SUPPLEMENTAL INFORMATION

Cellular senescence modulates progenitor cell expansion during axolotl limb regeneration

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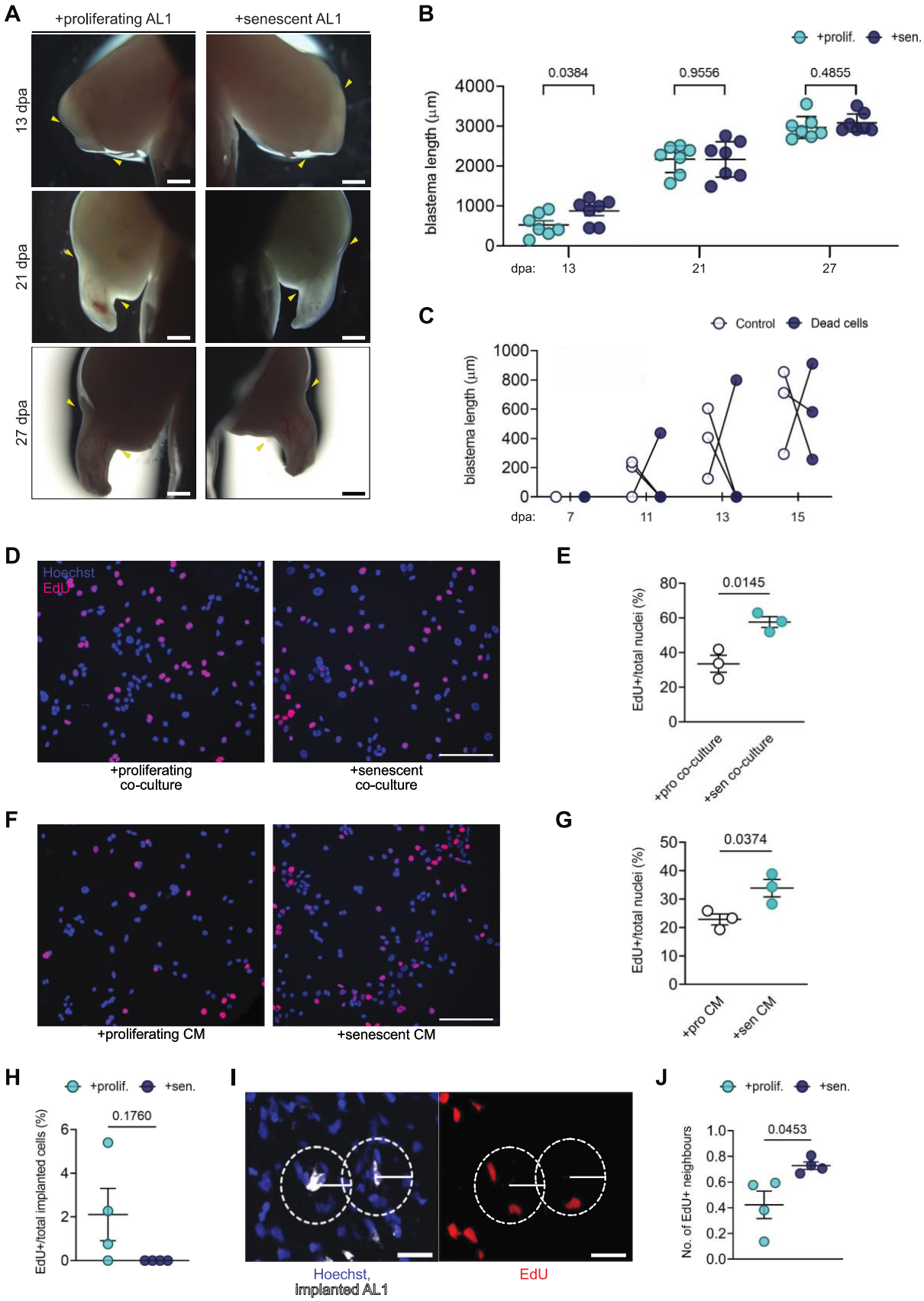

A
+proliferating AL1
+senescent AL1
13 dpa
21 dpa
27 dpa
B
+prolif.
+sen.
blastema length (µm)
0.0384
0.9556
0.4855
dpa:
13
21
27
C
Control
Dead cells
blastema length (µm)
dpa:
7
11
13
15
D
Hoechst
EdU
+proliferating co-culture
+senescent co-culture
E
EdU+/total nuclei (%)
0.0145
+pro co-culture
+sen co-culture
F
+proliferating CM
+senescent CM
G
EdU+/total nuclei (%)
0.0374
+pro CM
+sen CM
H
+prolif.
+sen.
EdU+/total implanted cells (%)
0.1760
I
Hoechst, implanted AL1
EdU
J
+prolif.
+sen.
No. of EdU+ neighbours
0.0453

Supplementary Fig. 1 (Related to Figure 1). Senescent AL1 cells enhance limb regeneration by stimulating proliferation in a paracrine manner.

