NOTCH1 mediates a switch between two distinct secretomes during senescence

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Senescence, a persistent form of cell-cycle arrest, is often associated with a diverse secretome, which provides complex functionality for senescent cells within the tissue microenvironment. We show that oncogene-induced senescence is accompanied by a dynamic fluctuation of NOTCH1 activity, which drives a TGF-β-rich secretome, while suppressing the senescence-associated pro-inflammatory secretome through inhibition of C/EBPβ. NOTCH1 and NOTCH1-driven TGF-β contribute to ‘lateral induction of senescence’ through a juxtacrine NOTCH–JAG1 pathway. In addition, NOTCH1 inhibition during senescence facilitates upregulation of pro-inflammatory cytokines, promoting lymphocyte recruitment and senescence surveillance \textit{in vivo}. As enforced activation of NOTCH1 signalling confers a near mutually exclusive secretory profile compared with typical senescence, our data collectively indicate that the dynamic alteration of NOTCH1 activity during senescence dictates a functional balance between these two distinct secretomes: one representing TGF-β and the other pro-inflammatory cytokines, highlighting that NOTCH1 is a temporospatial controller of secretome composition.

Cellular senescence is an autonomous tumour suppressor mechanism, whereby various triggers drive a stable proliferative arrest. Senescence is accompanied by diverse biochemical changes including upregulation of CDK inhibitors, the accumulation of senescence-associated β-galactosidase (SA-β-gal) activity, and expression of a wide variety of secretory proteins\textsuperscript{1,2}. These features of senescence have been recapitulated by \textit{in vivo} models, including both pathological and physiological contexts\textsuperscript{3}.

Senescent cells have profound non-autonomous functionality in the tissue microenvironment through the senescence-associated secretory phenotype (SASP)\textsuperscript{2}. Previous studies have demonstrated heterogeneous effects of the SASP on tumorigenesis. The SASP can reinforce the senescent phenotype in both an autocrine and paracrine fashion\textsuperscript{4,5} and activate immune clearance of senescent cells\textsuperscript{7–9} from tissues, thereby contributing to tumour suppression. Some tumorigenic activities of the SASP have also been shown through promoting cellular growth and epithelial–mesenchymal transition in neighbouring immortalized or transformed epithelial cells\textsuperscript{10,11}. In addition, SASP components, among others, include inflammatory cytokines and matrix-modifying enzymes, which play key roles in the clearance of senescent or damaged cells and resolution of tissue injury, respectively. Thus, it is conceivable that both the relative and absolute expression of SASP components is dynamic and under tight regulation. However, the basis for the regulation of different SASP components or controlling the net function of the SASP is unclear.

NOTCH signalling is evolutionarily conserved and involved in a wide range of developmental and physiological processes, controlling cell-fate specification and stem cell homeostasis\textsuperscript{12}. In addition, alterations of the NOTCH pathway have been linked to stress response and tumorigenesis, where it can be oncogenic or tumour suppressive depending on tissue and context\textsuperscript{13}. There are four NOTCH receptors, which bind the Jagged (JAG) and Delta-like family of ligands\textsuperscript{12}. Upon ligand binding, the NOTCH receptors undergo a series of proteolytic cleavage events liberating the intracellular domain (ICD), which subsequently translocates to the nucleus to bind a multi-molecular complex, including both the DNA-binding protein RBPJ and Mastermind-like (MAML) co-activators\textsuperscript{12} and drive transcription of NOTCH-target genes, such as the HES/HEY family of transcription factors.

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factors. Importantly, NOTCH ligands are also transmembrane proteins; thus, signalling is thought to be restricted to adjacent cells through juxtacrine interaction, and the role of NOTCH in autocrine or paracrine signalling through secreted factors remains unclear.

Through a quantitative cell surface proteome of oncogene-induced senescent (OIS) cells and subsequent validation, we have identified a global upregulation of NOTCH1 that is accompanied by dynamic alteration of its downstream activity during senescence. We describe how NOTCH1 functions as a master regulator of SASP composition through a temporal and functional switch between two distinct secretomes, representing TGF-β or pro-inflammatory cytokines, in part through downregulation of C/EBPβ. We show that inhibiting Notch signalling promotes clearance of OIS cells in the liver, implying a unique therapeutic opportunity to target senescent cells through modulation of immune surveillance.

RESULTS
Plasma membrane proteome in OIS
To gain a better understanding of the phenotype of OIS cells, particularly potential mediators of non-cell-autonomous signalling, we conducted a proteomic screen of plasma membrane (PM) surface proteins using a quantitative SILAC (stable isotope labelling with amino acids in cell culture) approach in IMR90 human diploid fibroblasts (HDFs) expressing oncogenic HRAS<sub>G12V</sub> in a 4-hydroxytamoxifen (4OHT)-inducible form (ER:HRAS<sub>G12V</sub>) (Fig. 1a and Supplementary Fig. 1a). We identified peptides from 1,502 proteins differentially expressed during RIS (P < 0.05), red dots indicate 94 proteins with more than twofold change. (d) Cell surface NOTCH1 expression by flow cytometry in indicated IMR90 cells: left, ER:HRAS<sub>G12V</sub> cells with (d6) or without (growing) 4OHT (iso-IgG, isotype control IgG); centre, cells with constitutive overexpression of either HRAS<sub>G12V</sub>, E1A, or both; right, DNA-damage-induced senescence (DDIS). To establish DDIS, cells were treated with 100 μM etoposide for 2 days, followed by 5 days of incubation in drug-free medium.

Figure 1 Plasma membrane proteomics (PMP) defines NOTCH1 as upregulated in OIS. (a) The workflow for quantitative PMP using differential SILAC labelling of growing and HRAS<sub>G12V</sub>-induced senescent (RIS) IMR90 cells. (b) GO cellular compartment term enrichment for all 1,502 identified proteins in both conditions. (c) Volcano plot of 521 high-confidence protein identifications from PMP demonstrating log<sub>2</sub> fold change (RIS(d6)/growing) against negative log<sub>10</sub> P value (n = 4 independent experiments). Among 167 proteins differentially expressed during RIS (P < 0.05), red dots indicate 94 proteins with more than twofold change. (d) Cell surface NOTCH1 expression by flow cytometry in indicated IMR90 cells: left, ER:HRAS<sub>G12V</sub> cells with (d6) or without (growing) 4OHT (iso-IgG, isotype control IgG); centre, cells with constitutive overexpression of either HRAS<sub>G12V</sub>, E1A, or both; right, DNA-damage-induced senescence (DDIS). To establish DDIS, cells were treated with 100 μM etoposide for 2 days, followed by 5 days of incubation in drug-free medium.
Figure 2 Dynamic canonical NOTCH1 signalling is responsible for reciprocal regulation of TGF-β ligands and pro-inflammatory cytokines during senescence. (a) Time series analysis of cell surface NOTCH1 expression during RIS in IMR90 cells by flow cytometry. Values are means relative to d0 ± s.e.m. from three independent experiments. (b, c) Time course of protein expression by immunoblotting during RIS (b) or DDIS (c). (d) ER:HRASG12V IMR90 cells, expressing dnMAML1–mVenus or matched control, were incubated with or without 4OHT for three days and analysed for expression of the indicated mRNA and proteins by qRT-PCR and immunoblotting respectively; n = 5 biologically independent experiments for TGFB1 and IL1B, n = 4 biologically independent experiments for IL1A; unpaired t-test. (e) ER:HRASG12V IMR90 cells, expressing a doxycycline-inducible N1ICD–FLAG construct (TRE-N1ICD), were analysed after 6 days of treatment with 4OHT with or without doxycycline at the indicated concentrations from d3 by qRT-PCR and immunoblotting; n = 6 biologically independent experiments for all conditions (except 4OHT/1 μM Doxy where n = 5); unpaired t-test. Values are mean ± s.e.m.; *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. Statistics source data for a, d and e are provided in Supplementary Table 2. Unprocessed original scans of blots are shown in Supplementary Fig. 9.

Of the 1,502 proteins, 521 were identified with 'high confidence' independent proteins with enrichment for localization in PM or extracellular compartments in Gene Ontology (GO) analysis (Fig. 1b). Of the 1,502 proteins, 521 were identified with 'high confidence' (see Methods) with 32 and 135 significantly up- and downregulated respectively in HRASG12V-induced senescent (RIS) cells (Fig. 1c and Supplementary Table 1).
Figure 3 NOTCH1 drives a cell-autonomous senescence with a distinct secretory profile. (a,b) ER-HRAS\textsuperscript{G12V} IMR90 cells, stably expressing N1ICD–FLAG or control vector (V), were incubated with or without 4OHT for 6 days and analysed for expression of the indicated proteins by immunoblotting (a) and SA-β-gal and BrdU incorporation (b). One-way ANOVA with Dunnett’s multiple comparison test; bars are means of ≥200 cells, n = 4 biologically independent experiments. ***P ≤ 0.001 versus control cells. Scale bar, 100 μm. (c) Time series analysis of the indicated transcripts after doxycycline (Dox) induction in TRE-N1ICD–FLAG IMR90 cells by qRT-PCR. Values are mean ± s.e.m., n = 3 biologically independent experiments. Inset, immunoblotting of fractionated chromatin in IMR90 cells expressing HRAS\textsuperscript{G12V} (d6) or TRE-N1ICD–FLAG (d3) for the downstream TGF-β phosphorylation target SMAD3 (phos-SMAD3). (d) TRE-N1ICD–mVenus IMR90 cells with or without 3 days of doxycycline were analysed for cell surface expression of the TGFB1 gene product latency-associated peptide by flow cytometry. (e) Differentially expressed transcripts in N1ICD-, HRAS\textsuperscript{G12V}- or etoposide-induced senescent IMR90 cells (NIS, RIS or DDIS, respectively), compared with normal control cells. The heat map shows z-score-normalized fold changes of 1,150 secretome genes differentially expressed in at least one comparison. Representative KEGG pathways enriched in four clusters (false discovery rate < 0.01) are shown. (f) TRE-N1ICD–FLAG IMR90 cells treated with or without doxycycline for three days with or without TGF-β receptor antagonists (no. 1, SB431542; no. 2, A83-01) were analysed by qRT-PCR and immunoblotting for the indicated genes. Values are mean ± s.e.m., n = 5 biologically independent experiments for CDK2a (CDK2); n = 4 biologically independent experiments for TGFB-induced (TGFB1). Statistics source data for b, c and f are provided in Supplementary Table 2. Unprocessed original scans of blots are shown in Supplementary Fig. 9.
To validate our proteomic findings, we compared the RIS-associated PM changes with transcriptomic data and identified a significant positive correlation between messenger RNA and protein changes during RIS (Supplementary Fig. 1b).

