

**Physiological role of senescent cells
during development in *Drosophila*
*melanogaster***

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Abstract

Cellular senescence, a state of irreversible cell cycle arrest, has long been associated with aging and tumor suppression. However, recent studies have highlighted its important role in vertebrate development. While programmed cell senescence—primarily functioning in tissue remodeling during embryogenesis—has been studied in vertebrate models, its existence and functional relevance in invertebrates remain largely unexplored. This study investigates programmed cell senescence in *Drosophila melanogaster* and demonstrates that this process is not only conserved across species—from invertebrates to vertebrates—but also essential for the proper development of sensory organs.

In this study, I identify and characterize programmed cell senescence in *Drosophila melanogaster*, establishing *Drosophila* as a new model to investigate conserved aspects of developmental senescence. Using SA- β -gal assays and fluorescent senescence markers, I found that two distinct clusters of cells exhibit classical hallmarks of senescence, including elevated SA- β -gal activity, cell cycle arrest (primarily at the G2 phase), upregulation of the cyclin-dependent kinase inhibitor Dacapo, heterochromatin formation marked by H3K9me3, oxidative stress, and cellular hypertrophy. Notably, these cells also express Unpaired3, a cytokine homologous to mammalian IL-6.

Mechanistic dissection revealed that programmed senescence in the wing disc is regulated by the EGFR-Ras signaling pathway, through its downstream effector Pointed (Pnt), a transcription factor regulated by Ras-MAPK activity.

I further demonstrate that the achaete-scute complex (AS-C), which is required for the formation of proneural clusters, could suppress sensory organ development without affecting senescence markers. It indicates that senescence promotes, but is not driven by, proneural gene expression.

Furthermore, the transcription factor Zfh2, a homolog of mammalian ATBF1, was found to be essential for pnt-regulated senescence and proper campaniform sensilla formation.

My work establishes that developmentally programmed cellular senescence is not only present in invertebrates but also essential for the formation of campaniform sensilla. The Ras-Zfh2-Pnt signal mediates this process, ensuring the proper number of sensory organs.

Abbreviations

A	anterior
<i>Ac</i>	<i>Achaete</i>
AS-C	achaete-scute complex
AER	Apical ectodermal ridge
A/P	anterior/posterior
APF	After puparium formation
CAFs	cancer-associated fibroblasts
CDK	cyclin-dependent kinase
EL3	early L3
D	dorsal
<i>Dap</i>	<i>Dacapo</i>
DDR	DNA damage response
DH	distal hinge
DSCs	dorsal senescent cells
IFN- γ	interferon- γ
LL3	Late L3
ML3	Middle L3
<i>mmp1</i>	<i>matrix metalloproteinase 1</i>
OIS	oncogene-induced cellular senescence
P	posterior
PDGF-AA	platelet-derived growth factor AA
pERK	Phosphor-p44/42 MAPK (Erk1/2)
PH	proximal hinge
pH3	phosphohistone H3
<i>Pnt</i>	<i>Pointed</i>
PP3	Pre-pupal phase 3
<i>rl</i>	<i>rolled</i>
ROS	reactive oxygen species
RNS	reactive nitrogen species
SA- β -gal	senescence-associated β -galactosidase
SAHF	senescence-associated heterochromatin foci
SASP	Senescence-Associated Secretory Phenotype
<i>Sca</i>	<i>Scabrous</i>
Tri-me H3K9	trimethylation of histone H3-K9
<i>upd</i>	<i>unpaired</i>
<i>upd3</i>	<i>unpaired3</i>
V	ventral
VSCs	ventral senescent cells
<i>zfh2</i>	<i>Zn finger homeodomain 2</i>

Chapter 1 Introduction

1-1 Cellular senescence

Cellular senescence was first observed by Hayflick and Moorhead who found that normal human fibroblast cells can only divide finite cell cycle, around 40 to 60 divisions, before they enter a state of permanent growth arrest when they were cultured in vitro (Hayflick and Moorhead, 1961). The number of times which normal somatic cells can divide is called Hayflick limit and the state of irreversible cell cycle arrest in aged cells is named senescence (Shay, J., Wright, 2000).

Senescence can be induced by various factors, including DNA damage, oxidative stress, and oncogenes activation. (Brandl et al., 2011; Kuilman et al., 2010; Schumacher et al., 2021; Serrano et al., 1997). DNA damage triggered by external factors, like ionizing radiation, or internal changes, such as telomeric shortening, will lead to activation of DNA damage response (DDR) to arrest the cell cycle progression (Shreeya et al., 2023). Oxidative stress, resulting from accumulation of reactive oxygen species (ROS) and reactive nitrogen species (RNS), also contribute to senescence, which in turn activates cell cycle arrested mechanism (Faraonio, 2022). Additionally, Oncogene-induced cellular senescence (OIS), which occurred through genetic mutations or overexpression, can control malignant cell proliferation (Liu et al., 2018). In general, the reason why cells undergo senescence is that cellular senescence serves primarily as a protective mechanism against damage and uncontrolled growth (Collado et al., 2007).

Additionally, an important feature of senescence is that senescent cells can adopt a phenotype characterized by the high expression of secretory proteins, including inflammatory cytokines, chemokines, growth factors, and metastasis-promoting factors, known as the Senescence-Associated Secretory Phenotype (SASP) (Ohtani, 2022). It plays beneficial roles in tissue repair by secretion of SASP factors, such as platelet-derived growth factor AA (PDGF-AA), IL-6, Gro- α , CXCL9, mediating immune cell recruitment to clear excess extracellular matrix production, thereby remove damaged tissues, prevent fibrosis and promote the proliferation (Demaria et al.,

2014; Yoshimoto et al., 2013). Moreover, during early stage of cancer, or called as precancerous cells, SASP factors secretion are the main suppressor against tumorigenesis as the SASP factors (interferon- γ (IFN- γ), IL-2, *et al*) recruited immune cells to clear those precancerous cells (Kang et al., 2011; Xue et al., 2007). However, SASP also has detrimental roles in accelerating tumorigenesis and aging. In advanced stage of tumor, which tumor is fully developed, senescent cancer-associated fibroblasts (CAFs) secrete SASP factor and promote tumorigenesis (Krtolica et al., 2001).

Besides SASP, cellular senescence is also characterized by several other distinct features (Kuilman et al., 2010). One hallmark of senescence is the elevation of senescence-associated β -galactosidase (SA- β -gal) activity, which is detectable under acidic conditions, pH6 (Debacq-Chainiaux et al., 2009). The increased activity of SA- β -gal is thought to reflect lysosomal expansion, which causes the elevation of lysosomal activity of β -gal (Varela-Eirín and Demaria, 2022). This process is accompanied by the upregulation of cyclin-dependent kinase (CDK) inhibitors, such as *p16/p19/p21*, which block cell cycle progression (Capparelli et al., 2012). Furthermore, senescent cells often accumulate senescence-associated heterochromatin foci (SAHF), silencing genes, like E2F targets, which is essential for proliferation, and display persistent DDR signals, marked by γ H2AX and 53BP1 foci (Meier et al., 2007; Narita et al., 2003). Morphologically, these cells are enlarged and flattened due to nuclear protein accumulation and microtubule and actin filaments remodeling (De Cecco et al., 2011; Davies et al., 2022; Moujabber et al., 2019). Together, these features make cellular senescence play a significant role in aging, tissue repair, and various age-related diseases.

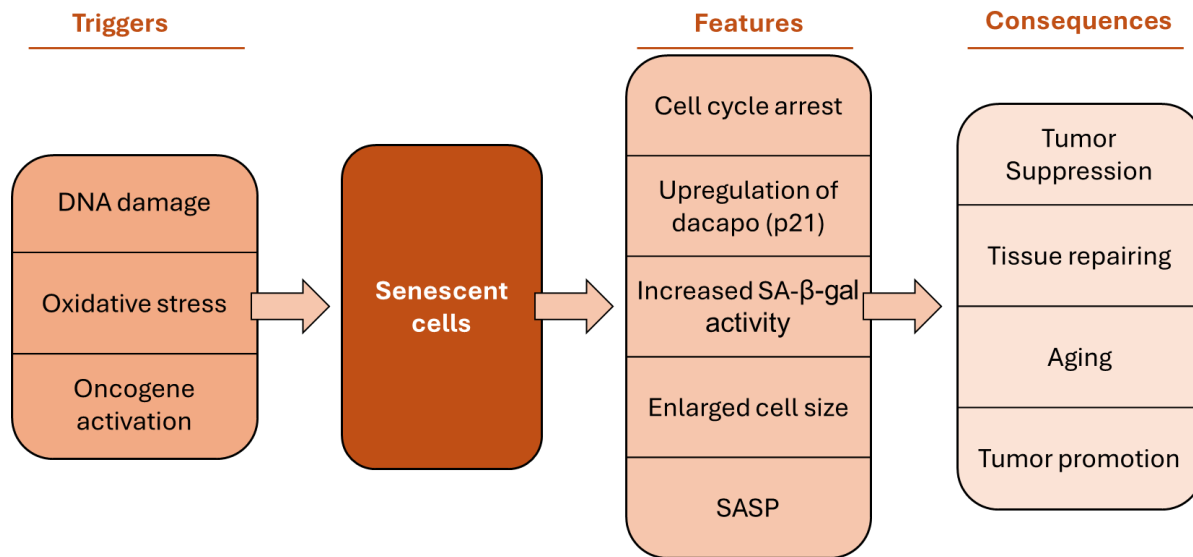


Figure 1-1 Schematic representation of cellular senescence.

Cellular senescence is a biological process in which cells lose their ability to divide and grow, acting as a response to stress or damage. Senescence can be triggered by various factors, including DNA damage, oxidative stress, and oncogene activation, all of which lead to the formation of senescent cells. These cells exhibit several key features, such as cell cycle arrest, upregulation of *dacapo* (*p21*), increased SA- β -gal activity, enlarged cell size, and senescence-associated secretory phenotype (SASP). The consequences of cellular senescence can be both beneficial and detrimental. On the one hand, it plays a crucial role in tumor suppression and tissue repair; on the other hand, it contributes to aging and tumorigenesis.

1-2 Programmed cell senescence

Although previous studies of cellular senescence are restricted to pathological environments, such as DNA damage, stress, tumorigenesis or aging, a surprising finding of senescence is that it also has beneficial roles during development. Senescence-like cells with increased SA- β -gal activity have first been observed in quail embryos (Nacher et al., 2006). Programmed cell senescence was first found in mouse and chick embryos (Muñoz-Espín et al., 2013; Storer et al., 2013). SA- β -gal activity has been observed in various tissues, including the hindlimb, tail, otic vesicle, hindbrain, and neural tube of mouse embryos, as well as the pharyngeal arches, neural tube, and eye of chick embryos. SA- β -gal activated structures like the mesonephros (a transient embryonic kidney), endolymphatic sac (which regulates inner ear pressure), apical ectodermal ridge (AER, a key limb bud structure), and neural tube (which develops into the central nervous

system) was confirmed through multiple markers, including lack of proliferation (evidenced by negative Ki67 staining and absence of BrdU incorporation), increased heterochromatin markers (H3K9me3 and HP1 γ /CBX3), and elevated expression of cell cycle inhibitors such as *p15*, *p21*, and *p27*. This highlights a developmental role for senescence beyond traditional associations with aging or stress. Furthermore, in amphibians, cellular senescence is mediated by TGF β signaling but is independent of ERK/MAPK activation, highlighting distinct regulatory pathways (Davaapil et al., 2017). The elimination of senescent cells through TGF β inhibition results in developmental defects, emphasizing the necessity of cellular senescence for proper tissue formation in amphibians. In addition, programmed cell senescence has been identified in the hair follicles of newborn naked mole rats, where it is proposed to play a crucial role in their unique hairless phenotype (Zhao et al., 2018). These observations collectively suggest that developmentally programmed cell senescence is a conserved mechanism across diverse vertebrate species.