(A-B) Dynamics of regeneration during AL1 cell implantation. **(A)** Representative bright-field images of regenerating limbs at the indicated times after implantation of proliferating (left panels) or senescent (right panels) AL1 cells. Scale bar = 1000 μm. **(B)** Quantification of blastema length. *P* values determined by paired two-tailed t-test. Error bars depict mean ± SEM.

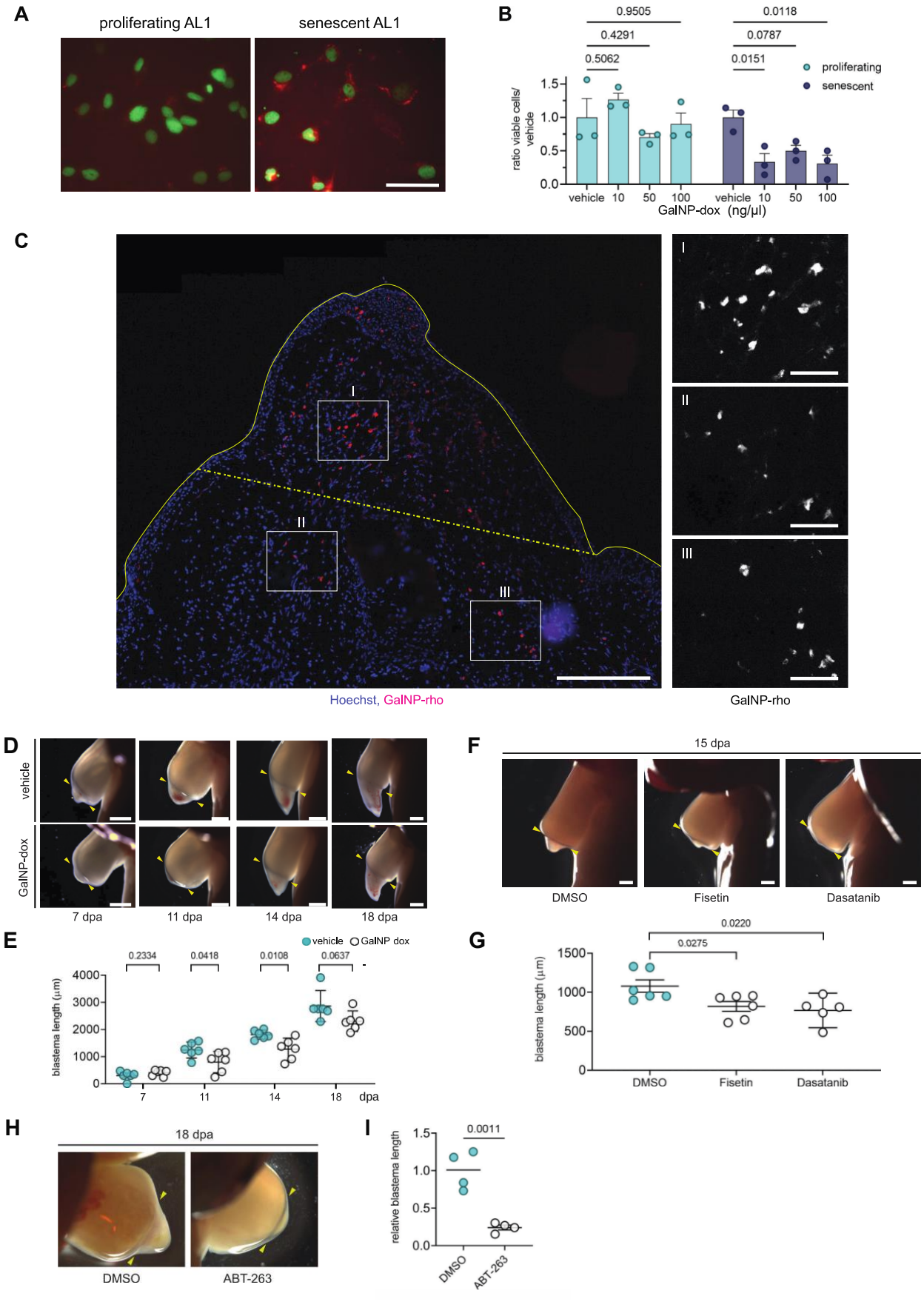
(C) Implantation of dead cells does not affect the rate of limb regeneration. Quantification of blastema length from regenerating limbs implanted with either control AL1 or ethanol-fixed AL1 cells at indicated days post amputation (dpa). No significant differences observed in blastema length at any timepoint as determined by paired two-tailed t-tests.