**NOTCH1 is upregulated in OIS**

To understand signalling networks involving senescence-associated PM proteins, we conducted network enrichment analysis, using both transcriptomic and proteomic data. The highest enriched network contained the NOTCH1 receptor as a major network hub and its canonical targets (HES1, HEY1, and HEYL) and binding partners (RBPJ and MAML3) (Fig. 1c and Supplementary Fig. 1c).

Using flow cytometry, we confirmed the substantial upregulation of cell surface NOTCH1 during senescence induced by different triggers (oncogenic MEK or DNA damage) or RIS in different HDFs (Fig. 1d and Supplementary Fig. 1d,e). In contrast, bypass of RIS through co-expression of the adenosival oncoprotein E1A failed to upregulate cell surface NOTCH1 on IMR90 cells (Fig. 1d). Although the NOTCH pathway has recently been implicated in senescence,

**NOTCH1 signalling is dynamically regulated during senescence**

We next investigated the temporal changes of cell surface NOTCH1 and its downstream activity after ER:HRAS<sup>G12V</sup> induction. In this system, senescence develops progressively from an initial mitotic phase (~d1) to senescence establishment (~d6) (Supplementary Fig. 1a)\(^9\). After a slight reduction at the mitotic phase, cell surface NOTCH1 continually increased during RIS (Fig. 2a). However, the cleaved, active NOTCH1 intracellular domain (N1ICD) and the canonical NOTCH1-target HES1 were transiently upregulated during the transition to senescence, but returned to near basal level at full senescence (Fig. 2b). The transient activation of NOTCH1 signalling, despite increased cell surface NOTCH1, was also observed during DNA damage-induced senescence\(^9\) (DDIS; Figs 1d right and 2c).

Characterized SASP components include multiple pro-inflammatory cytokines, such as IL-1, IL-6 and IL-8\(^{11,20,21}\). More recently, TGF-β ligands have been identified as SASP components, which are involved in senescence induction, in part through inducing p15 and p21\(^4,5\). IL-6 and IL-8 were primarily upregulated at full senescence. However, we found a transient induction of TGF-β ligands during both RIS and DDIS, reminiscent of the N1ICD expression pattern (Fig. 2b,c and Supplementary Fig. 2a), suggesting that NOTCH signalling temporally correlates with the reciprocal induction of TGF-β and pro-inflammatory cytokines during senescence.

**NOTCH1 reciprocally regulates TGF-β and pro-inflammatory cytokines**

To examine the relationship between NOTCH1 and regulation of secretory factors during RIS, we first introduced a dominant negative form of MAML1, fused to mVenus (dnMAML1-mVenus), into ER:RAS<sup>G12V</sup>-expressing IMR90 cells. At d3 after ER:RAS<sup>G12V</sup> induction, expression of dnMAML1 had minimal effect on proliferation, but completely blocked the induction of HES1 (Fig. 2d). This inhibition of NOTCH signalling significantly reduced the upregulation of TGFBI, suggesting that NOTCH is upstream of HRAS<sup>G12V</sup>-driven TGF-β induction (Fig. 2d). Conversely, upregulation of pro-inflammatory cytokines (IL-8, IL1A and IL1B) was enhanced by dnMAML1, suggesting that activated NOTCH1, during senescence transition, negatively regulates the expression of pro-inflammatory cytokines. Similar results were obtained pharmacologically with DAPT, a gamma secretase inhibitor, which blocks cleavage and release of the N1ICD (Supplementary Fig. 2b). Notably, the endogenous levels of N1ICD were modestly increased in the presence of dnMAML1 regardless of induction of HRAS<sup>G12V</sup> (Fig. 2d). This is consistent with previous studies showing that N1ICD levels are controlled by negative feedback through MAML-dependent proteasomal degradation, providing a potential mechanism for the decoupling of surface NOTCH1 and N1ICD levels\(^{22}\).

We next introduced a doxycycline-inducible N1ICD-FLAG system into IMR90 cells (Supplementary Fig. 2c). Restoration of N1ICD at the late phase of RIS (d6) led to a dose-dependent decrease in IL1A, IL1B and IL-8 expression and a dose-dependent increase in TGFBI expression, with minimal impact on senescence arrest (Fig. 2e). Therefore, during RIS, the dynamic alteration of NOTCH1 controls the temporally reciprocal pattern of TGF-β1 and pro-inflammatory cytokines and manipulating NOTCH signalling allows for SASP modulation with senescence arrest being maintained.

**Enforced activation of NOTCH1 induces a unique senescence phenotype in HDFs**

Consistent with recent reports\(^{16,18}\), expression of ectopic N1ICD drove a senescence-like morphological change with stable cell-cycle arrest, although accumulation of SA-β-gal activity was relatively modest (Fig. 3a,b and Supplementary Fig. 2d,e). Note, proliferative arrest was maintained even after removal of ectopic N1ICD, the hallmark of senescence (Supplementary Fig. 2e). Overexpression of N1ICD was sufficient for reduction of basal IL-8 levels as well as induction of TGF-β1 and its downstream effector phosphorylated SMAD3 (Fig. 3a,c,d). Thus, ectopic N1ICD induces senescence that is distinct from RIS or DDIS, particularly in its SASP composition.

To understand the broader implications of NOTCH1 in the control of secretome composition, we performed mRNA-seq analysis of senescent IMR90 cells driven by HRAS<sup>G12V</sup>, DNA damage or N1ICD. Transcriptional profiling of secretory factors of RIS and DDIS shared large clusters (Fig. 3e). Gene set enrichment analysis (GSEA) showed that all types of senescence shared a common cell-cycle signature (Supplementary Fig. 3a). However, the secretome expression profile of N1ICD-induced senescence (NIS) exhibited an almost mutually exclusive pattern with RIS and DDIS, particularly in those shared clusters (Fig. 3e). Many secretory factors that have been associated with RIS or DDIS, such as pro-inflammatory cytokines and matrix metalloproteinases (MMP1/3/10), were repressed by ectopic N1ICD. Downregulated secretory factors at d6 of RIS, including TGF-β ligands (TGFBI/2/3), were upregulated by ectopic N1ICD in IMR90 cells. GSEA revealed a close association of TGF-β1 signatures with NIS (Supplementary Fig. 3b). To understand the relative dominance of RAS and N1ICD upon the secretome composition we analysed secretome transcriptional data from IMR90 cells undergoing RIS, NIS or expressing both RAS and N1ICD (N+RIS). Unsupervised clustering revealed the similarity between NIS and N+RIS secretomes, where ectopic N1ICD mostly overcame the RIS
Figure 4 NOTCH1 drives non-cell-autonomous senescence partly dependent on TGF-β. (a) The proliferative ability of mRFP-labelled cells was analysed during co-culture with unlabelled senescent cells by proliferation analysis; representative images demonstrating co-cultured cells. Scale bar, 150 µm. NIS, doxycycline was added at d0 to induce N1ICD; RIS, ER:HRASG12V was pre-induced for 4 days before co-culture; DDIS, senescence was induced by etoposide as in Fig. 2c for 4 days before co-culture. (b) mRFP-labelled cells were co-cultured with doxycycline-inducible TRE-N1ICD cells treated with or without doxycycline for three days before flow sorting and expression analysis of the two cell populations for the indicated transcripts by qRT-PCR; unpaired t-test; bars are means, n = 3 independent biological replicates. (c) The proliferative ability of mRFP-labelled IMR90 cells was analysed during co-culture with TRE-N1ICD IMR90 cells treated with or without doxycycline and TGF-β receptor antagonists; representative result from five biologically independent experiments with similar results. (d, e) mRFP-labelled (puromycin-resistant) cells were co-cultured with cells stably expressing N1ICD–FLAG (hygromycin-resistant) for seven days before puromycin selection to selectively remove N1ICD-expressing cells, yielding populations that were ~99% mRFP-positive by flow cytometry. mRFP-labelled cells were then analysed for expression of indicated proteins by immunoblotting (d), and SA-β-gal and DNA synthesis by BrdU incorporation (e); unpaired t-test; values are mean ± s.e.m. of ≥200 cells from 8 high-power fields, n = 7 biologically independent experiments. **P <0.01, ***P <0.001. Scale bar, 200 µm. Statistics source data for b and e are provided in Supplementary Table 2. Unprocessed original scans of blots are shown in Supplementary Fig. 9.
Figure 5 NOTCH1 drives juxtacrine senescence through JAG1-mediated lateral induction in IMR90 cells. (a) Time series analysis of JAG1 expression by immunoblotting (upper) and at the cell surface by flow cytometry (lower) after doxycycline induction in TRE-N1ICD cells. (b) The proliferative ability of mRFP-labelled cells was analysed during co-culture with TRE-N1ICD cells treated with or without doxycycline and with or without DAPT. (c) The proliferative ability of TRE-N1ICD cells was analysed with or without doxycycline and DAPT at the indicated concentrations; representative result from four biologically independent experiments with similar results. (d) The proliferative ability of mRFP-labelled cells with stable expression of dnMAML1-mVenus or mVenus alone was analysed during co-culture with TRE-N1ICD cells treated with or without doxycycline; representative result from four biologically independent experiments with similar results. (e,f) Expression of JAG1 and proliferation of TRE-N1ICD cells stably expressing vector or indicated shRNAs targeting JAG1, demonstrated by immunoblot (e) and proliferation analysis with or without doxycycline (f); representative result from four biologically independent experiments with similar results. (g) The proliferative ability of TRE-N1ICD cells treated with or without JAG1 shRNA mRFP and with or without doxycycline. For proliferation assays (b-g), lines and bars represent means and s.e.m. (h) mRFP-labelled cells were analysed for BrdU incorporation, when physically separated from TRE-N1ICD cells treated with or without doxycycline in a Transwell chamber; unpaired t-test; ≥200 cells from eight high-power fields; n = 5 independent biological replicates. (i) TRE-N1ICD cells treated with or without doxycycline and TGF-β receptor antagonists (left) or co-transfected with vector or dnSMAD4 (right) were analysed for JAG1 expression by qRT-PCR; n = 3 biologically independent experiments; 1, SB431542; 2, A83-01. One-way ANOVA with Dunnett’s multiple comparison test (left) or unpaired t-test (right); bars are means (h,i) ± s.e.m. (h). Statistics source data for h and i are provided in Supplementary Table 2. Unprocessed original scans of blots are shown in Supplementary Fig. 9.
pattern (Supplementary Fig. 3c). Interestingly, such dominance of NOTCH over RAS also applied to GLB1, encoding the lysosomal enzyme responsible for SA-β-Gal activity23, potentially explaining the modest SA-β-Gal activity of NIS (Fig. 3b). Altogether, our data suggest that NIS and RIS are associated with reciprocal secretory profiles, and that dynamic NOTCH1 activity during senescence determines the balance between two extremities: one representing TGF-β ligands and the other representing ‘classical’ SASP components including pro-inflammatory cytokines.