However, in contrast to damage-induced senescence, which is characterized by persistent DNA damage and is often associated with pathological conditions, developmental senescence occurs in the absence of detectable DNA damage (Muñoz-Espín and Serrano, 2014). Instead, it serves essential physiological roles, such as guiding tissue remodeling and morphogenesis during normal development. This process ensures proper organ formation and structural refinement by enabling the controlled removal of specific cell populations and coordinating interactions between senescent cells and their microenvironment. By functioning as a regulatory mechanism during development, developmental senescence highlights a fundamental distinction from the stress-induced senescence observed in response to cellular damage or aging.

1-3 Senescent cells in *Drosophila*

Drosophila serves as an invaluable genetic model for investigating the physiological roles and underlying mechanisms of various biological processes during normal development. A previous study by Nakamura et al. (2014) demonstrated that ectopic activation of the Ras oncogene,

coupled with mitochondrial defects, induces cellular senescence in the developing *Drosophila* imaginal epithelia (Figure1-2).

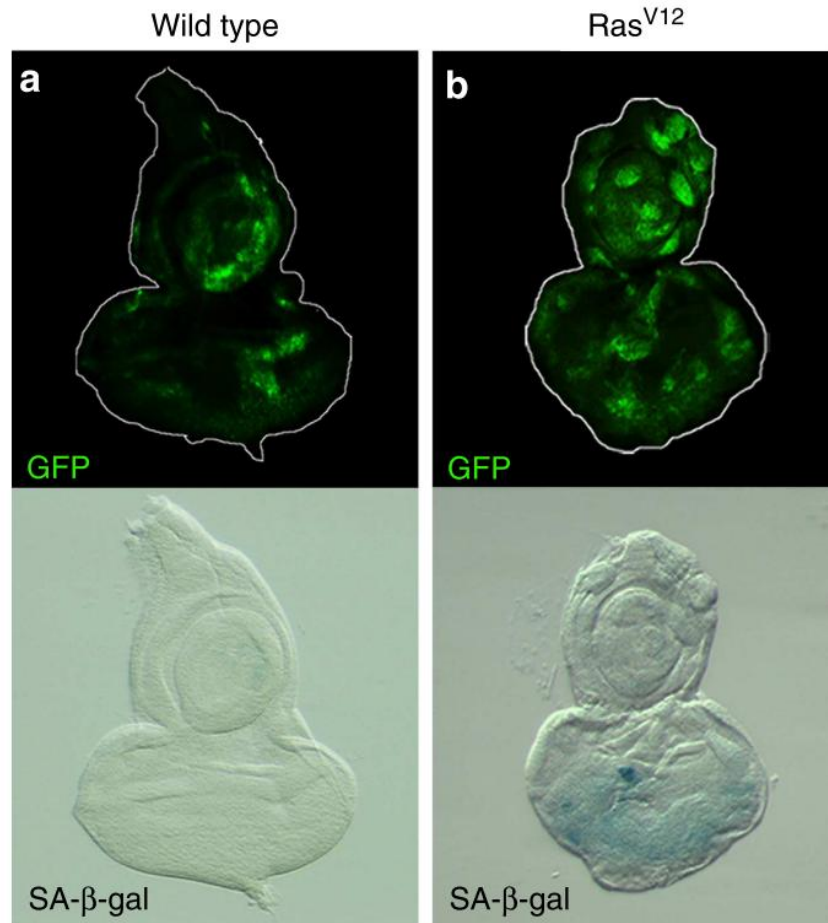


Figure 1-2 Ras activation induces cellular senescence markers in *Drosophila*.

(a, b) Eye-antennal discs containing green fluorescent protein (GFP)-labeled clones, either wild-type (a) or expressing *Ras*^{V12} (b), were analyzed using the SA-β-gal assay. (Adapted from Nakamura et al., 2014)

This senescence phenotype exhibited all the hallmark features of senescent cells, such as cell cycle arrest, upregulation of cell cycle inhibitors, altered chromatin structure, and increased SA-β-gal activity. These findings suggest that, under specific pathological conditions, *Drosophila* senescent cells exhibit a senescence program similar to that observed in vertebrates. This highlights the potential conservation of cellular senescence mechanisms across species,

particularly in response to oncogenic stress and mitochondrial dysfunction, providing further insights into the roles of senescence in development and disease across the animal kingdom.

To examine the presence of programmed cell senescence during *Drosophila* development, my collaborator Mr. Yoshimoto conducted SA- β -gal staining across multiple tissues in third instar larvae. An intense SA- β -gal signal at pH6.0 was reproducibly observed around the center of the wing imaginal discs (Figure 1-3A, yellow arrowheads). Notably, no β -gal activity was observed at pH7.5 (Figure 1-3A), indicating that the elevated SA- β -gal signal was not simply due to increased β -gal expression but specifically to senescence-associated β -gal activation. These results indicate that a group of cells displaying SA- β -gal activity, a marker of cellular senescence, are present in the wing discs of *Drosophila* during development.

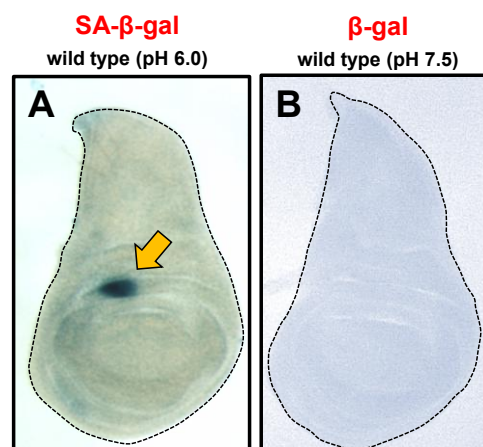


Figure 1-3. Presence of SA- β -gal activity in normal *Drosophila* wing discs

(A) SA- β -gal staining of the wing disc from a third instar larva of wild-type *Drosophila*. Cells exhibiting SA- β -gal activity, indicating β -galactosidase activity under pH 6.0 conditions, are observed (yellow arrow).

(B) X-gal staining of the wing disc from a third instar larva of wild-type *Drosophila*. No β -galactosidase activity is detected under pH 7.5 conditions. (Adapted from Yoshimoto's master thesis, 2016)

1-4 The aims of the programmed cell senescence in *Drosophila*

Those findings prompt us to further investigate whether programmed cell senescence also occurs in *Drosophila* during normal development. Given the potential roles of senescence in tissue

remodeling and organogenesis across various species, it is important to consider whether this process is conserved in *Drosophila*, a model organism widely used in developmental biology. Exploring whether *Drosophila* undergoes programmed senescence during its developmental stages could provide valuable insights into the evolutionary conservation of senescence mechanisms and their functional significance in different species.

Chapter 2 Materials and Methods

2-1 *Drosophila* strain and culture condition

The following fly stocks were used in this thesis: *w¹¹¹⁸*, *Ubi-GFP-E2F11-230 Ubi-mRFP-NLS-CyclinB1-260* (III) (BL 55099), *gstD-GFP* (X) (a gift from D. Bohmann), *upd3-Gal4* (II) (a gift from H. Agaisse), *ci-Gal4* (II), *nub-Gal4* (II), *ap-Gal4* (II), *UAS-Ras^{N17}* (X) (Kyoto Stock Center), *UAS-rl-RNAi^{TRiP.HMS00173}* (III), *UAS-Ras^{V12}* (II), *UAS-zfh2-RNAi^{TRiP.HMC0304}* 3 (III), *PntP1-lacZ^{HZ20}* (a gift from B. Shilo), *UAS-pnt-RNAi^{VDRC.v105390}* (II), *UAS-ac-RNAi^{TRiP.HMS02646}* (II), *UAS-sc-RNAi^{TRiP.JF02104}* (III), *sca-Gal4* (II) (BL6479), *pnt-Gal4^{NP112814}* (III).

Flies were maintained at 25°C with a standard cornmeal-yeast food, unless otherwise stated.

All experiment used larvae or adults of both sex except for *Ras^{N17}* experiment (only females), as *UAS-Ras^{N17}* transgene is located on the X chromosome.

Larvae were collected at wandering third instar larval stage except for age-related assays.

2-2 SA-β-gal staining

Senescent Cells Histochemical Staining Kit (CS0030, Sigma-Aldrich) was used to detect SA-β-gal activity. Larvae were dissected in PBS and then fixed with PFA. After washing with PBS 3 times, larvae were incubated in the Staining Mixture for 7 days at 37°C. Images were taken with a stereomicroscope.

2-3 Ex vivo culturing of wing disc

L3 larvae were dissected and cultured in Shields and Sang M3 insect medium (Sigma) with 2% FBS, 0.5% penicillin-streptomycin and 0.1 μg/mL Ecdysone (dissolved with 10% isopropanol first) for 24h in room temperature (Aldaz et al., 2010).

2-4 SPiDER-βgal staining

Cellular Senescence Detection Kit SPiDER-βGal (SG-03, Dojindo Molecular Technologies) was used to analyze SA-β-gal activity. After dissecting in PBS, larvae or pupae were fixed with PFA and washed with PBS, then incubated in SPiDER-βgal working solution for 1h at 37°C. Images were taken with a Leica SP8 confocal microscope (Leica).

2-5 Histology

Larval tissues were stained with standard immunohistochemical procedures using chick anti-β-galactosidase polyclonal antibody (Abcam, 1:500), rabbit anti-Histone H3 (trimethyl K9) polyclonal antibody (Abcam, 1:50), mouse anti-Dacapo (Developmental Studies Hybridoma Bank, 1:6, for 7 days), rabbit anti-upd antibody (a gift from D. Harrison, 1:500), mouse anti-mmp1 (DSHB, 1:100 from 1:1:10 cocktail of 3A6B4, 3B8D12 and 5H7B11), mouse anti-achaete antibody (Developmental Studies Hybridoma Bank, 1:500), mouse anti-phospho histone H3 antibody (Cell Signaling, 1:50), Capicua polyclonal antibody (a gift from Iswar Hariharan, 1:300), Rabbit anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (Cell Signaling, 1:50), rat anti-Pnt antibody (1:10) or Alexa546-conjugated phalloidin (Molecular Probes, 1:50) and were mounted with DAPI-containing SlowFade Gold Antifade Reagent (Molecular Probes). For EdU labeling, larvae were dissected in Schneider's medium and incubated in 1x EdU solution (Click-iT® EdU Imaging Kits; Thermo Fisher Scientific C10340) for 10 min. After fixation and washing, tissues were incubated in EdU cocktail for 30 min and were mounted as described above. Images were taken with a Leica SP8 confocal microscope (Leica).