(D-E) Co-culture with senescent cells promotes proliferation *in vitro*. **(D)** Representative AL1 cultures co-cultured either with proliferating- or senescent-AL1 cells and stained for EdU. Scale bar = 300 μm Blue: Hoechst-stained nuclei; magenta: EdU. **(E)** Quantification of EdU+/total nuclei reveals significantly higher levels of EdU-incorporation in cells co-cultured with senescent AL1 cells. Each datapoint represents a technical replicate. *P* values determined by paired two-tailed t-test. Error bars depict mean ± SEM.

(F-G) Senescent cell conditioned media promotes proliferation *in vitro*. **(F)** Representative AL1 cultures treated with either proliferating- or senescent-derived conditioned media and stained for EdU. Scale bar = 300 μm. Blue: Hoechst-stained nuclei; magenta: EdU. CM = conditioned media. **(G)** Quantification of EdU+/total nuclei reveals significantly higher levels of EdU-incorporation in cells cultured with senescent-derived conditioned media. Each datapoint represents a technical replicate. *P* values determined by paired two-tailed t-test. Error bars depict mean ± SEM.

(H) Quantification of proliferation of implanted cells. Implanted cells were identified through histology using Vybrant CM labelling DiI and the percentage of EdU+/total implanted cells quantified per blastema. No EdU+ nuclei were detected in implanted senescent AL1 cells.

(I-J) Implanted senescent AL1 cells promote proliferation of blastema progenitors in a short-ranged manner. **(I)** Histology of 16 dpa blastemas implanted with AL1 cells. Implanted cells were identified by Vybrant CM DiI labelling and tissue collected at 16 dpa. Average number of EdU+ cells within a 50 μm radius was assessed. **(J)** Quantification of number EdU+ neighbouring cells, based on **(I)**. *P* values determined by paired two-tailed t-test. Error bars depict mean ± SEM.


A
proliferating AL1
senescent AL1
B
0.9505
0.4291
0.5062
0.0118
0.0787
0.0151
proliferating
senescent
ratio viable cells/ vehicle
1.5
1.0
0.5
0.0
vehicle 10 50 100 vehicle 10 50 100
GalNP-dox (ng/µl)
C
I
II
III
Hoechst, GalNP-rho
GalNP-rho
D
vehicle
GalNP-dox
7 dpa
11 dpa
14 dpa
18 dpa
E
vehicle GalNP dox
0.2334
0.0418
0.0108
0.0637
blastema length (µm)
4000
3000
2000
1000
0
7 11 14 18 dpa
F
15 dpa
DMSO
Fisetin
Dasatanib
G
0.0220
0.0275
blastema length (µm)
1500
1000
500
0
DMSO
Fisetin
Dasatanib
H
18 dpa
DMSO
ABT-263
I
0.0011
relative blastema length
1.5
1.0
0.5
0.0
DMSO
ABT-263

Supplementary Fig. 2 (Related to Figure 2). Elimination of senescent cells through senescence-specific GalNP or senolytic treatments attenuates regeneration.

(A) GalNP-rho specifically labels senescent cells *in vitro*. Representative images of proliferating or senescent AL1 cells *in vitro* treated with a 24 h pulse of GalNP-rho. Green: Hoechst; red: rhodamine. Scale bar = 50 μm.

(B) GalNP system enables specific killing of senescent cells *in vitro*. Number of live cells as assessed by calcein green AM staining for indicated doses of GalNP-dox in proliferating or senescent AL1 cells *in vitro*. Data displayed as ratio of live cells normalised against vehicle treatment. *P*-values determined by unpaired two-tailed t-test. Data points represent technical replicates.