NOTCH1-driven cell-autonomous senescence is partly dependent on TGF-β signalling

To understand how N1ICD induces senescence, we expressed N1ICD in the presence or absence of inhibitors of the TGF-β receptor (TGFBR1). Inhibition of TGF-β signalling prevented upregulation of TGF-β targets, CDKN2B (also known as p15) and TGFB-induced (TGFBI), in N1ICD-expressing cells, and partly rescued the anti-proliferative effect of N1ICD (Fig. 3f and Supplementary Fig. 4a, left). Similar results were also obtained by expression of a dominant negative form of SMAD4 (dnSMAD4; Supplementary Fig. 4b, left)24. Importantly, recombinant TGF-βs alone had no anti-proliferative effect on IMR90 cells (Supplementary Fig. 4c,d), suggesting that NOTCH-driven TGF-β signalling contributes to senescence cooperatively with other NOTCH1-downstream factor(s), as yet to be elucidated.

Non-cell-autonomous effects of NOTCH1 on normal cells

To investigate the non-cell-autonomous effects of differing forms of senescence, we set up co-culture experiments of mRFP-labelled, N1ICD-expressing IMR90 cells co-cultured with NIS, but not late phase RIS or DDIS cells, at least in IMR90 cells, underwent a growth arrest, suggesting a key role for the NOTCH1-driven secretome in the transition of senescence (Fig. 4a). To understand signalling pathways that might underpin N1ICD-mediated non-autonomous growth arrest, we co-cultured NIS and mRFP-labelled IMR90 cells for 72 h before flow sorting and then analysing gene expression in both cell populations. Consistent with N1ICD-mediated induction of TGF-β ligands in the monoculture experiments (Fig. 3c–e), both N1ICD-expressing and mRFP-labelled cells exhibited upregulation of the TGF-β targets CDKN2B (p15) and TGFBI (Fig. 4b). Similarly to autonomous NOTCH1 activation, TGFBR1 inhibitors or dnSMAD4 partially rescued the non-autonomous growth arrest in mRFP-labelled cells when co-cultured with N1ICD-expressing cells (Fig. 4c and Supplementary Fig. 4a,b).

To further examine whether N1ICD-expressing cells induce senescence in neighbouring cells, we took advantage of the difference in the drug selection markers of retroviral vectors expressing either N1ICD (or control vector) or mRFP: after co-culturing N1ICD-expressing, but not with vector-expressing cells, exhibited a senescent phenotype (Fig. 4d,e). Importantly, this phenotype was maintained even after the removal of the signal-sending cells, indicating that the N1ICD-expressing cells transmitted a senescent phenotype to the neighbouring cells.

N1ICD-induced ‘lateral induction’ of senescence

The role of NOTCH in biological patterning during development is attributed to processes termed ‘lateral inhibition’ and ‘lateral induction’25. NOTCH-mediated downregulation of NOTCH ligands in the same cells will negatively regulate NOTCH signalling in neighbouring cells (lateral inhibition), whereas NOTCH-mediated upregulation of NOTCH ligands will positively regulate NOTCH activity in neighbouring cells (lateral induction)26.

Interestingly, activation of downstream NOTCH signalling was observed not only in the N1ICD-expressing cells, but also in the co-cultured target cells with increased expression of HES1 (Fig. 4b). In addition, basal levels of ILLA were repressed in both cell populations (Fig. 4b), suggesting that NOTCH signalling was transmitted from N1ICD-expressing cells to neighbouring cells. Among the five canonical NOTCH ligands27, we found a strong, unique upregulation of JAG1 after ectopic N1ICD expression (Fig. 5a and Supplementary Fig. 5a). Although shedding of the extracellular domain of JAG1 has been reported, we did not detect this in conditioned media from NIS cells27 (Supplementary Fig. 5b). Induction of JAG1 was also observed during the transition to RIS with up- and subsequent downregulation mirroring the dynamic expression of N1ICD (Supplementary Fig. 2a). Induction of senescence with increased JAG1 was confirmed in N1ICD-expressing hTERT-RPE1 cells (Supplementary Fig. 5c). These results suggest that N1ICD activation induces a cell-contact-dependent growth arrest through a process similar to embryonic lateral induction. To further corroborate this, we examined how downstream inhibition of NOTCH signalling in the mRFP-labelled target cells affected non-cell-autonomous suppression of proliferation in the co-culture system. Consistent with our hypothesis, use of DAPT led to a dose-dependent inhibition of the non-cell-autonomous growth arrest of mRFP-labelled cells co-cultured with N1ICD-expressing cells (Fig. 5b and Supplementary Fig. 5d). As expected, it had no effect on autonomous cell growth in cells expressing N1ICD, which acts downstream of gamma secretase activity (Fig. 5c). More specifically, dnMAML1-mediated inhibition of NOTCH signalling only in the mRFP-labelled target cells also led to resistance to the non-cell-autonomous growth arrest in the co-culture system (Fig. 5d).

We next inhibited NOTCH ligand activity in N1ICD-expressing cells. RNA-interference-mediated knockdown of JAG1 in the N1ICD-expressing IMR90 cells had no effect on cell-autonomous growth of these cells (Fig. 5e,f), but led to a dose-dependent inhibition of the non-cell-autonomous growth arrest in the co-cultured mRFP-labelled cells (Fig. 5g and Supplementary Fig. 5d). Culturing the N1ICD- and mRFP-labelled cells apart using a Transwell chamber led to only a marginal decrease in proliferation of the mRFP-labelled cells (Fig. 5h), supporting the critical role for cell–cell contact in activation of NOTCH signalling and subsequent senescence induction in cells adjacent to N1ICD-expressing cells. Similar NOTCH-mediated senescence transmission was also observed in mRFP-labelled IMR90.
Figure 6 NOTCH1 is dynamically upregulated within NRAS-senescent hepatocytes and inhibits senescence surveillance. (a) Livers were harvested from mice 12 days after hydrodynamic tail-vein injection of NRAS<sup>G12V</sup> or inactive NRAS<sup>G12V/D38A</sup>-bearing transposons, and analysed by immunohistochemistry for NRAS and Notch1 expression in serial sections; quantification of NRAS<sup>+</sup> hepatocytes expressing NOTCH1; values are mean ± s.e.m. from manual counting of ≥200 cells; n = 3 mice per condition. Insets, magnified pictures of the dashed areas. Scale bar, 200 μm. (b) Time series analysis of hepatic NRAS expression by immunohistochemistry after injection of NRAS<sup>G12V</sup>–IRES–mVenus or NRAS<sup>G12V</sup>–IRES–dnMAML1–mVenus. Scale bar, 200 μm. (c) Quantification of NRAS<sup>+</sup>, p21- or CD3 (T-lymphocyte marker)-positive cells within livers of mice treated as in b; unpaired t-test; values are mean ± s.e.m. from manual counting (NRAS) or automated image analysis of ≥10<sup>5</sup> cells (p21 and CD3) from liver sections (see Methods); for NRAS<sup>G12V</sup>-injected animals at d6, 9 and 12, n = 3, 3 and 4 mice respectively; for NRAS<sup>G12V</sup>–IRES–dnMAML1-injected animals at d6, 9 and 12, n = 4, 3 and 5 mice respectively; *P < 0.05, **P < 0.01. (d) Lateral induction of Notch signalling in mouse livers treated as in b. Representative immunohistochemistry of NRAS and Hes1 at d9 in serial sections. Insets, magnified pictures of the dashed areas. Asterisks demonstrate Hes1-expressing, NRAS-negative cells adjacent to NRAS-expressing hepatocytes. Arrowheads indicate positive internal control staining of Hes1 within cholangiocytes. The percentage of NRAS-positive cells with adjacent Hes1-positive (but not NRAS-positive) cells was manually counted; n = 3 mice per condition; bars are means; unpaired t-test. Similar results were also obtained using dual staining in the same section (Supplementary Fig. 6c). Scale bar, 200 μm. (e) Flow-based assay of peripheral blood lymphocyte (PBL) adherence (adherent cells per mm<sup>2</sup> per 10<sup>6</sup> perfused cells) to human liver sinusoidal endothelial cells (HSECs) from three separate individuals pre-incubated with conditioned media from IMR90 cells expressing ER:HRAS<sup>G12V</sup> and TRE-N1ICD with or without 4OHT (d6) and/or doxycycline (d3) (left; n = 3 biologically independent replicates or conditioned media from ER:HRAS<sup>G12V</sup> IMR90 cells, expressing dnMAML1–mVenus or matched control and incubated with or without 4OHT for 3 days (right; n = 3 biologically independent replicates) (see Supplementary Fig. 7a,b). Representative images (bottom) demonstrating adherent PBLs (arrows) to HSECs after pre-incubation with the indicated conditioned media. One-way ANOVA with Dunnett’s multiple comparison test; bars are mean; *P < 0.05, **P < 0.01. Scale bar, 50 μm. Statistics source data for a,c–e are provided in Supplementary Table 2.
Figure 7 Co-expression of NRAS<sup>G12V</sup> and N1ICD drives short-term apoptosis and long-term tumorigenesis in the liver. (a–c) Livers from mice injected with either NRAS<sup>G12V</sup> or NRAS<sup>G12V</sup>–IRES–N1ICD were subjected to immunohistochemistry for NRAS and cleaved caspase 3 staining at the indicated time points in serial sections. Relatively fewer NRAS-positive hepatocytes were detected in the NRAS<sup>G12V</sup>–IRES–N1ICD cohort (a), and these NRAS-positive cells were mostly positive for cleaved caspase 3 (d6) (b,c). Insets are magnified pictures of the dashed areas (b). Bars are means from automated image analysis of ≥10<sup>4</sup> cells from each liver section; d6 NRAS<sup>G12V</sup> n = 4 mice; d12 NRAS<sup>G12V</sup> n = 6 mice; d6 NRAS<sup>G12V</sup>–IRES–N1ICD n = 4 mice; d12 NRAS<sup>G12V</sup>–IRES–N1ICD n = 7 mice; unpaired t-test. Scale bar, 200 μm. (d) Mice injected with NRAS<sup>G12V</sup> (n = 7 mice) or NRAS<sup>G12V</sup>–IRES–N1ICD (n = 9 mice) underwent long-term follow-up; necropsy was performed in all to confirm the presence of liver tumours. Kaplan–Meier plots of cancer-free survival from the two cohorts; survival analysis by log-rank test. (e) Example images of gross liver pathology at two months post-HDTV injection of one mouse from each cohort revealing a large tumour (arrow) and multiple small cystic lesions in the liver injected with NRAS<sup>G12V</sup>–IRES–N1ICD. (f,g) Immunohistochemical and haematoxylin and eosin (H&E) staining of serial liver sections from each cohort for the indicated proteins. H&E staining demonstrating tumour (T) infiltrating the surrounding normal parenchyma (N) and strong tumoral immunohistochemical staining for the proliferative marker Ki-67 in serial sections (g). Images in g are magnified views of the dashed areas in f. *P < 0.05. Scale bar, upper panels, 5 mm; lower panels, 200 μm. Statistics source data for a and c are provided in Supplementary Table 2.