2-6 Wing image

Adult flies were incubated in a glycerol/ethanol mixture (1:1) for 1 day. Then wings were dissected

from adult flies then mounted with the glycerol/ethanol mixture and imaged with microscope.

2-7 Quantification and Statistical Analysis

Percentage of antibody staining/fluorescence marker-positive cells in SA- β -gal-elevated clusters was calculated in each disc by dividing the marker-positive area by SA- β -gal-elevated area.

The intensity of Tri-methylated H3K9 was quantified within individual nuclei, identified by DAPI staining, and normalized to the corresponding DAPI intensity.

Cell sizes were measured by ImageJ based on the outline of phalloidin staining.

The normalization of SA- β -gal assay intensities was conducted by dividing the intensities of senescence markers by the intensity of background.

The number of campaniform sensilla was counted and was compared with the mean number of campaniform sensilla on wild-type wings.

Percentage of antibody staining/fluorescence marker-positive cells in senescent clusters was calculated in each disc by dividing the marker-positive area by FUCCI GFP and RFP positive area.

Data were analyzed using Welch's t-test for single comparisons. Bar graphs represent the mean $\pm$ SE. All n values correspond to biological replicates. Statistical significance is indicated as follows: ***P < 0.001, **P < 0.01, *P < 0.05, and n.s. (not significant) for P > 0.05.

2-8 Detailed genotypes in Figures

Detailed genotypes in Figure 3-1

w1118

Detailed genotypes in Figure 3-2

(A-D) *w1118*

Detailed genotypes in Figure 3-3

(A, B) *Ubi-GFP-E2F1*, *Ubi-mRFP-NLS-CycB* (III)

Detailed genotypes in Figure 3-4

(A-D) *Ubi-GFP-E2F1*, *Ubi-mRFP-NLS-CycB* (III)

Detailed genotypes in Figure 3-5

(A, B) *Ubi-GFP-E2F1*, *Ubi-mRFP-NLS-CycB* (III)

Detailed genotypes in Figure 3-6

(A-B) *w1118*

Detailed genotypes in Figure 3-7

(A-C) *w1118*

Detailed genotypes in Figure 3-8

(A) *gstD-GFP* (X)

Detailed genotypes in Figure 3-9

(A-B) *w1118*

Detailed genotypes in Figure 3-10

(A-B) *w¹¹¹⁸*

Detailed genotypes in Figure 3-11

(A) *gstD-GFP (X)*

Detailed genotypes in Figure 3-12

(A-B) *ci-Gal4 UAS-GFP / +;*

Ras^{N17} / Y; ci-Gal4, UAS-GFP / +;

Detailed genotypes in Figure 3-13

(A-B) *Ras^{N17} / Y; ap-Gal4, UAS-GFP / +; Sb or + / TM6B*

Detailed genotypes in Figure 3-14

(A-C) *w¹¹¹⁸*

Detailed genotypes in Figure 3-15

(A) *upd3-Gal4, UAS-GFP(II)*

Detailed genotypes in Figure 3-16

(A-F) *w¹¹¹⁸*

Detailed genotypes in Figure 3-17

(A-F) *w¹¹¹⁸*

Detailed genotypes in Figure 3-18

(A) *ci-Gal4, UAS-GFP / CyO; Sb / TM6B*

(B) *nub-Gal4 UAS-GFP / CyO; Sb / TM6B*

Detailed genotypes in Figure 3-19

(A) *Ubi-GFP-E2F1, Ubi-mRFP-NLS-CycB (III)*

Detailed genotypes in Figure 3-20

(A) *w¹¹¹⁸*

Detailed genotypes in Figure 3-21

(A) *nub-Gal4 / CyO; Ubi-GFP-E2F1, Ubi-mRFP-NLS-CycB / TM6B*

(B) *sca-Gal4 UAS-GFP (II)*

Detailed genotypes in Figure 3-22

(A) *ci-Gal4 UAS-GFP / +;*

(B) *Ras^{N17} / Y; ci-Gal4, UAS-GFP / +;*

Detailed genotypes in Figure 3-23

(A) *ci-Gal4 UAS-GFP / +; PntP1-lacZ^{HZ20} / +*

(B) *Ras^{N17} / Y; ci-Gal4, UAS-GFP / +; PntP1-lacZ^{HZ20} / TM6B*

(C) *ci-Gal4 UAS-GFP / +; PntP1-lacZ^{HZ20} / UAS-rl-RNAi^{TRiP.HMS00173}*

Detailed genotypes in Figure 3-24

(A) *ci-Gal4 UAS-GFP / +*

ci-Gal4 UAS-GFP / pnt-RNAi^{VDRC. v105390}

Detailed genotypes in Figure 3-25

(A) *ap-Gal4, UAS-GFP / pnt-RNAi^{VDRC. v105390}*

Detailed genotypes in Figure 3-26

(A) *Ras^{N17} / Y; ci-Gal4 / +; Ubi-GFP-E2F1, Ubi-mRFP-NLS-CycB/TM6B*

(B) *ci-Gal4 / pnt-RNAi^{VDRC.v105390}; Ubi-GFP-E2F1, Ubi-mRFP-NLS-CycB / +*

(E) *Ras^{N17} / Y; ci-Gal4 / +; Ubi-GFP-E2F1, Ubi-mRFP-NLS-CycB/TM6B*

(F) *ci-Gal4 / pnt-RNAi^{VDRC.v105390}; Ubi-GFP-E2F1, Ubi-mRFP-NLS-CycB / +*

Detailed genotypes in Figure 3-27

(A, D) *ci-Gal4, UAS-GFP / +; UAS-string / TM6B*

Detailed genotypes in Figure 3-28

(A) *ci-Gal4 UAS-GFP / pnt-RNAi^{VDRC. v105390}*

Detailed genotypes in Figure 3-29

(A) *ci-Gal4 UAS-GFP / +*

(B) *ci-Gal4 UAS-GFP / pnt-RNAi^{VDRC. v105390}*

Detailed genotypes in Figure 3-30

(A) *nub-Gal4 UAS-GFP / UAS-ac-RNAi^{TRiP.HMS02646}; Sb / UAS-sc-RNAi^{TRiP.JF02104}*

(B) *nub-Gal4 UAS-GFP / UAS-ac-RNAi^{TRiP.HMS02646}; Sb / UAS-sc-RNAi^{TRiP.JF02104}*

Detailed genotypes in Figure 3-31

(A, B) *w; UAS-GFP / CyO; PntP1-Gal4 / TM6B*

Detailed genotypes in Figure 3-32

(A) *nub-Gal4 UAS-GFP / +; Sb / +*

nub-Gal4 UAS-GFP / +; Sb / UAS-zfh2-RNAi^{TRiP.HMC03043}

Detailed genotypes in Figure 3-33

(A) *ap-Gal4, UAS-GFP / +; Sb / UAS-zfh2-RNAi^{TRiP.HMC03043}*

Detailed genotypes in Figure 3-34

(A) *nub-Gal4 UAS-GFP / UAS-Ras^{V12}; Sb / +*

nub-Gal4 UAS-GFP / UAS-Ras^{V12}; Sb / UAS-zfh2-RNAi^{TRiP.HMC03043}

Detailed genotypes in Figure 3-35

(A) *nub-Gal4 UAS-GFP / +; Sb / +*

nub-Gal4 UAS-GFP / +; Sb / UAS-zfh2-RNAi^{TRiP.HMC03043}

Detailed genotypes in Figure 3-36

(A) *nub-Gal4 UAS-GFP / +; Sb / UAS-zfh2-RNAi^{TRiP.HMC03043}*

Chapter 3 Results

3-1 In *Drosophila* wing disc, two clusters of cells are observed with intense SA- β -gal activity. To explore the existence of programmed cell senescence during *Drosophila* development, I conducted SA- β -gal staining in the 2nd and the 3rd instar larvae in *Drosophila*. This approach allowed us to assess SA- β -gal activity, a widely recognized marker for senescence, within the developmental context of various larval stages.

A cluster of cells with elevated SA- β -gal activity began to appear at the middle L3 (ML3) stage and the signal intensity increased as larvae grew into late L3 (LL3).

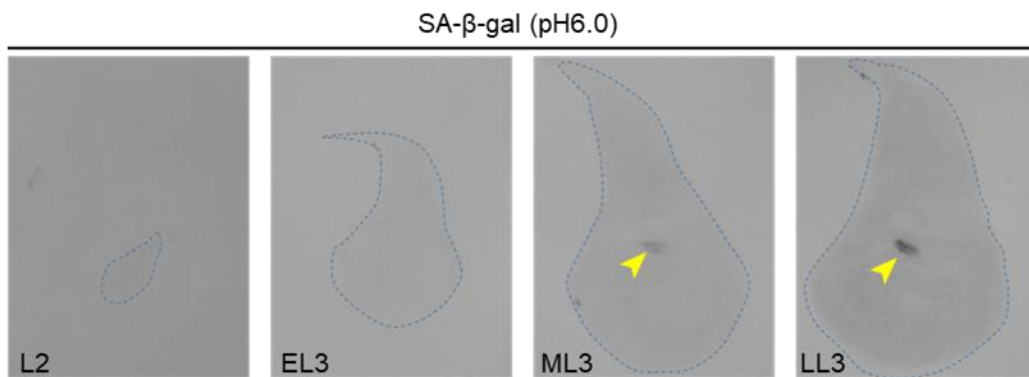


Figure 3-1. Wild-type wing discs at different stages subjected to SA- β -gal assay with X-gal staining. L2, EL3, ML3, LL3 stage larvae. Arrowheads indicate SA- β -gal-activated clusters. EL3, early L3; ML3, middle L3; LL3, late L3. (Adapted from Zang et al, 2025)

To further examine SA- β -gal activity in the wing discs during the pupal stage, I utilized the more sensitive fluorescent probe SPiDER- β Gal at pH 6.0. Although the morphology of the wing disc undergoes significant changes following puparium formation (APF), SA- β -gal activity remained detectable even at 5 hours APF (Figure 3-2 A-C, yellow arrowheads). At later pupal stages, wing discs recruit numerous hemocytes, which exhibit strong staining with SPiDER- β Gal and SA- β -gal, complicating the analysis. To circumvent this issue, I cultured L3 wing discs ex vivo in hemocyte-free media for 24 hours. This approach confirmed that a distinct cluster of cells with elevated SA- β -gal activity persisted in the wing disc during the pre-pupal phase 3 (PP3) (~9 hours APF) (Figure 3-2 D) (Aldaz et al., 2010; Classen et al., 2008)..