(C) Representative sections of 15 dpa blastemas at 48 h after GalNP-rho injection. Solid yellow line: outline of blastema; yellow dotted line: amputation plane. Blue: Hoechst; red: rhodamine. Scale bar = 500 μm for main; 50 μm for insets.

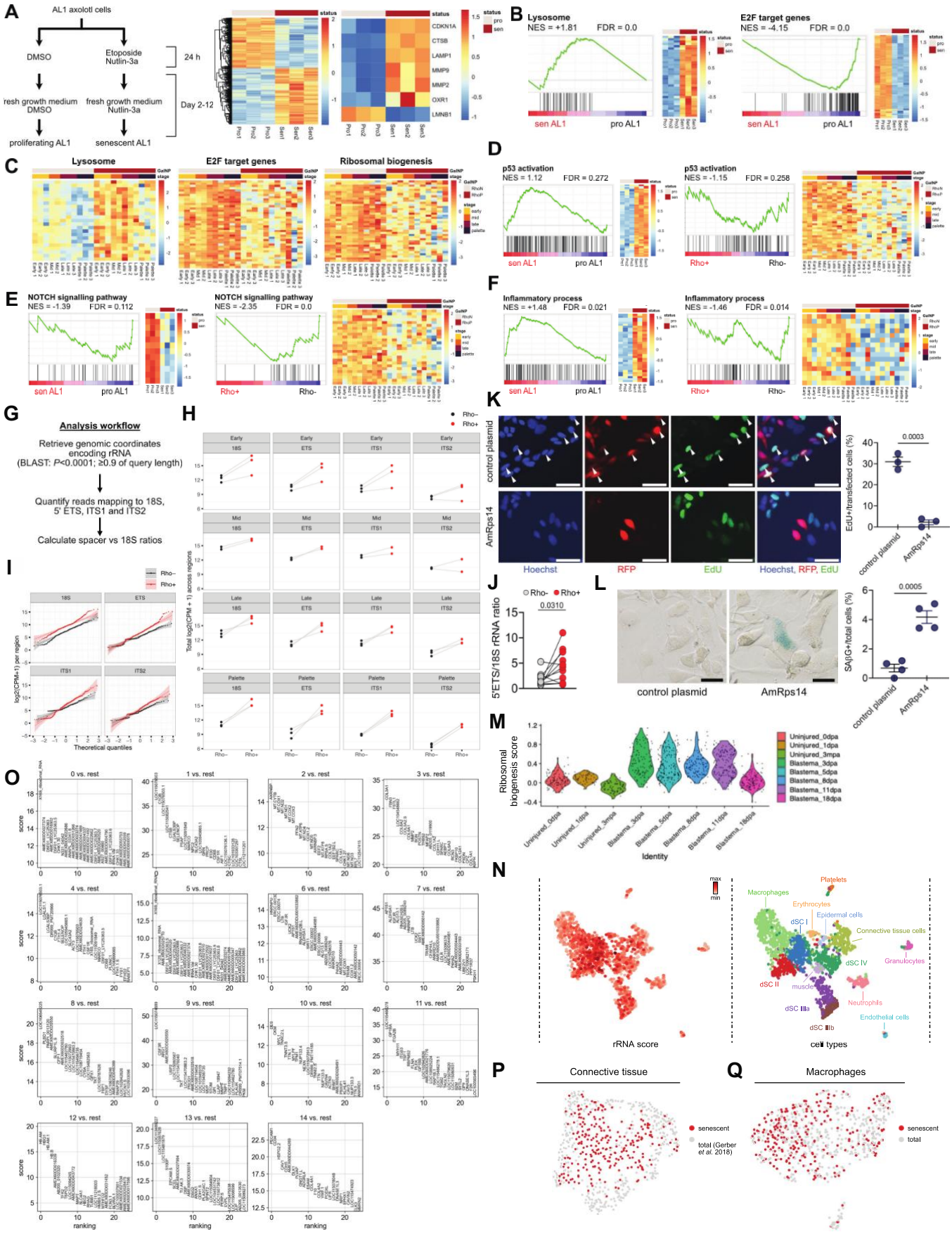
(D-E) Dynamics of regeneration during GalNP-mediated depletion. **(D)** Bright-field images of regenerating limbs at indicated timepoints, treated with vehicle (top panels) or GalNP-dox (bottom panels). Scale bar = 1000 μm. Arrowheads indicate amputation site. **(E)** Quantification of blastema length across indicated times. *P* values determined by unpaired two-tailed t-tests. Error bars depict mean ± SEM.