cells co-cultured with N1ICD-expressing RPE1 cells, where JAG1 was upregulated (Supplementary Fig. 5c,e). Although it is known that TGF-β signalling can induce JAG1 expression<sup>28</sup>, neither TGFBR1 inhibitors or expression of dnSMAD4 affected the N1ICD-mediated upregulation of JAG1 in HDFs (Fig. 5i), reinforcing that NOTCH is an upstream regulator of TGF-β. Together, these data indicate...
that N1ICD expression leads to cell-autonomous upregulation of both JAG1 and TGF-β ligands; the former triggers lateral induction of NOTCH signalling, and together with TGF-β signalling, induces senescence in neighbouring cells (Supplementary Fig. 5f).

Interestingly, the NOTCH-mediated transmission of senescence was blocked by coexisting RIS cells, which were expressing dnMAML1 to minimize the inhibitory effect of NOTCH on the ‘RIS-secretome’, highlighting the functional distinction between non-autonomous activities of the two phases of RIS. This might also be involved in the negative feedback of NOTCH activity observed in the late phase of RIS in culture (Supplementary Fig. 5g).

**NOTCH1 activation during OIS in vivo**

To test whether NOTCH signalling is involved in senescence in vivo, we first examined KrasG12D-driven pancreatic intraepithelial neoplasia (PanIN) in KrasG12D–/–; p48−cre mice, previously demonstrated to show evidence of senescence. It was shown that Hes1 is upregulated in KrasG12D−/−-driven mouse PanIN30–32. While most cells in adult wild-type pancreas exhibited low levels of Notch1 (Supplementary Fig. 6a), Notch1 was highly upregulated in PanIN cells that were positive for the senescence marker Dec129, although the nuclear staining of Notch1 appeared heterogeneous (Supplementary Fig. 6a). Notch1 was also upregulated in acinar to ductal metaplasia, a potential histological precursor for PanIN, previously linked to senescence33 (Supplementary Fig. 6a).

**Non-cell-autonomous effects of NOTCH1 on immune clearance of senescent cells**

We also examined the level of Notch1 in a mouse liver OIS model, in which transposable elements containing oncogenic NRASG12V are stably transduced to hepatocytes through the hydrodynamic tail-vein injection (HDTV): it was shown that NRASG12V−/− hepatocytes are often surrounded by immune cells, and progressively cleared by a CD4+ T-cell-dependent immune reaction. We found that cellular levels of Notch1 were upregulated in hepatocytes expressing NRASG12V, but not in hepatocytes expressing the non-functional NRASG12V/D38A (Fig. 6a).

To test whether Notch inhibition during NRASG12V−/−-driven senescence would modulate immune-mediated clearance of these cells, we compared two cohorts of mice, injected with NRASG12V or NRASG12V combined with dnMAML1. Consistent with previous reports, we observed a time-dependent clearance of NRASG12V-induced senescent hepatocytes (Fig. 6b,c). In the presence of Notch inhibition, this clearance was accelerated with a reduction in NRASG12V−/− and p21-expressing hepatocytes at d12 post-HDTV (Fig. 6b,c). Strikingly, in NRASG12V-expressing hepatocytes, the frequency of nuclear Hes1-positive cells progressively increased over time (Fig. 6d and Supplementary Fig. 6b), while, at d12 when most NRASG12V-expressing hepatocytes had been eliminated, the frequency was more variable between mice. Thus, the dynamic regulation of Notch activity observed during in vitro OIS and DDS (Fig. 2b,c) was recapitulated in vivo. Moreover, NRAS-expressing hepatocytes were often associated with neighbouring Hes1- or p21-expressing hepatocytes that did not express NRAS, at least at d9 (Fig. 6d and Supplementary Fig. 6c,d), providing in vivo evidence for senescence-associated lateral induction of Notch signalling. Note, we failed to observe any inhibition of NRASG12V−/−-driven senescence (probed by p21) by dnMAML1, particularly up to d9, in both NRAS-positive and -negative hepatocytes (Fig. 6c and Supplementary Fig. 6e): we speculate that dnMAML1 is likely to inhibit juxtacrine-mediated, but not paracrine-mediated, senescence. These data reinforce the immune-modulating function of Notch expression.

We confirmed the recruitment of immune cells into the liver injected with NRASG12V; recruitment of CD3+ T-lymphocytes, but not B220+ B-lymphocytes, was significantly accelerated in livers injected with NRASG12V−/−-IRES-dnMAML1 when compared with animals injected with NRASG12V−/−-IRES-mVenus (Fig. 6c and Supplementary Fig. 6f.g).

Leukocyte recruitment to the liver requires a leukocyte adhesion cascade to sinusoidal endothelial cells, which separate the liver parenchyma from sinusoidal blood flow. To examine the effect of NOTCH1-modulated secretomes on lymphocyte recruitment, we performed an in vitro flow adhesion assay35; human sinusoidal endothelial cells (HSECs), derived from explanted livers, were incubated in differentially conditioned media from IMR90 cells, before analysis of the ability of peripheral blood lymphocytes (PBLs) from healthy volunteers to adhere to HSECs under conditions of shear stress, recapitulating the physiological context of liver sinusoids (Supplementary Fig. 7a,b). Conditioned media from late phase (d6) of RIS IMR90 cells led to a significant increase in PBL adherence to HSECs and this effect was abrogated by co-expression of N1ICD (Fig. 6e). Similarly, inhibition of the NOTCH-regulated secretome at RIS transition (d3) led to significant increases in PBL adherence to HSEC when compared with HRASG12V−/−-conditioned medium (Fig. 6e). Therefore, RIS-driven secreted factor(s) act upon HSECs to facilitate lymphocyte adhesion, which is negatively regulated by NOTCH through modulation of the SASP.

We next injected NRASG12V or NRASG12V-IRES-N1ICD into mice; surprisingly, the number of NRAS-positive hepatocytes was much lower in the presence of ectopic N1ICD even at d6 (Fig. 7a). To understand potential reasons for this, we stained the livers for cleaved caspase 3 (CC3), an apoptosis marker, and found that hepatocytes expressing NRASG12V−/−-IRES-N1ICD were often CC3-positive (Fig. 7b,c). Nevertheless, in longer-term cohorts, most NRASG12V−/−-IRES-N1ICD−, but no NRASG12V−/−-, injected mice developed liver tumours (Fig. 7d–f). Thus, despite the efficient induction of apoptosis, ectopic NRASG12V and N1ICD cooperate to drive tumorigenesis. It remains to be elucidated whether this tumour formation is due to escape from senescence arrest and/or senescence surveillance, but the results underscore the context-dependent interaction between RAS and NOTCH signalling during tumorigenesis.

Our data collectively suggest that, at the endogenous level, Notch signalling modulates SASP composition in senescent hepatocytes, controlling the immune reaction in the liver and thereby negatively regulating the elimination of senescent hepatocytes, at least in part through suppressing T-lymphocyte recruitment to the liver.

**NOTCH1 regulates senescence secretome through repression of C/EBPβ**

To examine how NOTCH1 controls secretome composition, we measured the impact of N1ICD on two transcription factors:
NF-kB and C/EBPβ, previously shown to cooperatively regulate the SASP\cite{6,21,36}.\footnote{\textsuperscript{6,21,36}} NF-kB activation is primarily regulated through nuclear translocation, and consistent with previous studies\cite{20,36}, the level of chromatin-bound RELA/p65, the major component of NF-kB, was increased in RIS cells with its level in whole-cell lysates being unchanged (Fig. 8a). In contrast, C/EBPβ was upregulated in both whole-cell and chromatin fractions during RIS treatment (Fig. 8a)\cite{6,21,36}. Strikingly, ectopic N1ICD expression diminished levels of C/EBPβ, but not RELA, in both whole and chromatin fractions in RIS cells (Fig. 8a, compare lanes 2 and 4), although ectopic N1ICD appeared to be sufficient to inhibit the basal level of chromatin-bound RELA (Fig. 8a, compare lanes 1 and 3, Supplementary Fig. 8a).