Additionally, I identified a smaller cluster of cells exhibiting elevated SA- β -gal activity at the ventral hinge of the wing disc (Figure 3-2 A and A', white arrowheads). Based on their consistent and reproducible presence, I designate these two cell populations as dorsal senescent cells (DSCs; yellow arrowheads) and ventral senescent cells (VSCs; white arrowheads), respectively.

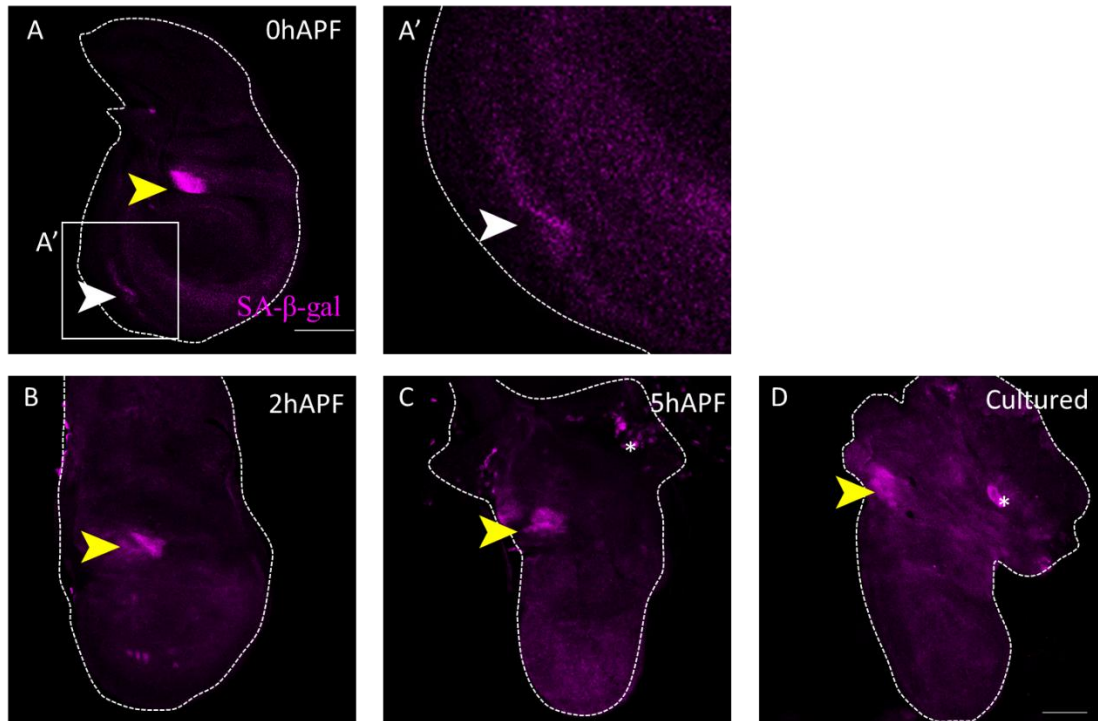


Figure 3-2. Wild-type wing discs were stained with SPiDER- β gal. (A-D) SA- β -gal activity of wild-type wing disc at 0h APF, 2h APF, 5h APF and cultured *ex vivo* for 24h, respectively. Yellow arrowheads and white arrowheads indicate dorsal SA- β -gal-activated clusters and ventral SA- β -gal-activated clusters, respectively. Asterisks represent hemocytes that attach to the wing disc. APF, after puparium formation. Scale bars, 100 μ m. (Adapted from Zang et al, 2025)

A key feature of cellular senescence is cell cycle arrest. To investigate this, I first analyzed the cell cycle phases of DSCs and VSCs using the cell cycle monitoring probe Fly-FUCCI (Zielke et al., 2014). My results showed that 85.8% of DSCs (yellow arrowheads) and 88.0% of VSCs (white arrowheads) were GFP-positive, indicating that these cells were in the G2/M/G1 phases of the cell cycle (Figure 3-3 A and B).

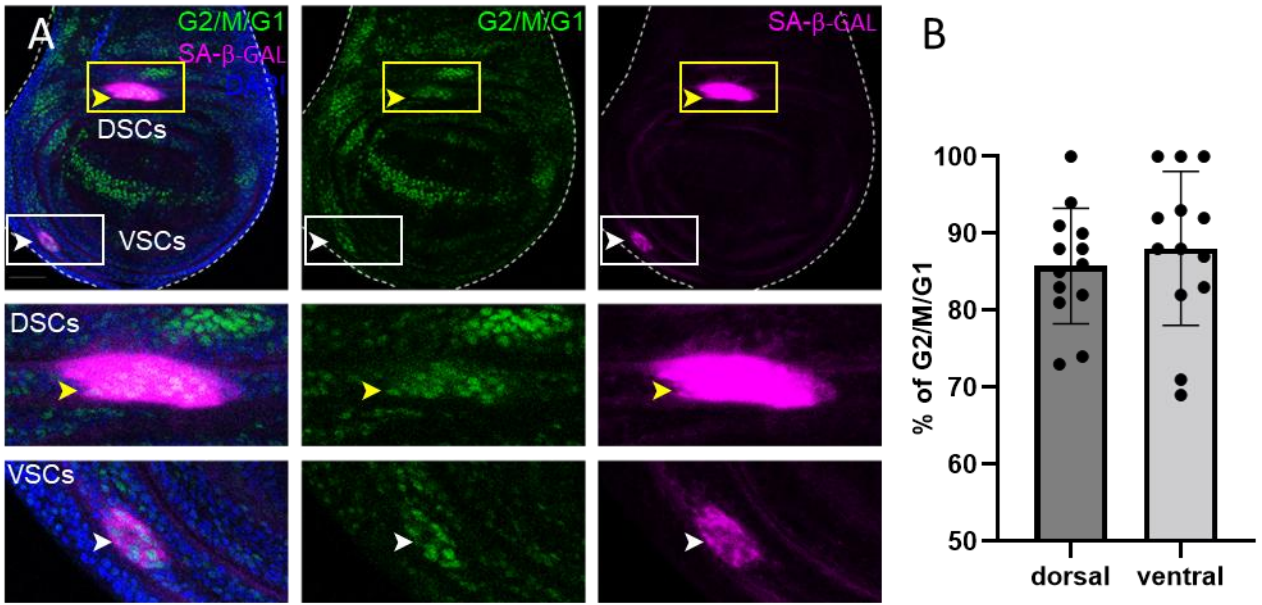


Figure 3-3. The percentage of G2/M/G1 positive cells in SA-β-gal-activated clusters. Wing discs expressing fly-FUCCI were stained with SPiDER-βgal (A) and percentage of G2/M/G1 cells (green) in SA-β-gal-activated clusters (magenta) was quantified in (B). Yellow arrowheads indicate dorsal senescent cells (DSCs), while white arrowheads indicate ventral senescent cells (VSCs). Scale bars, 50 μm. (Adapted from Zang et al, 2025)

Both DSCs and VSCs exhibited GFP (G2/M/G1) and RFP (S/G2/M) signals (Figure 3-4 A-B), suggesting that these cells were in either the G2 or M phase. Furthermore, the absence of EdU staining, a widely used marker for the S phase, in DSCs and VSCs confirmed that these cells were not undergoing DNA synthesis (Figure 3-4 C).

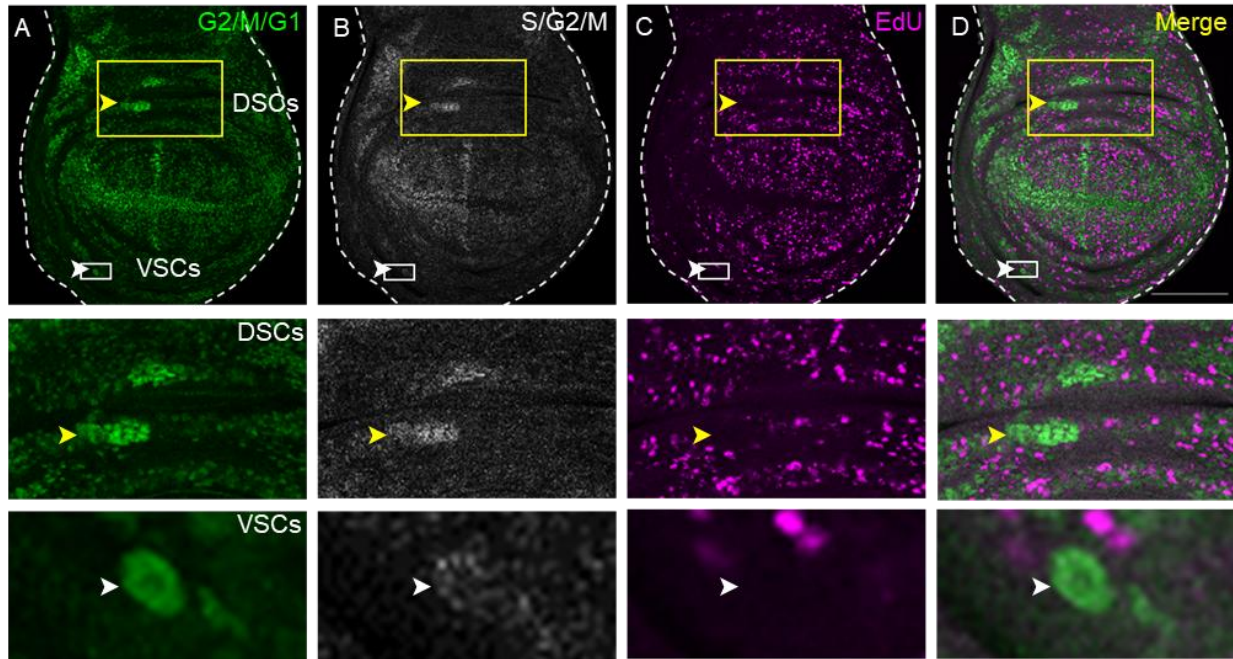


Figure 3-4. Proliferation is not detected in SA-β-gal-positive clusters. (A-D) Wing discs expressing fly-FUCCI were stained with EdU. GFP-E2F1 represents G2/M/G1 phase (panel A, green) and mRFP-NLS-CycB represents S/G2/M phase (panel B, white). The proliferated cells are marked with Edu (panel E, magenta). All signals are merged in panel D. Yellow arrowheads indicate dorsal senescent cells (DSCs), while white arrowheads indicate ventral senescent cells (VSCs). Scale bars, 100 μm. (Adapted from Zang et al, 2025)

Moreover, phosphohistone H3 (pH3) staining, which specifically marks cells in the M phase, did not label DSCs or VSCs, indicating that these cells are arrested in the G2 phase (Figure 3-5, yellow and white arrowheads). These findings suggest that the majority of SA-β-gal-positive cell clusters in the wing discs undergo cell cycle arrest at the G2 phase.