(F) Bright-field images of regenerating limbs at 15 dpa treated with DMSO, or indicated senolytics. Scale bar = 1000 μm.

(G) Quantification of blastema length, based on **(C)**. *P* values determined by unpaired two-tailed t-test against DMSO-treated animals. Error bars depict mean ± SEM.

(H) Bright-field images of regenerating limbs at 18 dpa treated with DMSO or ABT-263.

(I) Quantification of blastema length, based on **(H)**. Data represented as relative blastema length normalised against mean DMSO blastema length. *P* values determined by unpaired two-tailed t-test. Error bars depict mean ± SEM.


A
AL1 axolotl cells
DMSO
Etoposide Nutlin-3a
24 h
Day 2-12
fresh growth medium DMSO
fresh growth medium Nutlin-3a
proliferating AL1
senescent AL1
CDKN1A
CTSB
LAMP1
MMP9
MMP2
OXR1
LMNB1
B
Lysosome
NES = +1.81
FDR = 0.0
sen AL1
pro AL1
E2F target genes
NES = -4.15
FDR = 0.0
C
Lysosome
E2F target genes
Ribosomal biogenesis
D
p53 activation
NES = 1.12
FDR = 0.272
p53 activation
NES = -1.15
FDR = 0.258
Rho+
Rho-
E
NOTCH signalling pathway
NES = -1.39
FDR = 0.112
NOTCH signalling pathway
NES = -2.35
FDR = 0.0
F
Inflammatory process
NES = +1.48
FDR = 0.021
Inflammatory process
NES = -1.46
FDR = 0.014
G
Analysis workflow
Retrieve genomic coordinates encoding rRNA (BLAST: P<0.0001; ≥0.9 of query length)
Quantify reads mapping to 18S, 5' ETS, ITS1 and ITS2
Calculate spacer vs 18S ratios
H
Rho−
Rho+
Early
Mid
Late
Palette
18S
ETS
ITS1
ITS2
I
Rho−
Rho+
log2(CPM+1) per region
Theoretical quantiles
Total log2(CPM + 1) across regions
K
control plasmid
AmRps14
Hoechst
RFP
EdU
Hoechst, RFP, EdU
0.0003
EdU+/transfected cells (%)
J
Rho- Rho+
0.0310
5'ETS/18S rRNA ratio
L
control plasmid
AmRps14
0.0005
SAβG+/total cells (%)
M
Ribosomal biogenesis score
Uninjured_0dpa
Uninjured_1dpa
Uninjured_3mpa
Blastema_3dpa
Blastema_5dpa
Blastema_8dpa
Blastema_11dpa
Blastema_18dpa
Identity
O
0 vs. rest
1 vs. rest
2 vs. rest
3 vs. rest
4 vs. rest
5 vs. rest
6 vs. rest
7 vs. rest
8 vs. rest
9 vs. rest
10 vs. rest
11 vs. rest
12 vs. rest
13 vs. rest
14 vs. rest
score
ranking
N
max
min
rRNA score
Platelets
Macrophages
Erythrocytes
Epidermal cells
dSC I
Connective tissue cells
dSC IV
Granulocytes
dSC II
muscle
Neutrophils
dSC IIIa
dSC IIIb
Endothelial cells
cell types
P
Connective tissue
senescent
total (Gerber et al. 2018)
Q
Macrophages
senescent
total

Supplementary Fig. 3 (Related to Figure 3). RNA-seq unveils core features of cellular senescence *in vitro* and *in vivo*.

(A) Schematic of senescence induction in AL1 cells *in vitro* and corresponding heatmaps of differentially expressed genes (DEGs) between proliferating and senescent AL1 cells for total DEGs (left) and senescent-related genes (right).

(B) Pre-ranked GSEA plots and corresponding heatmaps for leading-edge genes comparing *in vitro* senescent and non-senescent cells for lysosome and E2F target genes in AL1 cells.

(C) Pre-ranked GSEA plots and corresponding heatmaps for leading-edge genes comparing *in vivo* senescent and non-senescent blastemal cells for lysosome, E2F target, and ribosomal biogenesis-associated genes, as in **Fig. 3 D & G**.

(D-F) Pre-ranked GSEA plots and corresponding heatmaps for leading-edge genes comparing senescent and non-senescent cells *in vitro* (left panels) and *in vivo* (right panels) for: **(D)** p53 transcriptional target genes; **(E)** NOTCH signalling pathway transcriptional targets; and **(F)** inflammatory process.

