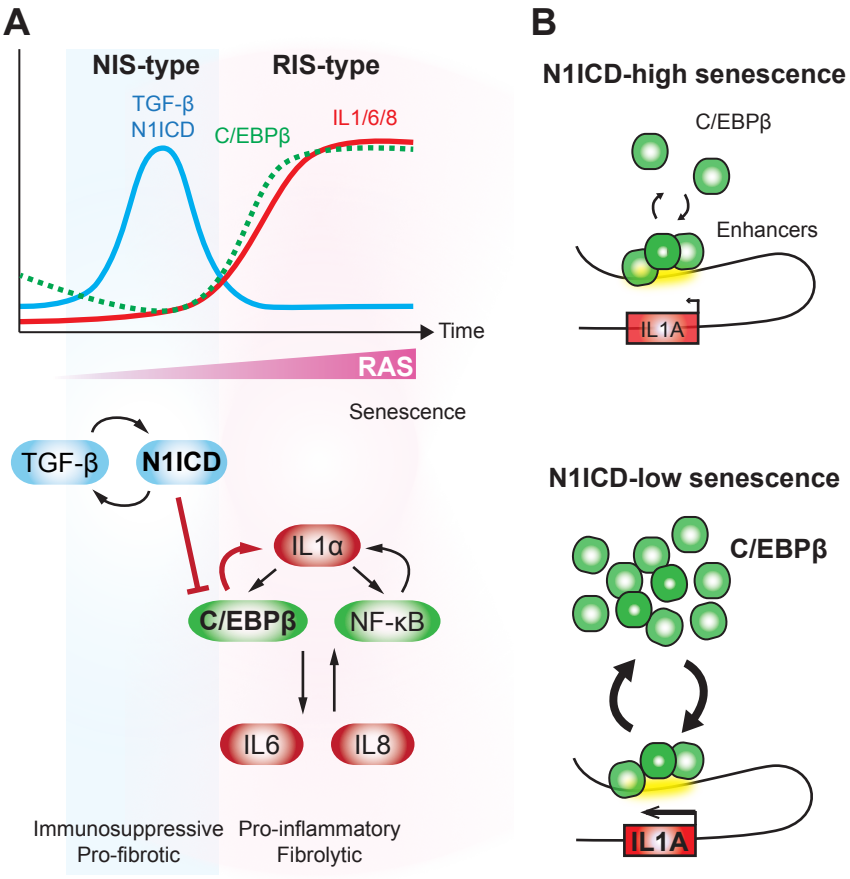
A
NIS-type
RIS-type
TGF-β
N1ICD
C/EBPβ
IL1/6/8
Time
RAS
Senescence
TGF-β
N1ICD
IL1α
C/EBPβ
NF-κB
IL6
IL8
Immunosuppressive
Pro-fibrotic
Pro-inflammatory
Fibrolytic
B
N1ICD-high senescence
C/EBPβ
Enhancers
IL1A
N1ICD-low senescence
C/EBPβ
IL1A

Figure I for Box