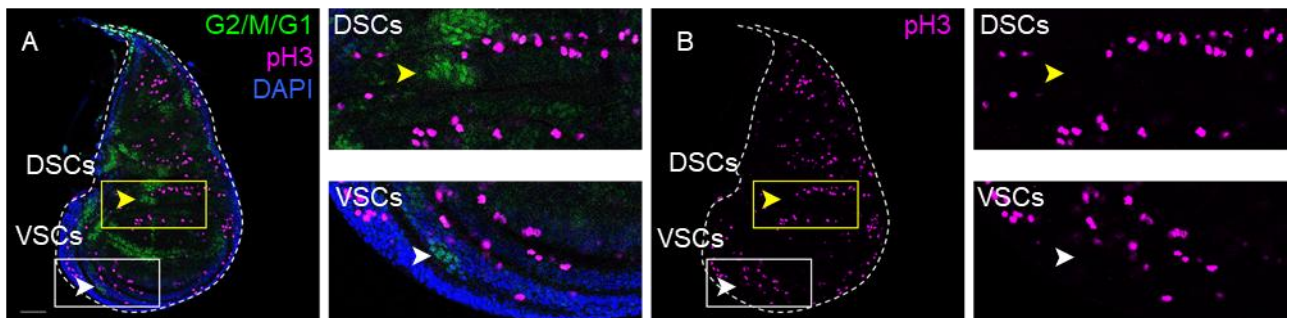


Figure 3-5. The SA-β-gal-activated clusters are not in M phase. (A-B) Wing discs expressing fly-FUCCI were stained with phosphohistone H3 (pH3) antibody. GFP-E2F1 represents G2/M/G1 phase (panel A, green) and pH3 represents M phase (panel B, magenta). Yellow

arrowheads indicate dorsal senescent cells (DSCs), while white arrowheads indicate ventral senescent cells (VSCs). Scale bars, 50 μ m. (Adapted from Zang et al, 2025)

3-2 The SA- β -gal-positive cells exhibit other senescence markers

To confirm whether DSCs and VSCs are indeed undergoing cellular senescence, I examined additional hallmarks of senescence. First of all, one key indicator is the upregulation of the cyclin-dependent kinase (CDK) inhibitor Dacapo (Dap), a homolog of p21, which plays a crucial role in cell cycle arrest during senescence. Immunostaining for Dap revealed its elevated expression in these cell clusters (Figure 3-6A, quantified in Figure 3-6B). This finding suggests that, in addition to G2 arrest, at least a subset of SA- β -gal-positive cell clusters also undergo G1 arrest, as Dacapo inhibits the Cyclin E/Cdk2 complex and thereby blocks the G1-to-S phase transition of the cell cycle.

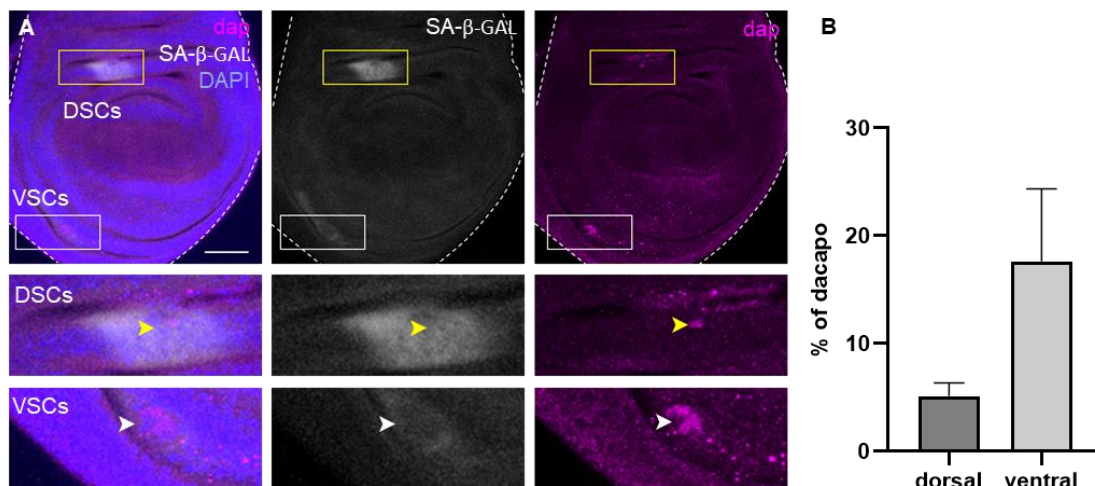


Figure 3-6. Percentage of dacapo positive cells in SA- β -gal clusters. (A, B) Wild-type wing discs were stained with SPiDER- β gal and anti-Dacapo (A). Percentage of positive cells (magenta) in SA- β -gal-activated clusters (white) were quantified in (B). Dorsal cells (n=8), ventral cells (n=8). Scale bars, 50 μ m. (Adapted from Zang et al, 2025)

Secondly, trimethylation of histone H3 at lysine 9 (H3K9me3), a hallmark of senescence-associated heterochromatin foci, was found to be elevated in both DSCs and VSCs (Figure 3-7A, quantified in Figure 3-7B and C).

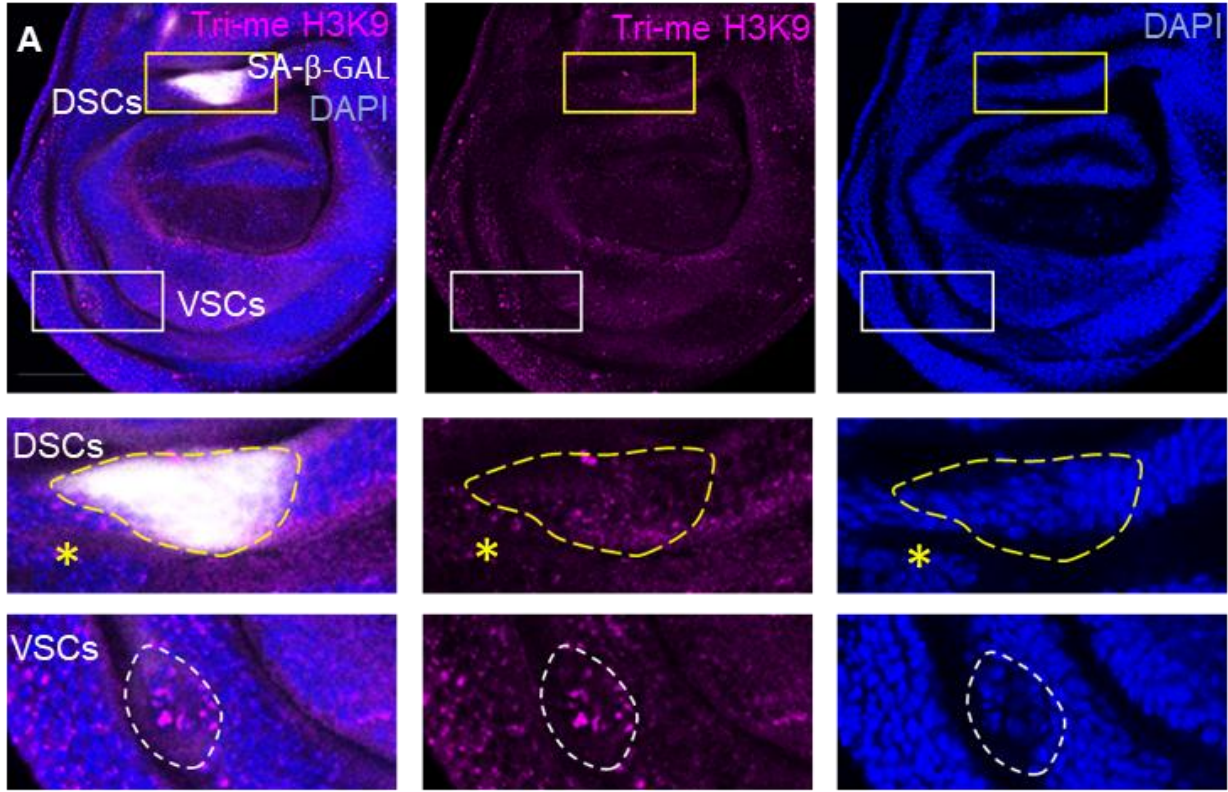


Figure 3-7. Intensity of Tri-me H3K9 positive cells in SA-β-gal clusters. (A-C) Wild-type wing discs were stained with SPiDER-βgal and anti-Histone H3 (trimethyl K9). Intensity of Tri-me H3K9 (magenta) was measured within each nucleus, marked by DAPI staining (blue), and normalized with corresponding DAPI intensity. Outlined areas indicate senescent cells and asterisks indicate neighboring normal cells. Data were collected as mean $\pm$ s.e., normal dorsal cells (DCs) (n=12); DSCs (n=12); normal ventral cells (VCs) (n=12); VSCs (n=12). Student's *t*-test, * $P < 0.05$, *** $P < 0.001$. Scale bars, 50 μ m. (Adapted from Zang et al, 2025)

Thirdly, oxidative stress, a common feature of senescent cells, was elevated in both DSCs and VSCs, as *gstD*-GFP are colocalized with (Figure 3-8).

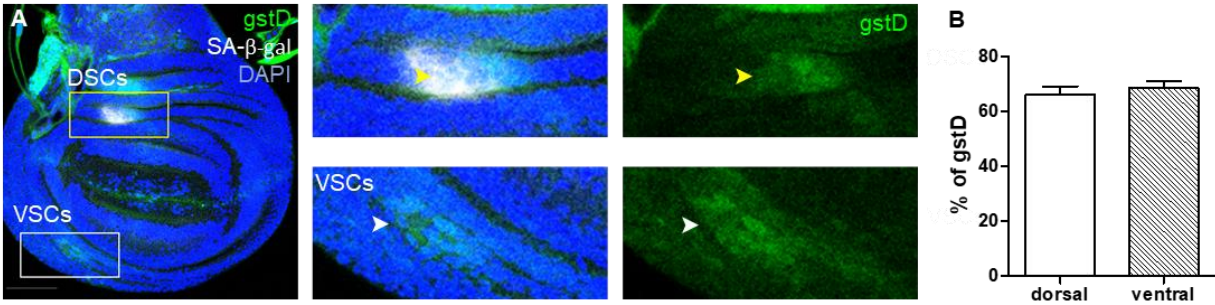


Figure 3-8. Percentage of oxidative positive cells in SA-β-gal clusters. (A, B) Wing disc carrying *gstD*-GFP was stained with SPiDER-β-gal (A). Percentage of *gstD*-GFP in SA-β-gal-activated clusters were quantified in (B). Dorsal cells (n= 9), ventral cells (n=9). Yellow arrowheads indicate DSCs and white arrowheads indicate VSCs. Scale bars, 50 μm. (Adapted from Zang et al, 2025)

Fourthly, both DSCs and VSCs in wild-type wing discs exhibited cellular hypertrophy, another key hallmark of senescent cells (Figure 3-9A and B).