In addition, N1ICD-mediated repression of C/EBPβ was abrogated in the presence of dnMAML1 (Fig. 8b). GSEA revealed enrichment of a C/EBPβ signature\cite{37} in NIS- or N4-RIS-downregulated genes, and RIS-upregulated genes (Supplementary Fig. 8b), suggesting that the transcriptional activity of C/EBPβ is broadly diminished in N1ICD-expressing IMR90 cells.
CEBPB translates from different in-frame start sites generating two transcriptional activators, LAP$^a$ and LAP (liver-activating protein), and an amino-terminally truncated transcriptional inhibitor, LIP (liver inhibitory protein)$^b$. We introduced full-length CEBPB complementary DNA (LAP, see Methods)$^b$ to inducible N1ICD-expressing IMR90 cells. The enforced expression of LAP$^a$ in N1ICD-expressing cells fully restored expression of IL-8 (Fig. 8c, compare lanes 2 and 4) and IL1A (Fig. 8d), suggesting that repression of pro-inflammatory cytokines by N1ICD is primarily mediated by inhibition of C/EBPB, although we do not exclude a role for N1ICD in qualitative regulation of the NF-xB pathway.

The preferential downregulation of C/EBPB was also observed when IMR90 cells were treated with recombinant TNF (also known as TNF$\alpha$). Ectopic N1ICD, which inhibited TNF-mediated pro-inflammatory cytokine induction, had no effect on the level of TNF-activated RELA (Fig. 8e) or other NF-xB family components (Supplementary Fig. 8c), whereas N1ICD efficiently downregulated C/EBPB$^+$ in both basal and TNF-treated conditions. Further, ectopic N1ICD-driven downregulation of C/EBPB was also observed in the HaCaT cells, where N1ICD failed to induce senescence, suggesting that NOTCH1-mediated C/EBPB inhibition is not limited to senescence (Supplementary Fig. 8d).

It is well established that IL-1$\alpha$ acutely activates NF-xB and C/EBPB to induce their targets, including IL1B, IL6 and IL8$^{36}$. In the context of senescence, it was shown that IL-1$\alpha$ is an upstream SASP effector, regulating a cytokine network through NF-xB and C/EBPB$^{37}$. Thus, it is possible that N1ICD negatively regulates IL-1$\alpha$ and thereby C/EBPB. However, overexpression of C/EBPB was sufficient for inducing IL1A even in the presence of ectopic N1ICD (Fig. 8d). In addition, when we treated N1ICD-expressing cells with recombinant IL-1$\alpha$, we observed only a modest increase of C/EBPB levels, whereas IL-6 was strongly upregulated to a level higher than control cells (Fig. 8f), suggesting that IL-1$\alpha$, like IL-1$\beta$/6/8, is also downstream of C/EBPB.

Unlike IL1B/6/8, the transcriptional regulation of IL1A is unclear. To test whether C/EBPB directly regulates IL1A expression during senescence, we first characterized the basal profile of C/EBPB$^+$ binding sites along with key epigenetic marks in IMR90 cells using external data sets$^{39,40}$. We found several C/EBPB peaks around the IL1A locus, including a modest ‘proximal’ C/EBPB peak at the transcriptional start site and a prominent ‘distal’ site ~8kb upstream of the transcriptional start site (Supplementary Fig. 8e). The proximal and distal sites were enriched for promoter and enhancer markers, respectively (Supplementary Fig. 8e). Interestingly, these two sites were recently identified as a promoter–enhancer pair, forming a looping interaction$^{41}$, suggesting that this distal site is an enhancer for IL1A. Next, we performed C/EBPB ChIP–qPCR, targeting these two regulatory regions of IL1A, as well as known C/EBPB$^+$-binding sites at the IL6/8 loci in IMR90 cells expressing N1ICD, HRAS$^{G12V}$, or both. Consistent with previous reports, C/EBPB$^+$ promoter binding at the IL6/8 loci was increased in RIS cells (Supplementary Fig. 8f).

Similarly, we found that C/EBPB$^+$ binding at promoter and, more prominently, enhancer regions of IL1A was also increased (Fig. 8g), reinforcing that IL1A is a direct C/EBPB target. In addition, co-expression of N1ICD resulted in reduced enrichment of C/EBPB at these regulatory regions in the context of HRAS$^{G12V}$ (Fig. 8g). We propose that NOTCH1 inhibits pro-inflammatory cytokines, including IL-1$\alpha$, primarily through repression of their C/EBPB$^+$-mediated transcription (Fig. 8h).

**DISCUSSION**

The data that we present here suggest that the SASP is not a singular entity, but a complex evolving entity with tightly regulated composition and spatial activity, dependent on the level of NOTCH activity. We provide evidence for an additional layer of non-autonomous activity of senescence: ‘lateral induction’, which was originally described in NOTCH-mediated control of boundary formation during embryonic development$^{25}$. Interestingly, recent studies have identified embryonic senescence as a mechanism for developmental patterning: these senescent cells are accompanied by upregulation of TGF-β signalling and subsequent immune clearance$^{42,43}$.

Another transcription factor involved in embryonic development, GATA4, positively regulates the SASP in part through upregulation of IL-1$\alpha$$^{44}$ and NOTCH signalling appears to have a negative impact on GATA4$^{45}$. It would also be interesting to test whether GATA4 plays a role in NOTCH-mediated inhibition of the C/EBPB$^+$–IL1a axis. Additional implications of our data include a possibility that constitutively active NOTCH signalling in tumour cells drives lateral induction of senescence in the stroma. Emerging evidence suggests the important role of bone marrow stroma in survival/maintenance of T-cell ALL, which is associated with activating mutations of NOTCH1$^{46}$. It would be important to test whether NOTCH signalling derived from T-ALL cells can induce a NIS-like phenotype in the bone marrow stromal cells, which might have a substantial impact on the T-ALL niche.

Finally, therapeutic elimination of senescent cells has been suggested to provide beneficial effects on tissue homeostasis or tumour suppression$^{7,8,47,48}$. Manipulation of NOTCH may provide a unique therapeutic opportunity for targeting senescent cells through modulation of senescence surveillance.

**METHODS**

Methods and any associated references are available in the online version of the paper.

*Note: Supplementary Information is available in the online version of the paper*

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METHODS

Cell culture. IMR90 (ATCC), WI38 (ATCC) and ES (embryonic skin fibroblast)9 (a gift from J. Gil, Imperial College, UK) human diploid fibroblasts were cultured as previously described in DMEM/10% fetal calf serum (FCS) in a 5% O2/5% CO2 atmosphere. mRFP1 cells (a telomerase-immortalized human retinal pigment epithelial cell line; ATCC) were grown in DMEM/F12/10% FCS in a 5% O2/5% CO2 atmosphere. HACAT cells (ATCC) were cultured in DMEM/10% FCS in a 21% O2/5% CO2 atmosphere. No cell lines used in this study were found in the database of commonly misidentified cell lines that is maintained by ICLAC and NCBI Biosample. Cell identity was confirmed through STR genotyping. Regular testing was always negative for mycoplasma contamination.

The following drugs and inhibitors were used: 4-hydrostaxofenix (40HT) (Sigma); N-[3.5-dfluorophenylacetyl]-L-alanyl-2-phenylglycine (GOCAT) (DAPT) (Sigma); SN541542 (Tocris); A 83-01 (Tocris); GW788838 (Tocris); etoposide (Sigma); recombinant human TGF-β1 (Cell Signaling); recombinant human TGF-β2 (Peprotech); recombinant human TGF-β3 (Peprotech); tumour necrosis factor (TNF); recombinant IL-1β (both R&D systems).

Vectors. The following retroviral vectors were used in this study: pBabe-puro for HRASG12V (ref. 50); C/EBPβ-LAP (alternative start codons were replaced with TGG; a gift from D. Peeper, NKI, Amsterdam); pLNCX2 (Clontech) for ER:HRASG12V (ref. 15); pLNCX (Clontech) for ΔMEK1:ER (A31, S218E, S222D); pWZLL-hygro for NIHCCD–FLAG (residues 1758–2556 of human NOTCH1, as described previously14); mRFP1; mECL–puro for dmMAML1–mVenUS (residues 12–74 of human MAML1); N1ICD–FLAG: mRFP1; pQCHXII–i for NIHCCD–FLAG, NIHCD–FLAG–mVenUS, C/EBPβ–LAP, dnSMAD4–mVenUS (residues 1–514 of human SMAD4, as described previously10); pQCVXII–i for NIHCCD–FLAG: mVenUS–miR30–puro for JAGI shRNA (target sequences: no. 1, 5′-GGTTGACCTGGATGACTAC-3′; and no. 4, 5′-GGTTTTGGAGTCGAGGACTCT-3′).

The tetracycline-inducible retroviral vectors (pQCHXII–i and pQCVXII–i) were cloned using the following strategies. A third-generation tet-responsive element (TRE3G) and a constitutively expressed rtIAT3 tet-transactivator cassette were PCR-amplified from pCLIP-i4. These two fragments were assembled by overlap-extension PCR and the product was cloned into pQCHXII or pQCVXII (Clontech).

Plasmids for hydrodynamic tail-vein injection: pPGK-SB13; pT/CAGGS for HRASG12V, NRASG12V, NRASG14; pWZL-C/EBPβ for N1ICD–FLAG; pQCXIH-i for N1ICDFLAG, N1ICD–iRES–dnMAML1–mVenUS, NRASG12V–IRES–dnMAML1–mVenUS, NRASG12V–IRES–NIHCD–FLAG.

SILAC labelling. Cells were cultured in SILAC DMEM (Thermo) supplemented with 10% dialysed FCS (Life Technologies), L-proline (280 mg l–1, Sigma), L-glutamine (Life Technologies) and either light (Arg 0, Lys 0) (Sigma), medium (Arg 6, Lys 4) or heavy (Arg 10, Lys 8) amino acids (CKGas) at 150 mg l–1 L-glutamine (Life Technologies) and either light (Arg 0, Lys 0) (Sigma), medium (Arg 6, Lys 4) or heavy (Arg 10, Lys 8) amino acids (CKGas) at 150 mg l–1.