(G) Overview of RNA processing analysis strategy. BLAST was filtered to return results within 90% of query length to return true ribosomal RNA regions.

(H) qqPlots reveal enrichment of rRNA regions in bSCs (Rho+) versus their non-senescent (Rho-) counterparts for all regions analysed (18S, 5' ETS, ITS1 and ITS2).

(I) Quantification of total reads mapping to indicated rRNA regions comparing senescent (Rho+) and non-senescent (Rho-) populations, stratified by regeneration stages.

(J) Quantitative PCR analysis of unprocessed and processed rRNA ratios, using primers specifically targeting 5' ETS and 18S rRNA regions respectively, against cDNA derived from senescent (Rho+) and non-senescent (Rho-) cells.

(K) Overexpression of Rps14 induces strong reductions in cellular proliferation. *Left:* AL1 cells lipofected with either pN2-CMV:RFP control plasmids (top row) or pN2-CMV:AmRps14-RFP plasmids (bottom row) at 6 days post-lipofection. Blue: Hoechst; red: RFP; green: EdU. Scale bar = 100 μm for main. *Right:* Quantification of cell-cycle induction between control and AmRps14-overexpressing AL1 cells. Data represented as percentage of EdU-positive of total transfected cells, as identified by RFP signal. Each data point corresponds to one individual transfection. *P* values determined by unpaired two-tailed t-test. Error bars depict mean ± SEM.

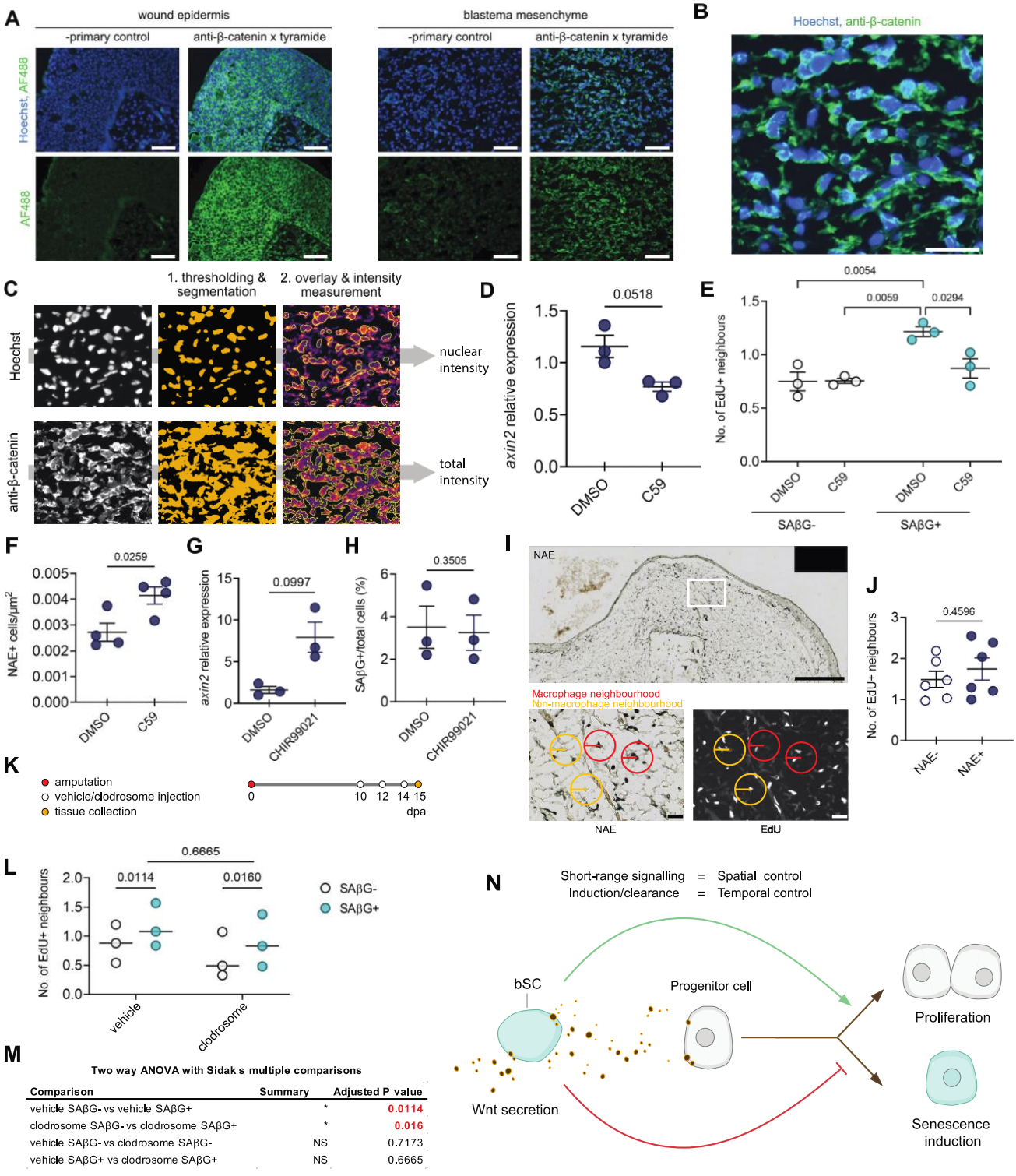
(L) Overexpression of Rps14 enforces induction of cellular senescence. *Left:* Representative bright-field images of AL1 cells lipofected with either pN2-CMV:RFP control plasmids or pN2-CMV:Rps14 plasmids at 17 days post-lipofection, stained with SAβG. Scale bar = 40 μm. *Right:* Quantification of senescence induction between control and AmRps14-overexpressing AL1 cells. Data represented as percentage of SAβG-positive cells of total cells. Each data point corresponds to one individual transfection. *P* values determined by unpaired two-tailed t-test. Error bars depict mean ± SEM.