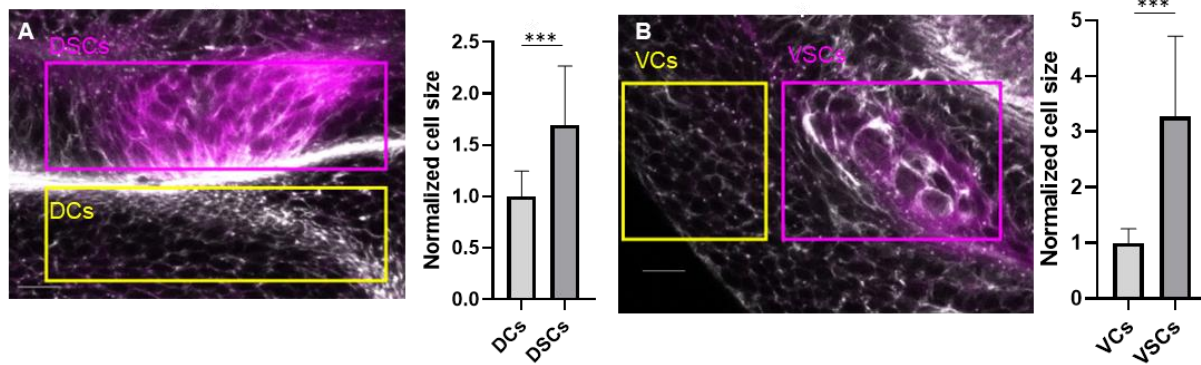


Figure 3-9. SA-β-gal positive clusters are hypertrophic cells. (A, B) Wild-type wing disc was stained with SPiDER-β-gal (magenta) and phalloidin (white). Cell size was collected from 3 biological replicates, measured with ImageJ and quantified as relative size to neighboring normal cells. Data were collected as mean ± s.e., normal dorsal cells (DC) (n=97); DSCs (n=97); normal ventral cells (VC) (n=58); VSCs (n=58). Student's *t*-test, ****P* < 0.001. Scale bars, 50 μm. (Adapted from Zang et al, 2025)

Next, I examined the upstream trigger of programmed cell senescence in the wing discs. EGFR-Ras signaling, a well-established inducer of cellular senescence in mammals, was found to be

upregulated in DSCs and VSCs. This was demonstrated by pERK staining, a marker of Ras signaling activation, which co-localized with SA- β -gal staining, indicating a potential role of Ras signaling in inducing programmed cell senescence in these cells (Figure 3-10A, quantified in Figure 3-10B).

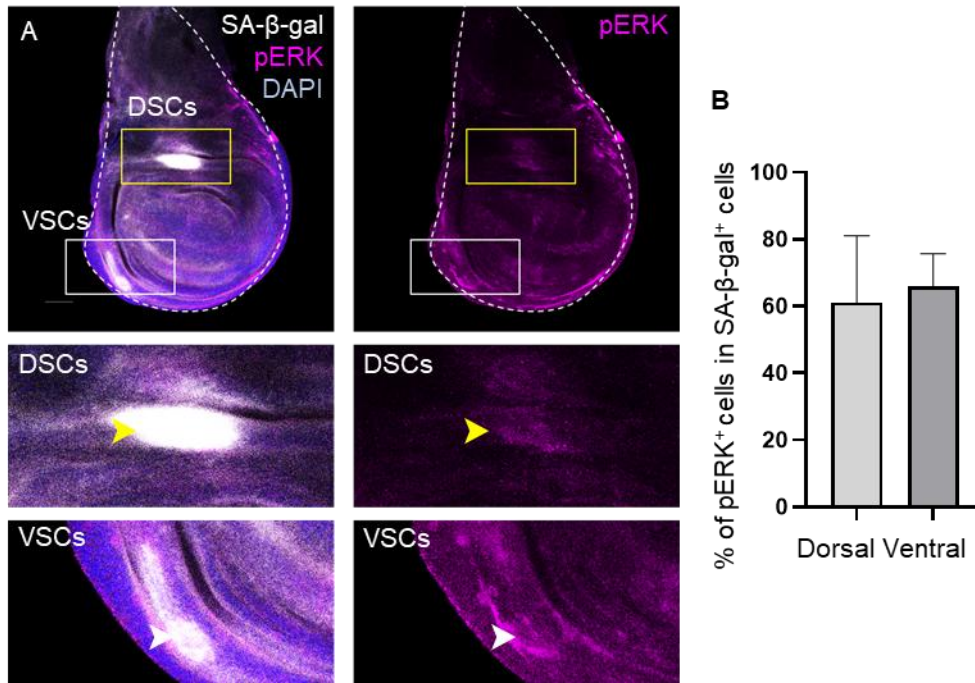


Figure 3-10. Percentage of pERK positive cells in SA- β -gal clusters. (A, B) Wild-type wing discs were stained with SPiDER- β gal and anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody. The percentage of pERK positive area (magenta) in SA- β -gal-activated clusters (white) were quantified in (B). Dorsal cells (n=12), ventral cells (n=12). Scale bars, 50 μ m. (Adapted from Zang et al, 2025)

Moreover, elevation of EGFR signaling was also verified by downregulation of a transcription factor Capicua (Roch et al., 2002) (Figure 3-11A).

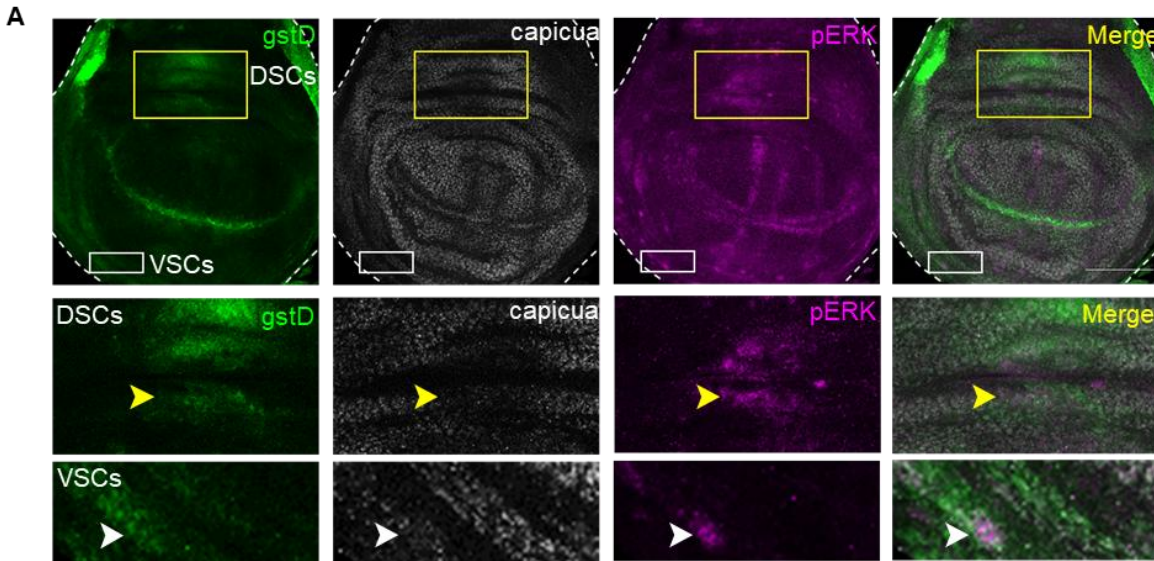


Figure 3-11. Capicua are downregulated in pERK positive cells. (A) Wing disc carrying *gstD*-GFP was stained with anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (magenta) and anti-Capicua (white) antibodies. Yellow arrowheads indicate DSCs and white arrowheads indicate VSCs. Scale bars, 50 μ m. (Adapted from Zang et al, 2025)

Additionally, the inhibition of Ras signaling using *Ras^{N17}*, which is a dominant-negative form of Ras, under the control of the *ci*-Gal4 driver, led to a suppression of endogenous SA- β -gal activity. This suggests that Ras signaling is required for the induction of programmed cell senescence in the wing discs. (Figure 3-12A, quantified in Figure 3-12B).

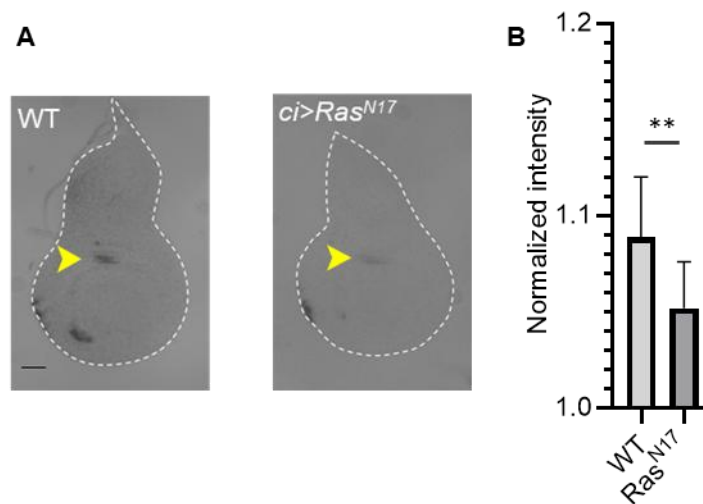


Figure 3-12. Inhibition of Ras signaling by *Ras^{N17}* leads to suppression of SA- β -gal activity.

(A, B) Wild-type or *ci-Gal4>Ras^{N17}* wing discs were subjected to SA-β-gal assay. Arrowheads indicate DSCs. Intensity of SA-β-gal was measured with ImageJ and normalized with background. Data were collected as mean ± s.e., wild type (n=10), *ci>Ras^{N17}* (n=6) Student's *t*-test, **P < 0.01. Scale bars, 50 μm. (Adapted from Zang et al, 2025)

To further validate this finding, I used the *ap-GAL4* driver and observed that *Ras^{N17}* expression similarly suppressed SA-β-gal activity (Figure 3-13A, quantified in Figure 3-13B). These results indicate that Ras signaling functions as an upstream trigger of programmed cell senescence and that the two cell clusters, DSCs and VSCs, in *Drosophila* wing discs undergo developmentally programmed cell senescence.