Gene expression profiling by mRNA sequencing. RNA was extracted using the Qiagen RNeasy plus kit according to the manufacturer’s instructions and RNA quality checked using a Bioanalyser Eukaryote Total RNA Nano Series II chip (Agilent). mRNA-Seq libraries were prepared from at least 6 biological replicates of each condition using the TruSeq Sample Prep Kit (Illumina) according to the manufacturer’s instructions. Single-end 40 bp reads generated on the Illumina HiSeq were aligned to the human genome version GRCh37.64 using TopHat v2.0.4 (ref. 54). Read counts were then obtained using HTSeq-count v0.5.3p9 (http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html), normalized and tested for differential gene expression using the Bioconductor package DESeq v1.10.1 (ref. 55). Multiple testing correction was applied using the Benjamini–Hochberg method. Genes were selected as differentially expressed with a false discovery rate of <0.01. Secretome genes were defined as previously described18.

Network analysis of prototermic and transcriptomic data. RIS-associated PMP data complemented by mRNA-Seq expression data were used to identify key senescence-associated membrane protein network hubs. Proteins detected through PMP were merged with genes, but genes annotated in the cellular membrane compartment were excluded. Log, fold ratios were used for both proteomics and transcriptomics data. Data were analysed using Ingenuity Pathway Analysis (IPA) (QIAGEN); the possible interaction networks were generated using Ingenuity knowledge base and included only direct relationships. Default settings were used, apart from restricting the networks to experimentally obtained interactions in human data. The highest scoring network as assigned by IPA, presented here, highlighted the importance of NOTCH1 as a key driver in the PMP data. For graph readability we removed interactions from non-hub genes.

Chromatin isolation. Chromatin isolation was performed as described previously9.

BrdU incorporation, colony formation and SA-β-gal assays. Cellular proliferation by BrdU incorporation, colony formation and SA-β-gal analysis have been described previously10.

Cellular proliferation by Incucyte. Analysis of short-term cellular proliferation was performed in either an Incucyte-HD or Incucyte-Zoom device (Essen Bioscience) in a 21% O2 atmosphere. Cells were plated, at 4 × 104 cells for monolucultures or at 3.5 × 105 signal-sending cells with 1.5 × 104 target cells, in a 12-well plate in 1 ml cell culture media. Cell proliferation was determined through repeated measures of confluency on phase or epifluorescent imaging.

Gene set enrichment analyses (GSEA). GSEA were performed as described previously2, P values derived from DESeq analyses of the RNA-Seq data were −log10 transformed and then signed according to whether genes were up(+) or down(−) regulated compared with control samples. These values were then used for ranking and weighting of genes in subsequent GSEA analyses9. Cell-cycle-related gene sets were obtained from the Molecular Signatures Database (http://software.broadinstitute.org/gsea/msigdb). Other gene signatures were used from data sets in the Gene Expression Omnibus (GEO).

Flow cytometry. Cells were washed once with cold PBS, before dissociation with Versene (Life Technologies), washed twice more in PBS/0.1% FCS, blocked in 1% mouse or rabbit serum before incubation with combinations of the following fluorochrome-conjugated antibodies: anti-NOTCH1 (Ebioscience, 17-9889, 1-50); anti-JAGGED1 (R&D systems, FB1726A, 1-8); anti-lateX-cytoplasmic antibody (Ebioscience, 17-9829, 1-20). Cells were then washed twice more, before fixation with 4% PFA and analysis on a FACSCalibur (Becton Dickenson). Flow-based cell sorting was conducted on a FACSaria II cytometer (Becton Dickenson). Flow data were analysed with FlowJo v10.

 Laser scanning cytometry. Cell-cycle profile analysis was performed using Laser Scanning Cytometry on an iCys Research Imaging Cytometer (CytoxEye) using anti-BrdU (BD, 555627, 1-500) and counterstaining with DAPI.

 mRNA expression by quantitative RT-PCR. RNA was extracted using the Qiagen RNeasy kit as above and reverse transcribed to cDNA using the high-capacity reverse transcription kit (Applied Biosystems). qRT-PCR was performed as described before14 with relative expression determined by the 2−ΔΔCt method13 using β-actin (ACTB) as an internal control. Primer sequences are as follows: ACTB forward primer 5′-GGACCTTGGAGCAAGAGATGG-3′; ACTB reverse primer 5′-AGGAAGAAAGGGCTAGAAAGC-3′; C/EBPβ forward primer 5′-GGTCACCACTTCTGCTGCTG-3′; C/EBPβ reverse primer 5′-CAGGGCATCTTCAGGCTTATT-3′; CDKN2B forward primer 5′-GGTGTCATCCTGCTGCTGCT-3′; CDKN2B reverse primer 5′-TCCACCTTGGCCTCAGTTCAGG-3′.
protein expression by immunoblotting and immunofluorescence. Immunofluorescence and immunoblotting, on SDS–PAGE on gels of various concentrations, were performed as described previously. The following antibodies were used in this study: anti-HRAS (Calbiochem, OP-23, 1:500); anti-cyclin A2 (Sigma, C4710, 1:500); anti-NOTCH1 (Cell Signaling, 4380, 1:1,000); anti-NICD (Cell Signaling, 4147, 1:100); anti-HES1 (Cell Signaling, 11988, 1:1,000); anti-TGF-β1 (Cell Signaling, 3709, 1:500); anti-IL-6 (R&D systems, MA2061, 1:250); anti-IL-8 (R&D Systems, MAB208, 1:500); anti-β-actin (Sigma, A5441, 1:5,000); anti-GFP (Clontech, 632377, 1:2000); anti-Rb (Cell Signaling, 9309, 1:1,000); anti-JAGGED1 (Cell Signaling, 2155, 1:1,000); anti-FLAG (Cell Signaling, 2368, 1:1,000); anti-c/EBPβ-LAP (Cell Signaling, 3087, 1:1,000); anti-c/EBPβ (Santa Cruz, sc-150, 1:500); anti-Histone H3 (Abcam, Ab-1791, 1:10,000); anti-RelA (Cell Signaling, 3034, 1:1,000); anti-RelB (Cell Signaling, 4922, 1:1,000); anti-c-Rel (Cell Signaling, 4727, 1:100); anti-NF-kB1 (Cell Signaling, 3035, 1:1,000); anti-NF-kB2 (Cell Signaling, 4882, 1:1,000); anti-IkBα (Cell Signaling, 4814, 1:1,000); anti-phospho-IkBα (Cell Signaling, 9246, 1:1,000); anti-p65 (Santa Cruz, sc-759, 1:500); anti-p21 (Santa Cruz, sc-397, 1:1,000); anti-SMAD2/3 (Cell Signaling, 6865, 1:1,000); anti-phospho-SMAD3 (Abcam, ab23993, 1:1,000); anti-TGF-induced (Cell Signaling, 5601, 1:1,000). Full scans of all immunoblots are included in Supplementary Fig. 9, including molecular weight markers.

Protein from conditioned media was obtained by plating 2.5 × 10^6 cells in media with 2% FCS for 16 h for filtration through a 0.22 μm filter and then centrifugation at 4,000 g for 40 minutes through a Vivashp 6 concentrator column (10kDa molecular weight cutoff, GE Healthcare). Coomassie staining of gels was performed as previously reported.

Hydrodynamic tail-vein injection. All animal experiments were approved by the German or UK legal authorities, and mice were kept under pathogen-free conditions in accordance with the institutional guidelines of the University of Tuebingen or University of Cambridge.

Male and female C57BL/6 mice were purchased from Charles River and injected at 5–8 weeks of age. Vectors for hydrodynamic injection were prepared with the Quagen EndoFree MaxiPrep kit. Transposon-mediated gene transfer was previously described, briefly 20 μg of appropriate vector and 5 μg of SB13 transposase-containing plasmid were diluted in sterile-filtered phosphate-buffered saline to a total volume of 10% of the body weight of the animal before being injected into the lateral tail vein in under 10 s.

Immunohistochemistry. Formalin-fixed paraffin-embedded mouse tissues were stained with the following antibodies: anti-NOTCH1 (Cell Signaling, 3608, 1:200); anti-Dec1 (a gift from A. Harris, 1:2,000); anti-NICD (Cell Signaling, sc-31, 1:100); anti-Hes1 (Cell Signaling, 11988, 1:250); anti-p21 (BD, 556431, 1:50); anti-CD3 (Dako, A0452, 1:1,000); anti-B220 (R&D systems, MAI217, 1:1,500); anti-RelB (Bethyl, IHC-00375, 1:1,000); anti-cleaved caspase 3 (Cell Signaling, 9664, 1:1,000) after heat-induced epitope retrieval in citrate (pH6) or Tris-EDTA (pH9) buffers before visualization using the DAKO Envision kit according to the manufacturer's instructions and counterstaining with haematoxylin. Dual chromogenic immunohistochemistry staining was performed on committee approval (LREC reference 06/Q2702/61, UK and 04/Q2708/41). Liver endothelial cells were isolated from explanted livers or donor tissue to surgical requirements using a collagenase digestion (collagenase type 1a, Sigma-Aldrich) as described previously. All tissue was collected from patients in the Liver Unit at Queen Elizabeth Hospital in Birmingham. Briefly, digested tissue was placed over a 33%/77% Percoll (Amersham Biosciences) density gradient. The endothelial cells were isolated by immunomagnetic selection using antibodies against CD31 conjugated to Dynabeads (Life Technologies). The endothelial cells were then cultured in medium composed of human endothelial basal growth medium (Life Technologies), 10% AB human serum (HD supplies), 10 ng ml⁻¹ vascular endothelial growth factor (VEGF), and 10 ng ml⁻¹ hepatocyte growth factor (HGF) (Peprotech). The cells were grown in collagen-coated culture flasks and were maintained at 37 °C in a humidified incubator with 5% CO₂ until confluent.

Isolation of peripheral blood lymphocytes (PBLs). PBLs were isolated as previously described by density gradient centrifugation over Lymphocyte (VH Bio) at 800 g for 25 minutes. Harvested lymphocytes were re-suspended in RPMI 1640 (Life Technologies) 10% FCS.

Flow adhesion assay. To study immune cell recruitment, HSECs were grown in ibidi μ-slide IV flow channels (Thistle Scientific) until confluent. HSECs were then cultured in conditioned media for 24 h before connection to the flow system previously described. Peripheral blood lymphocytes were perfused through the microvessels over the endothelial cells at a shear stress of 0.05 Pa. Phase-contrast video recordings made during lymphocyte perfusion were analysed offline to determine adherence. Adherence is expressed as numbers of adherent cells per square millimetre per million perfused cells (cells per mm² per 10⁴).