(M) Ribosomal biogenesis expression during axolotl limb regeneration. Violin plot representation of ribosomal protein score (Table S3) across mature or limb blastema connective tissue single cells[35] at the indicated days post amputation (dpa). Mpa=months post amputation.

(N) UMAP of single sorted bSCs showing overlaid rRNA score expression (left) or cluster annotations.

(O) Marker gene expression of individual clusters.

(P-Q) Integrated scRNA-seq datasets used to generate plots in Fig. 3, L-M. **(P)** Integration of senescent connective tissue (subsetted from bSCs) with total connective tissue[35]. **(Q)** Integration of senescent macrophages (subsetted from bSCs) with total (*mpeg:mCherry*-sorted) macrophages.



Comparison	Summary	Adjusted P value
vehicle SAβG- vs vehicle SAβG+	*	0.0114
clodrosome SAβG- vs clodrosome SAβG+	*	0.016
vehicle SAβG- vs clodrosome SAβG-	NS	0.7173
vehicle SAβG+ vs clodrosome SAβG+	NS	0.6665

Supplementary Fig. 7 (Related to Figure 4). Blastemal senescent cell-derived effects are mediated by Wnt signalling.

(A-C) Analysis of β-catenin nuclear translocation in the axolotl blastema. **(A)** Validation of anti-β-catenin antibody in axolotl tissues. Scale bars = 100 μm. Blue: Hoechst-stained nuclei; green: anti-β-catenin antibody. **(B)** Example input image for quantification used in **(C)**. Scale bar = 50 μm. Blue: Hoechst-stained nuclei; green: anti-β-catenin antibody. **(C)** Left panels: from Fig. S9B, images separated by channels as indicated. Middle panels: result of thresholding and segmentation. Right panels: segmentation results overlaid onto original β-catenin images; β-catenin signal is measured within segmentation-defined boundaries (shown as yellow outline).

(D) Validation of C59 efficacy *in vivo*. mRNA levels of *axin2* in DMSO or C59-treated blastemas measured via qRT-PCR. *axin2* expression values calculated using the cycle threshold method (ΔΔCT) method normalised against large ribosomal protein 4 (*Rpl4*). Data points correspond to biological replicates. *P* values determined by unpaired two-tailed t-test. Error bars depict mean ± SEM.

(E) Quantification of neighbouring cell proliferation reveals bSC effects are dependent on Wnt signalling. Blastema sections from DMSO or C59-treated animals were co-stained for SAβG and EdU. The average numbers of EdU^+ cells surrounding senescent versus non-senescent cells within a 50 μm radius were quantified per section. Data points correspond to biological replicates. *P* values determined by paired two-tailed t-test. Error bars depict mean ± SEM (n=3).