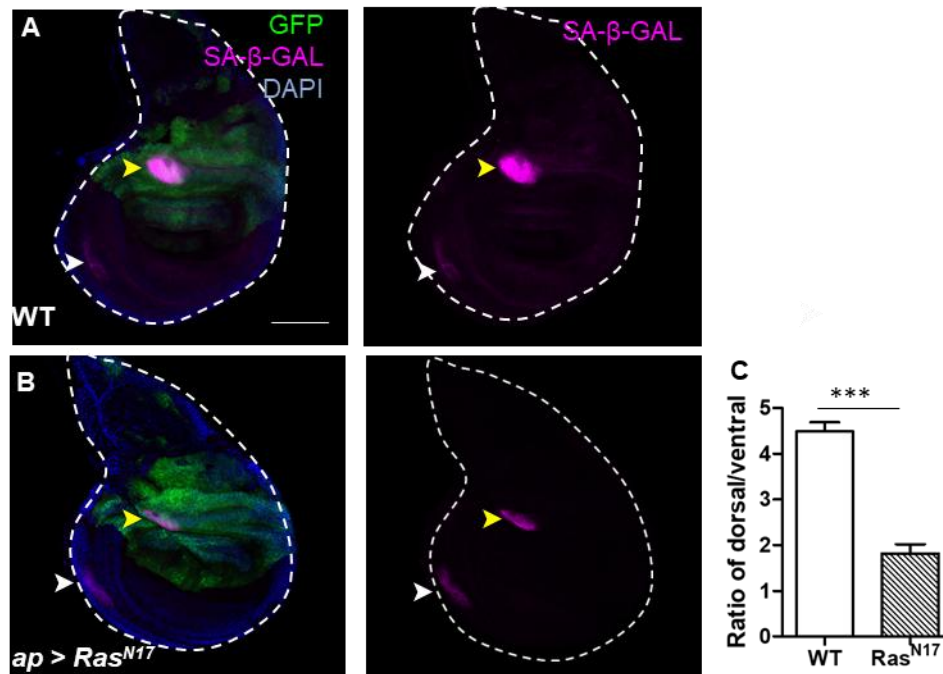


Figure 3-13. Activity of SA-β-gal in DSCs is significantly suppressed by *Ras^{N17}*. (A-C) Wild type (A) and *ap > Ras^{N17}* (B) wing disc was stained with SPiDER-βgal. Yellow arrowheads indicate DSCs and white arrowheads indicate VSCs. Intensity of SA-β-gal in dorsal senescent cells was measured with ImageJ and normalized with ventral senescent cells. Data were collected as mean ± s.e., wild type (n=14), *ap > Ras^{N17}* (n=6) Student's *t*-test, ***P < 0.001. Scale bars, 50 μm. (Adapted from Zang et al, 2025)

3-3 SASP in programmed cell senescence

I further explored whether senescence-associated secretory phenotype (SASP) is induced in the senescent clusters. I did not detect expression of *unpaired* (*upd*, an *IL-6* homologue) or *matrix*

metalloproteinase 1 (mmp1), typical SASP factors in mammals, in the wing disc (Figure 3-14A, B and C).

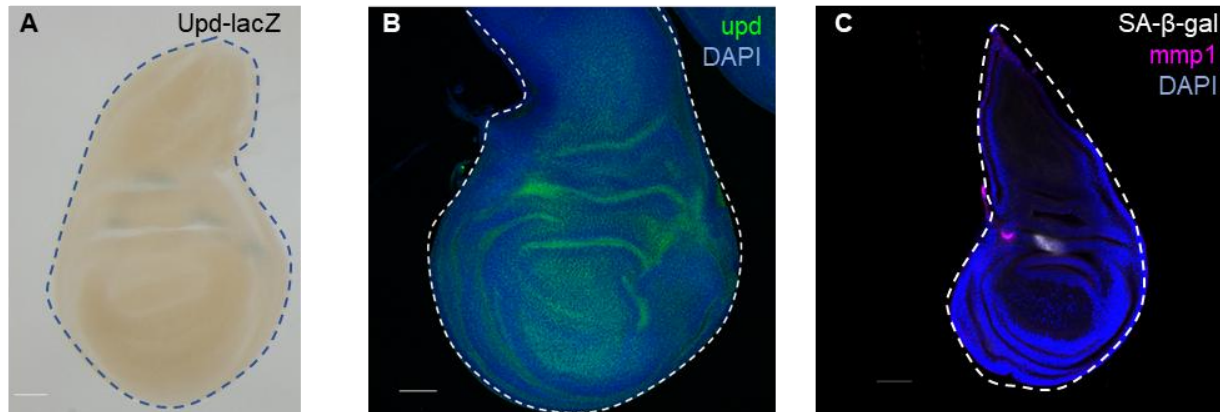


Figure 3-14. *upd* and *mmp1* are not observed in wing disc. (A) X-gal staining of wing imaginal discs expressing *upd-lacZ*. (B) Wild type wing disc was stained with anti-*upd*. (C) Wild type wing disc was stained with SPiDER- β -gal (white) and anti-*mmp* (magenta) antibody. Scale bars, 50 μ m. (Adapted from Zang et al, 2025)

However, the *IL-6* homologue *unpaired3 (upd3)*, a key activator of *Drosophila* JAK/STAT signaling, was significantly upregulated in DSCs and VSCs, as indicated by the *upd3-GAL4* reporter (Figure 3-15). These findings suggest that an interleukin-like cytokine is actively induced in programmed senescent cells within the wing discs.

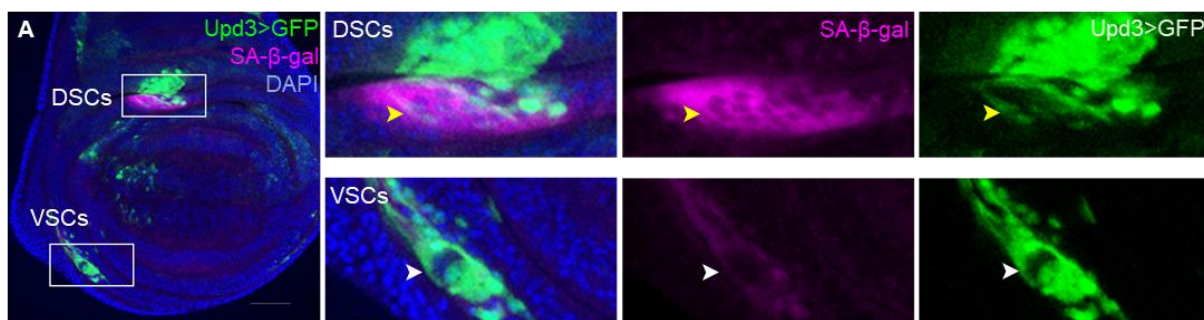


Figure 3-15. *upd3* are colocalized with SA- β -gal positive cells. (A) Wing discs carrying *Upd3>GFP* were stained with SPiDER- β -gal. Scale bars, 50 μ m. (Adapted from Zang et al, 2025)

3-4 Programmed cell senescence is also found in other tissues

Other tissues, including leg discs, haltere discs, and the brain, exhibited some SA- β -gal activity, whereas the salivary gland, fat bodies, and eye discs (except for hemocytes attached to the discs) did not (Figure 3-16 A-F). I focused my analysis on the SA- β -gal-positive clusters in wing discs, as they consistently displayed intense SA- β -gal staining with minimal background noise, making them highly suitable for investigation.

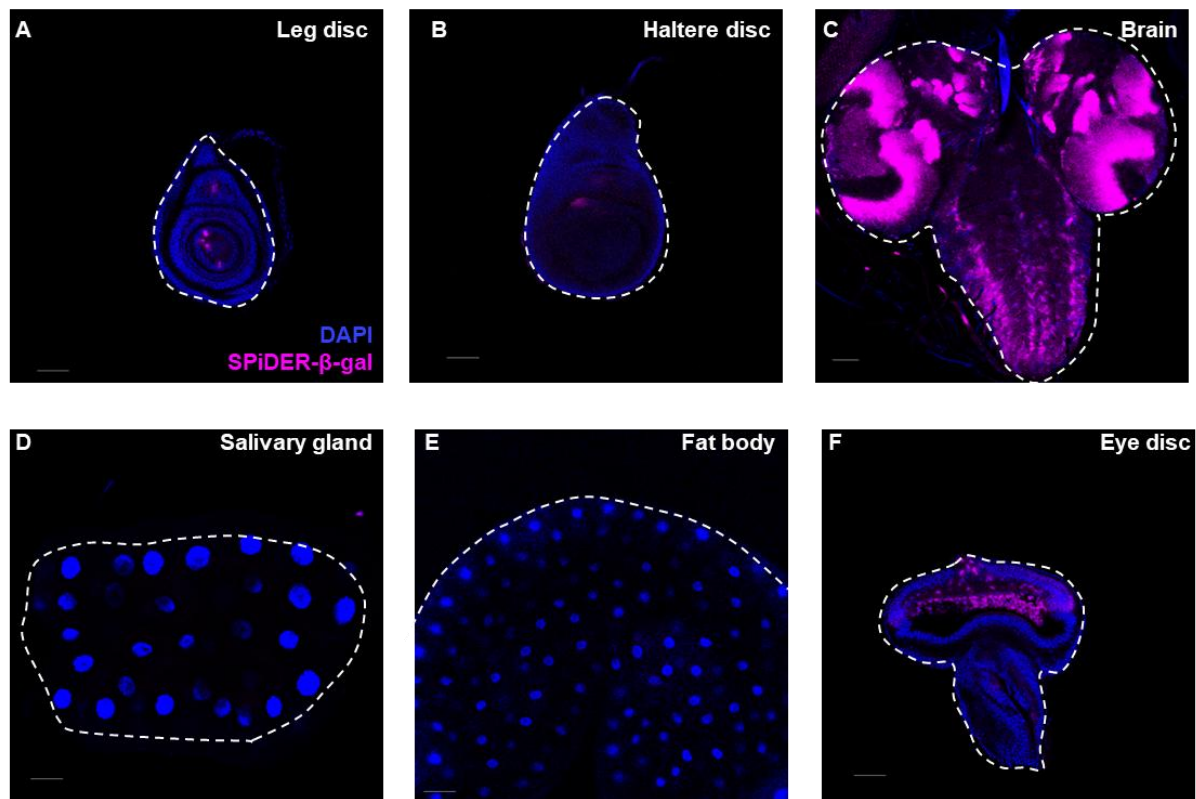


Figure 3-16. Wild-type tissues subjected to SA- β -gal assay. (A) SPiDER- β -gal staining (magenta) of the leg disc (A), haltere disc (B), brain (C), salivary gland (D), fat body (E), and eye-antennal disc (R) are shown. Note that the SPiDER- β -gal-signals in the eye-antennal disc is due to the attached hemocytes. Scale bars, 100 μ m, 50 μ m. (Adapted from Zang et al, 2025)

I conducted senolytic drug experiments using Navitoclax, a potent and broad-spectrum senolytic agent. However, as shown in Figure 3-17, feeding larvae with Navitoclax (1–100 μ M) did not impact the induction of programmed cell senescence in the wing discs. This suggests that *Drosophila* senescent cells, or specifically programmed senescent cells, may be resistant to

senolytic drug treatment.