Analysis of the IL1A locus. All sequence data were obtained from IMR90 cells. IL1A is shown with both the hg19 RefSeq annotation. GenCode version 19 annotation. C/EBPβ ChiP-seq and DNAase-seq data are from the Encode Project, and Histone data are from the Roadmap Epigenomics Project. The data were visualized using the Gviz Bioconductor library.

Chromatin immunoprecipitation (ChiP). ChiP was performed as described previously with modifications. Briefly, 50 μg of chromatin and 10 μg of antibody (C/EBPβ; Santa Cruz sc-150) were applied to each IP. For the negative control no antibody was added to the IP. Three replicate ChiPs were carried out for each condition followed by qPCR. Primer sequences used in qPCR are as follows:

**IL6** (−176) R (target): 5'-GCAATGCATAAGACAGCCTAC-3'
**IL6** (−167) R (target): 5'-GGGGCTATGTTGAAAGATTAAAGA-3'
**IL6** (−1158) R (nonspecific): 5'-GAATCCTGAGAGGAAACAGG-3'
**IL6** (−1158) R (nonspecific): 5'-GGTGGGACCAAACTTATT-3'
**IL6** (−134) / -45 (F target): 5'-AATGCTGGATGTCAGGATTTC-3'
**IL6** (−134) / -45 (R target): 5'-GCACCTCTATTTTTTCAATAG-3'
**IL6** (−134) / -45 (R non specific): 5'-GGGTCATTGGGAAGACATACA-3'
**IL1A proximal F (target): 5'-CTGGCAAGTTAAGCCTGGT-3'
**IL1A proximal R (target): 5'-TAAAATCCCCATTTTGGACG-3'
**IL1A distal F (target): 5'-GGGCGAGACATGTGGAGAGG-3'
**IL1A distal R (target): 5'-TGCACTAGGGAGGTTATGG-3'
**IL1A nonspecific F (nonspecific): 5'-GGGTCATGTTTGGGAAGG-3'
**IL1A nonspecific R (nonspecific): 5'-ATTCACTCCTGGACCAACATC-3'

The primer sets for **IL6** and **IL8** were previously reported. For **IL1A** Promximal (promoter) and 'Distal' (enhancer), qPCR primers were designed on the basis of C/EBPβ ChiP-seq data (ENCODE). In this case, 'nonspecific' is upstream of the IL1A promoter. The locations of the primer sets for **IL1A** are illustrated in Supplementary Fig. 8e.

Statistics and reproducibility. No statistical method was used to predetermine sample size. The experiments were not randomized and the investigators were not blinded to allocation during experiments. Unless otherwise stated, data are presented as mean ± s.d. All slides were scanned on a Leica AT2 at x 20 magnification and a resolution of 0.5 μm per pixel. Following digitization, image analysis was performed using the HALO (Indiclab), using the Cytonuclear v1.4 algorithm. Each stain was trained independently to provide the best accuracy for cell counting and all of the slides were reviewed manually for quality control. Analysis of the training was counted manually from 4 random high-power fields containing a median of 1,457 hepatocytes (range 1,304–1,678) as described previously, due to problems segmenting individual cells when staining was very intense.
as the mean ± s.e.m. n values represent the number of independent experiments performed or the number of individual mice per condition. For each independent in vitro experiment a minimum number of three experiments were performed to ensure reproducibility and adequate statistical power. For in vivo experiments all conclusions were based on a minimum of three mice per condition or time point. Analyses were conducted using Graphpad Prism 6. Student’s t-test was used for two-condition comparisons; one-way ANOVA with Dunnett’s multiple comparison test for more than two conditions. In the statistical analyses, two-tailed tests were used throughout; a P value of 0.05 was taken as significant. All of the study data including statistical tests and exact P values are provided in Supplementary Table 2.

Data availability. The RNA-sequencing data generated for this study have been deposited at the Gene Expression Omnibus (GEO) with the accession numbers GSE72404, GSE72407 and GSE72409. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE 82 partner repository with the data set identifier PXD004168.

The TGF-β1 signature was derived from previously published data available from GEO under accession codes GSE12493 (ref. 65) and GSE29660 (ref. 66). The C/EBPβ signature was derived from previously published data available from GEO under accession codes GSE47777 and GSE30834. Chromatin immunoprecipitation data sets were obtained from GEO with the following accessions: C/EBPβ, GEO ID GSM935519; DNase-Seq, GEO ID GSM1008586; H3K27ac, GEO ID GSM469966; H3K4me1, GEO ID GSM521895; H3K4me3, GEO ID GSM521901.

Proteomics data from Fig. 1 and Supplementary Fig. 1 have been provided as Supplementary Table 1. Source data for Figs 2–8 and Supplementary Figs 1, 2, 4–6 and 8 have been provided as Supplementary Table 2. All other data supporting the findings of this study are available from the corresponding author on request.