(F) Quantification of macrophage recruitment (detected through α-napthtyl esterase [NAE] enzymatic activity) to the blastema mesenchyme following Wnt inhibition using C59. Data is represented as the number of macrophages per um^2 of blastema tissue. Data points correspond to biological replicates. *P* values determined by unpaired two-tailed t-test. Error bars depict mean ± SEM (n=4).

(G) Validation of CHIR99021 efficacy *in vivo*. mRNA levels of *axin2* in DMSO or CHIR99021-treated blastemas derived from contralateral limbs measured via qRT-PCR. *axin2* expression values calculated using the cycle threshold method (ΔΔCT) method normalised against large ribosomal protein 4 (*Rpl4*). Data points correspond to biological replicates. *P* values determined by paired two-tailed t-test. Error bars depict mean ± SEM.

(H) Quantification of percentage of SAβG+/total cells within blastema sections in DMSO or CHIR99021-treated blastemas derived from contralateral limbs. *P* values determined by paired two-tailed t-test. Error bars depict mean ± SEM (n=5).

(I-J) Macrophages do not exhibit a neighbouring-cell effect. **(I)** Representative sections of 15 dpa blastemas co-stained for EdU and macrophages detected by NAE enzymatic activity. Top panel: bright-field image of overview of blastema. Scale bar = 500 μm. Bottom panels: close ups of region demarcated by white box; bottom left – bright-field; bottom right – EdU; scale bar = 50 μm. **(J)** Quantification of neighbouring cell proliferation reveals macrophages do not exhibit a neighbouring cell effect. 15 dpa blastema sections were co-stained for NAE and EdU. The average numbers of EdU^+ cells surrounding macrophages versus non-macrophage cells were quantified within a 50 μm radius. Data points correspond to biological replicates. *P* values determined by paired two-tailed t-test. Error bars depict mean ± SEM (n=6 biological replicates).

(K-L) Senescent-cell associated neighbouring cell effect persists after macrophage depletion. **(K)** Experimental timeline for macrophage-depletion using clodrosome treatments. **(L)** Quantification of senescent cell-neighbouring cell proliferation. Blastema sections from vehicle or clodrosome-treated animals were co-stained for SAβG and EdU. The average numbers of EdU^+ cells surrounding senescent versus non-senescent cells within a 50 μm radius were quantified per section. Data points correspond to biological replicates. *P* values determined by two-way ANOVA with Sidak's multiple comparisons. Error bars depict mean ± SEM (n=3). **(M)** Table of results of two-way ANOVA with Sidak's multiple comparisons, corresponding to data in **(L)**.

(N) A model for the role and regulation of cellular senescence in axolotl limb regeneration. Senescent-derived Wnt promotes the proliferation of progenitor cells in a paracrine manner, whilst simultaneously preventing their induction into the senescence state.

Table S1. DGE analysis of proliferating and senescent AL1 cells (separate Excel file), related to Figure 3.
Results of DGE analysis using DESeq2. Genes are listed in rows. The first 3 columns list gene name, log2-fold change between senescent versus non-senescent cells, and adjusted p values. The following columns provide EdgeR normalised counts for proliferating and senescent AL1 cells.

Table S2. DGE analysis of non-senescent and senescent blastema cells at early, mid, late and palette stages (separate Excel file), related to Figure 3.
Results of DGE analysis using DESeq2. Genes are listed in rows. The first 3 columns list gene name, log2-fold change between senescent versus non-senescent cells, and adjusted p values. The following columns provide EdgeR normalised counts for proliferating and senescent blastema cells.

Table S3. Intersection of commonly upregulated genes between *in vitro* and *in vivo* senescent cells and GSEA gene list (separate Excel file), related to Figure 3.
Sheet 1 shows significantly upregulated genes in senescent AL1 cells, endogenous bSCs identified through DESeq2 with an FDR cut-off of 0.1 and a minimum fold change of 1.5, and results from ribosomal BLAST query. Sheet 2 lists commonly upregulated genes shared between senescent AL1s and bSCs, highlighting ribosomal transcripts. Related to Figure 3E. Sheet 3 onwards: lists the genes used for GSEA on senescent and non-senescent cells, and their sources.

Table S4. Marker gene lists used for cluster annotation of bSC scRNA-seq (separate Excel file), related to Figure 3.
Sheet 1 provides selected marker genes used for annotation of bSC scRNA-seq clusters. Clusters with expression of 45S rRNA were designated as deep senescent cells. Sheet 2 provides full marker gene lists of all clusters. Cluster correspond to those shown in Fig. 3, J-K and Fig. S8A.