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**Supplementary Figure 1** Plasma membrane proteomics (PMP) defines the cell surface signature of senescence including NOTCH1. (A) ER:HRAS\textsuperscript{12V} IMR90 cells with RAS-induced senescence (RIS), induced by 6 days of 40HT or DNA-damage induced senescence (DDIS) induced by Etoposide, were analysed for SA-\beta-GAL staining (upper panels and lower left), presence of senescence-associated heterochromatic foci (SAHF) and DNA synthesis by BrdU incorporation (lower right). Manual counting of >200 cells; bars are means; unpaired t-test; n = 3 biologically independent experiments; *** P \leq 0.001. Scale bar 200 \mu m. (B) Correlation of log\_2 fold expression changes of 521 high-confidence PMP ‘hits’ at cell surface protein level by PMP (x axis) with transcriptomic changes by mRNA-Seq (y axis). Hits are colour-coded by significance level: green dots indicate a proteomic significance of P < 0.05; yellow indicate a transcript q < 0.01; red indicates significant in both PMP and transcript datasets. Correlation was calculated for all 521 (all), those with significance at transcript or protein level (anySig) or both transcript and protein (Commsig). FDR, false discovery rate. (C) Network enrichment analysis, with networks defined by the presence of a PMP hit and populated by transcript level data revealed the highest enriched network contained NOTCH1 as a major network hub. Other network members are shown by subcellular localisation; red indicates upregulation and green downregulation. Among four NOTCH receptors, only NOTCH1 was significantly upregulated in both PM proteomic and transcriptomic data from RIS cells. (D) ESF and WI38 human diploid fibroblast cell lines stably expressing oncogenic HRAS\textsuperscript{12V} were analysed for cell surface NOTCH1 expression by flow cytometry. (E) ΔMEK1:ER IMR90 cells with and without induction by 40HT were analysed for cell surface NOTCH1 expression by flow cytometry. Statistics source data for A are provided in Supplementary Table 2.
**Supplementary Figure 2** Dynamic canonical NOTCH1 signalling is responsible for reciprocal regulation of TGF-β ligands and proinflammatory cytokines during senescence. (A) ER:HRAS<sup>G12V</sup> IMR90 cells were examined in a time series analysis of TGF-β ligand expression by qRT-PCR (upper) and JAG1 expression by immunoblotting (lower) after RAS induction by 4OHT; values are mean ± SEM; the experiment was performed four times for day 0 through 6 and three times for day 8 with similar results. (B) ER:HRAS<sup>G12V</sup> IMR90 cells with and without 3 days treatment with 4OHT and DAPT at the indicated concentrations were analysed by qRT-PCR; One-way ANOVA with Dunnett’s multiple comparison test; values are mean ± SEM; n = 6 biologically independent experiments for HES1, IL1A and IL8, n = 5 biologically independent experiments for TGFBI. * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001. (C) Time-dependent behavior of TRE-N1ICD-FLAG IMR90 cells after induction of N1ICD expression by addition of doxycycline: immunoblot demonstrating expression of indicated proteins. (D) TRE-N1ICD IMR90 cells with and without canonical NOTCH-inhibitor dnMAML1 were analysed for colony forming capacity. (E) TRE-N1ICD IMR90 cells treated with or without doxycycline for 7 days were then either cultured with continued doxycycline treatment or in regular media for a further 10 days before analysis of colony forming capacity. Statistics source data for A & B are provided in Supplementary Table 2.
**Supplementary Figure 3** Activation of NOTCH is associated with cell-cycle arrest and antagonism of the RAS-driven secretome (A) Multiple cell-cycle-related genesets were significantly enriched within downregulated genes from IMR90 cells with N1ICD- (NIS), HRAS<sup>G12V</sup>- (RIS), or Etoposide-induced senescence (DDIS). Upper: example Geneset enrichment analysis (GSEA) plots for the ‘Reactome – cell cycle’ geneset in the indicated senescence dataset (NES, normalised enrichment score; FDR, false discovery rate); lower: table demonstrating enrichment of multiple cell-cycle genesets in down-regulated genes. (B) GSEA demonstrating enrichment of publically available TGF-β1-signatures in transcriptomic data from IMR90 cells stably expressing N1ICD (NIS). (C) Differentially expressed transcripts in cells expressing N1ICD-, HRAS<sup>G12V</sup>, or both (NIS, RIS, or N+RIS, respectively), compared to normal control cells. Heat map shows z-score normalised fold changes of 1150 secretome genes differentially expressed in at least in one comparison. Representative KEGG pathways enriched in five clusters (FDR < 0.01) are shown. Note, *GLB1* encodes the lysosomal enzyme responsible for SA-β-Gal activity.
Supplementary Figure 4 NOTCH1 drives autonomous and non-autonomous cell-cycle arrest in part through TGF-β. (A) Left: proliferation analysis of TRE-N1ICD IMR90 cells treated with or without doxycycline with or without the TGF-β receptor antagonist GW788388. Right: The proliferative ability of mRFP IMR90 cells was analysed during co-culture with TRE-N1ICD IMR90 cells treated with or without doxycycline and GW788388; Values are mean ± SEM, representative of three independent experiments with similar results. (B) Left: proliferation analysis of TRE-N1ICD IMR90 cells treated with or without doxycycline with or without dnSMAD4-mVenus or vector control treated with or without doxycycline; Values are mean ± SEM, representative of three independent experiments with similar results. Right: Immunofluorescence for TGFBI in IMR90 cells with dnSMAD4-mVenus or vector control treated with 10 ng/ml TGF-β1 or 1 ng/ml TGF-β1, 2 or 3; values are mean ± SEM; representative of three independent experiments with similar results. (C) IMR90 cells were subject to short-term proliferation analysis with or without the indicated concentrations of recombinant TGF-β1, 2 or 3; values are mean ± SEM; representative of three independent experiments with similar results. (D) Representative immunofluorescence of SMAD2/3 after 24h treatment with 10 ng/ml TGF-β1, 2, 3 or vehicle. Scale bar 50 μm.
**Supplementary Figure 5** NOTCH1 drives juxtacrine senescence through JAG1-mediated lateral induction. (A) IMR90 cells stably expressing N1ICD underwent transcriptional profiling by mRNA-seq; differential expression of canonical NOTCH ligands was then analysed. (B) Immunoblot for JAG1 and coomassie gel staining of whole cell lysate (WCL) and conditioned media (CM) from TRE-N1ICD IMR90 cells treated with or without doxycycline for 3 days. (C) RPE1 cells expressing ectopic N1ICD were analysed for markers of senescence; expression of the indicated proteins by immunoblotting; SA-β-gal and DNA synthesis by BrdU incorporation; and expression of p21 by immunofluorescence in mRFP-positive cells per condition; values are mean ± SEM; representative of three independent experiments with similar results. (D) Analysis of proliferation, by BrdU incorporation, and expression of p21 by immunofluorescence in mRFP-IMR90 cells when cocultured with TRE-N1ICD IMR90 cells with vector or shJAG1-4 and with or without 10 μM DAPT (See analogous experiment in Fig. 5b and g). Bars are means ±200 mRFP-positive cells per condition; n = 3 biologically independent experiments; one-way ANOVA with Dunnett’s multiple comparisons test; **P ≤ 0.01, ***P ≤ 0.001. (E) The proliferative ability of RPE1 cells expressing N1ICD or vector was analysed (upper). The proliferative ability of mRFP-IMR90 cells during co-culture with N1ICD or vector-expressing RPE1 cells, with and without DAPT, was analysed (lower) by proliferation assay; values are mean ± SEM; representative of three independent experiments with similar results. (F) Model of NOTCH-induced lateral induction of NOTCH-signalling through JAG1 and TGF-β. How exactly the NOTCH activation induces JAG1 and TGF-β remains to be elucidated (dotted line). Activation of NOTCH signalling in the left-hand cell leads to the expression of both JAG1 and TGF-β. JAG1 acts in a cell-contact dependent fashion to induce activation of endogenous NOTCH in the right-hand cell and in concert with TGF-β to induce juxtacrine senescence. (G) Proliferation of mRFP1-IMR90 cells was analysed during co-culture with TRE-N1ICD cells treated with or without doxycycline and ER:RAS cells expressing dnMAML1-mVenus treated with or without 4OHT; representative of three independent experiments with similar results. Statistics source data for A, C & D are provided in Supplementary Table 2.
Supplementary Figure 6 Notch1 is upregulated during in vivo Ras-induced senescence (RIS) and mediates lateral induction of senescence and Notch signaling. (A) Pancreatic tissue from p48-cre; LSL-KrasG12D and matched control mice was analysed by immunohistochemistry (IHC) for Dec1 and Notch1; insets demonstrate the staining pattern within normal pancreatic ducts (upper, arrowhead), pancreatic intraepithelial neoplasia (PanIN) (middle) and acinar-ductal metaplasia (ADM) (lower). Scale bar 200 μm. (B) Time series analysis of serial sections of murine liver tissue 9 days after HDTV-injection of NRASG12V-IRES-mVenus or NRASG12V-IRES-dnMAML1-mVenus stained by IHC for NRAS and Hes1; quantitation of positivity for Hes1 in NRAS-positive cells; bars are means; unpaired t-test; n = 3 mice per time point; * P ≤ 0.05. (C) Representative images of two-colour IHC staining of murine liver sections 9 days after HDTV-injection of NRASG12V-IRES-mVenus for NRAS (brown) and Hes1 (red) demonstrating Hes1-positive / NRAS-negative cells (asterisk) adjacent to NRAS-positive hepatocytes. Arrows demonstrate Hes1-negative cells for comparison. Scale bar 50 μm. (D & E) IHC for NRAS and p21 on serial murine liver sections at indicated time points after HDTV injection of NRASG12V-IRES-mVenus or NRASG12V-IRES-dnMAML1-mVenus. Representative IHC images; Insets, magnified picture of dotted rectangular areas. Asterisk demonstrates p21-positive / NRAS-negative cells adjacent to NRAS-positive hepatocytes (D). Scale bar 200 μm. Quantitation of p21 expression in NRAS-positive and negative hepatocyte populations (E). Hepatocytes were dichotomised into NRAS-positive (top) or negative (bottom) populations by automated image analysis and then p21 expression analysed in these 2 populations; values are mean ± SEM from automated image analysis of ≥105 cells per liver section; for NRASG12V injected animals at D6, 9 & 12, n = 3, 3 & 4 mice respectively; for NRASG12V-IRES-dnMAML1 injected animals at D6, 9 & 12, n = 4, 3 & 5 mice respectively; unpaired t-test. (F) Representative immunohistochemistry (upper) and immunofluorescence (lower) of CD3-expressing T-lymphocytes within the livers of mice injected with either NRASG12V-IRES-mVenus or NRASG12V-IRES-dnMAML1-mVenus. Arrows indicate CD3+ T-lymphocytes. (See also Fig. 6c) Scale bar upper panels 200 μm, lower panels 25 μm. (G) Quantification of B220 (B-lymphocytes) marker positive cells within indicated livers as in Fig. 6c. Values are mean ± SEM from automated image analysis of ≥105 cells from liver section; for NRASG12V injected animals at D6, 9 & 12, n = 3, 3 & 4 mice respectively; for NRASG12V-IRES-dnMAML1 injected animals at D6, 9 & 12, n = 4, 3 & 5 mice respectively. Statistics source data for B, E & G are provided in Supplementary Table 2.
**Supplementary Figure 7** Experimental setup for human sinusoidal endothelial cell (HSEC) flow-adhesion assay. (A) Workflow for production of conditioned media (CM) from ER:HRAS\(^{G12V}\)/TRE-N1ICD IMR90 cells with induction timing of HRAS and N1ICD and final incubation in serum free media for 16 hours prior to harvesting; similar schema for ER:HRAS\(^{G12V}\) IMR90s with or without dnMAML1 and harvesting at d3 of HRAS expression. (B) Diagram illustrating the set-up of the flow adhesion assay: HSEC from human liver tissue were plated within the flow cell and cultured until confluent, before incubation for 24 hours in differentially conditioned media. Then peripheral blood lymphocytes (PBLs) from healthy individuals were perfused over the HSEC in fresh media and analysed for their ability to adhere to the HSEC.
**Supplementary Figure 8** NOTCH1 controls the pro-inflammatory SASP through repression of C/EBPβ. (A) IMR90 cells expressing HRAS<sup>G12V</sup>, N1ICD-FLAG or both were analysed for expression of C/EBPβ by qRT-PCR; One-way ANOVA with Dunnett’s multiple comparison test; values are mean ± SEM; n = 6 biologically independent experiments; *** P ≤ 0.001. (B) Genes upregulated by C/EBPβ are highly enriched by geneset enrichment analysis amongst genes upregulated by HRAS<sup>G12V</sup> (RIS) and downregulated by N1ICD (NIS) and the co-expression of N1ICD and HRAS<sup>G12V</sup> (N+RIS); FDR, false discovery rate; NES, normalised enrichment score. (C) TRE-N1ICD IMR90 cells treated with or without doxycycline for 3 days, then with or without 100ng/ml TNFα for 1 hour were analysed for expression and chromatin binding of indicated NFκB family components by immunoblot. (D) Ectopic expression of N1ICD-FLAG in HaCaT immortalised human keratinocyte cells leads to a downregulation of C/EBPβ in Fig. 8g) and subsequent qPCR for loci at IL6 and IL8 promoters; n = 3 biologically independent experiments; *** P ≤ 0.001. (E) Genome browser snapshot of the IL1A locus demonstrating ChIP-Seq binding profiles for C/EBPβ, key histone marks and accessibility of chromatin by DNase-Seq (derived from ENCODE). There is a small C/EBPβ binding peak at the Gencode-annotated TSS (proximal), but much larger binding peaks at a distal enhancer region 8kb upstream (distal), recently demonstrated to be tethered to the IL1A core promoter by 3D chromatin interactome analysis. Representative RefSeq and Gencode annotated IL1A transcripts are shown. Solid vertical lines represent C/EBPβ ChIP-qPCR 'target' sites at the 'Proximal' and 'Distal' regions, whereas the dotted vertical line represents 'non-specific' site. (F) Chromatin immunoprecipitation of endogenous C/EBPβ (from experiment described in Fig. 8g) and subsequent qPCR for loci at IL6 and IL8 promoters; n = 3 biologically independent experiments; one way ANOVA with Dunnett’s multiple comparison test; values are mean ± SEM. *** P ≤ 0.001. Statistics source data for A, D & F are provided in Supplementary Table 2.
Supplementary Figure 9 Full scans of immunoblots used in figures, including molecular weight markers.
Uncropped immunoblots used in original figures

Fig. 4d

Fig. 5a

Fig. 8a

Fig. 5e

Fig. 8b

Fig. 8c

Fig. 8e

Fig. 8f

Supplementary Figure 9 continued
Supplementary Figure 9 continued
Supplementary Table Legends

**Supplementary Table 1** Plasma membrane proteomics (PMP) defines the cell surface signature of senescence. PM proteomics of 4 independent replicates of HRAS$^{G12V}$-senescent IMR90 HDFs compared to control cells demonstrating 521 high confidence protein ‘hits’: identifications with peptides identified in at least 2 independent replicates with at least 1 replicate having 2 or more peptides. Subcellular localisation by GO localisation terms including those with a Short Go (ShG) term: tagged with ‘integral to membrane’ but lacking a subcellular assignment. Proteins are ordered by the mean log$_2$ fold change of intensity ratios between senescent and control conditions.

**Supplementary Table 2** Statistical source data, tests used and exact p-values.