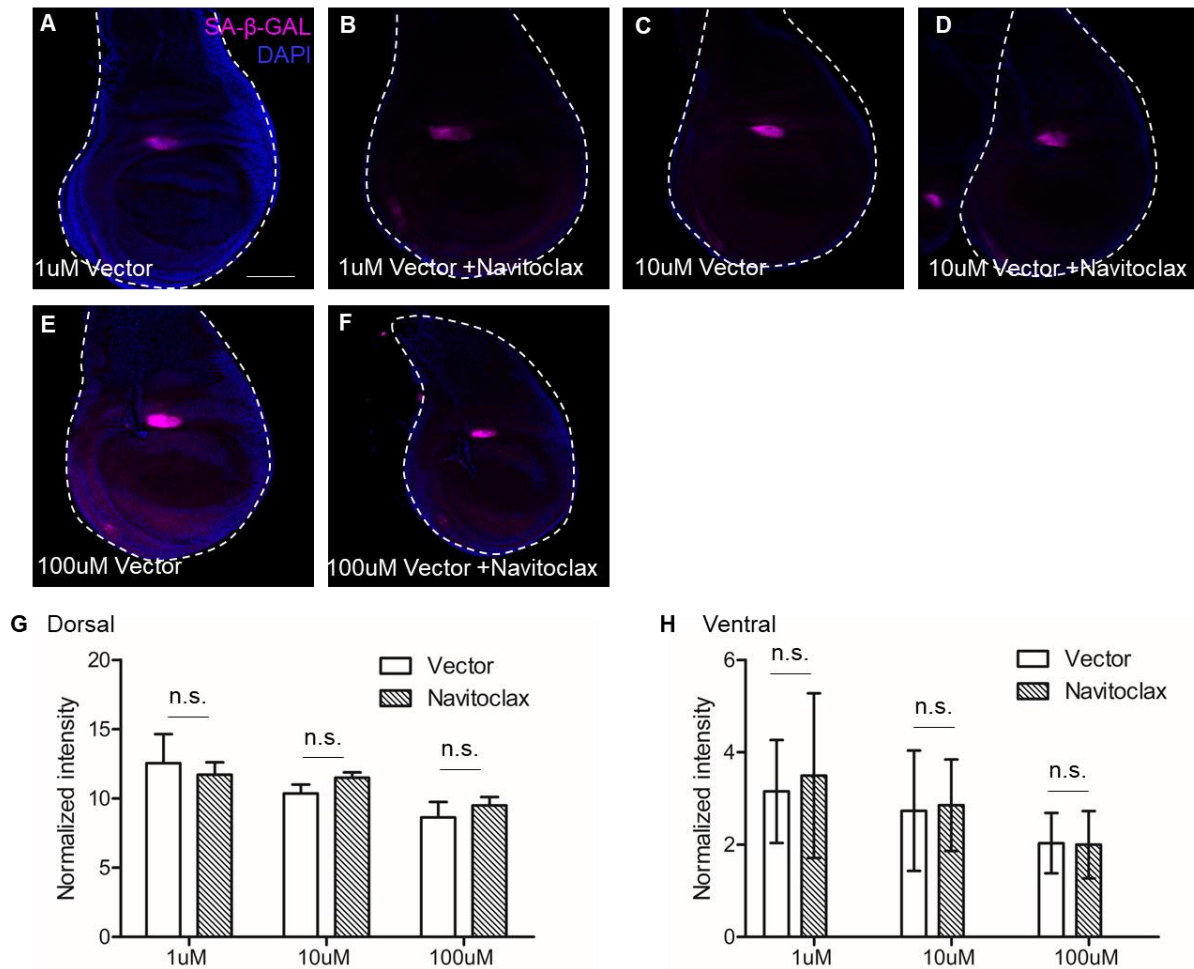


Figure 3-17. Navitoclax does not suppress programmed cell senescence in *Drosophila*

(A-H) Fly larvae were fed with 1 mM, 10 mM or 100 mM Navitoclax (B, D, F) or DMSO as a control (A, C, E) in the fly food. Intensities of the SA-β-gal staining in senescent cells were measured using ImageJ and normalized with background (G, H). Data were collected as mean ± s.e., 1 mM vector dorsal cells (n= 10), 1 mM Navitoclax-treated dorsal cells (n= 24), 10 mM vector dorsal cells (n=17), 10 mM Navitoclax-treated dorsal cells (n=32), 100 mM vector dorsal cells (n=21), 100 mM Navitoclax-treated dorsal cells (n=39), 1 mM vector ventral cells (n= 10), 1 mM Navitoclax-treated ventral cells (n= 24), 10 mM vector ventral cells (n=17), 10 mM Navitoclax-treated ventral cells (n=32), 100 mM vector ventral cells (n=21), 100 mM Navitoclax-treated ventral cells (n=39). Student's t-test, P >0.05. Scale bars 50 μm. (Adapted from Zang et al, 2025)

3-5 Location of programmed cell senescence

Next, I aimed to determine the cell type of the clusters undergoing programmed cell senescence

in the wing discs. The DSC and VSC clusters were found in the anterior region of the wing disc, near the anterior/posterior (A/P) boundary (Figure 3-18A, arrowheads), as indicated by *ci*-Gal4, which is expressed throughout the anterior compartment. Further analysis using *nub*-GAL4, which drives gene expression in the wing pouch and part of the hinge, confirmed that both DSCs and VSCs are located in the hinge region, adjacent to the wing pouch (Figure 3-18B, arrowheads).

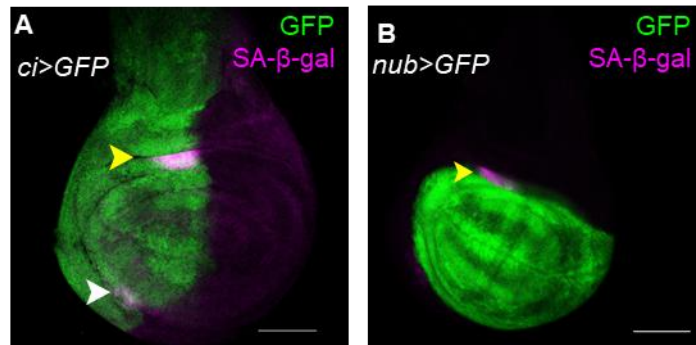


Figure 3-18. Location of senescent cells in wing disc. (A, B) Wing discs with *ci*>*GFP* (D, GFP is expressed at the anterior half of the wing disc) or *nub*>*GFP* (E, GFP is expressed in the wing pouch and a part of hinge regions) were stained with SPiDER-βgal. Scale bars 50 μm. (Adapted from Zang et al, 2025)

Additional analysis using DAPI staining, which highlights the folding structure of the wing disc, revealed that both DSCs and VSCs are situated in the distal hinge (DH) region (Figure 3-19A). Notably, these two locations correspond to the future dorsal and ventral compartments of the adult wing hinge, specifically the dorsal and ventral radiuses, which are positioned near the wing blade.

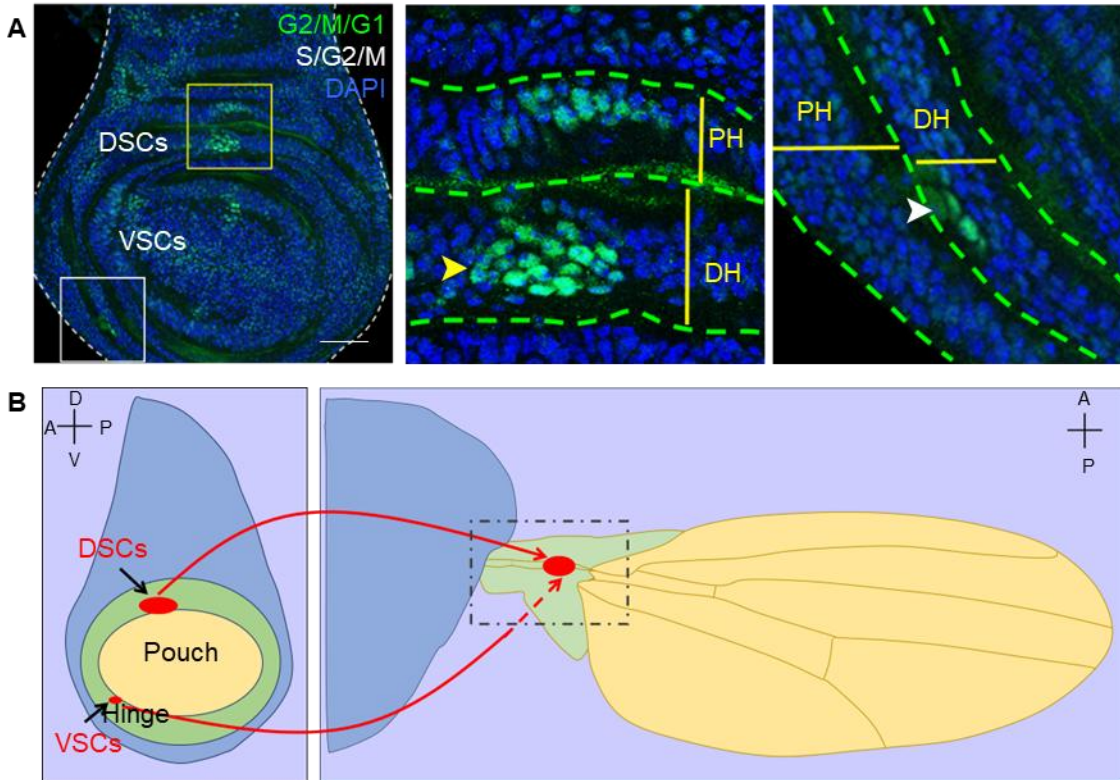


Figure 3-19. Location of senescent cells from wing disc to adult wing. (A) Wing disc expressing fly-FUCCI was stained with DAPI. PH, proximal hinge; DH, distal hinge. (B) The location of senescent cells in the larval wing disc (left) and adult wing (right). A, anterior; P, posterior; D, dorsal; V, ventral. Scale bars 50 μ m. (Adapted from Zang et al, 2025)

3-6 Development of campaniform sensilla requires programmed cell senescence

The dorsal and ventral radii contain specialized sensory organs known as campaniform sensilla, which function as mechanoreceptors and originate from proneural clusters in the wing disc (Figure 3-20, arrows). Notably, although various types of campaniform sensilla exist, those associated with DSCs and VSCs share a distinct morphological characteristic: they appear as circular, low-profile sensilla with a socket structure (Cole and Palka, 1982).

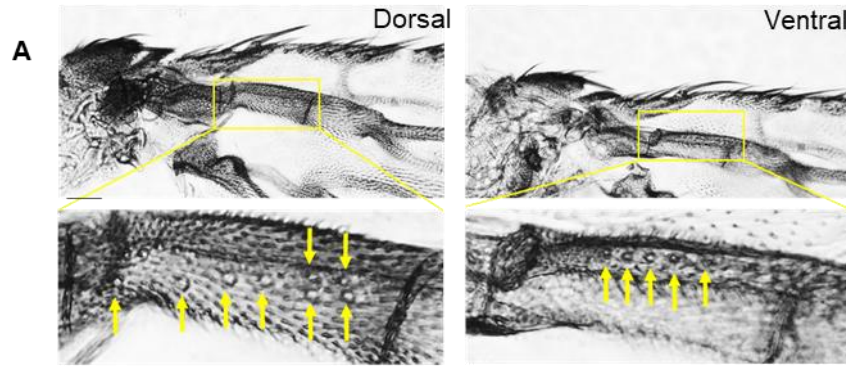


Figure 3-20. Campaniform sensilla on adult wing. (A) Campaniform sensilla (arrowheads) on dorsal and ventral radii of wild-type adult wing. Scale bars 50 μ m. (Adapted from Zang et al, 2025)

Further analysis of DSCs and VSCs in the wing disc revealed that these cell clusters were co-localized with the proneural markers Achaete (Ac) and Scabrous (Sca) (Figure 3-21 A and B).

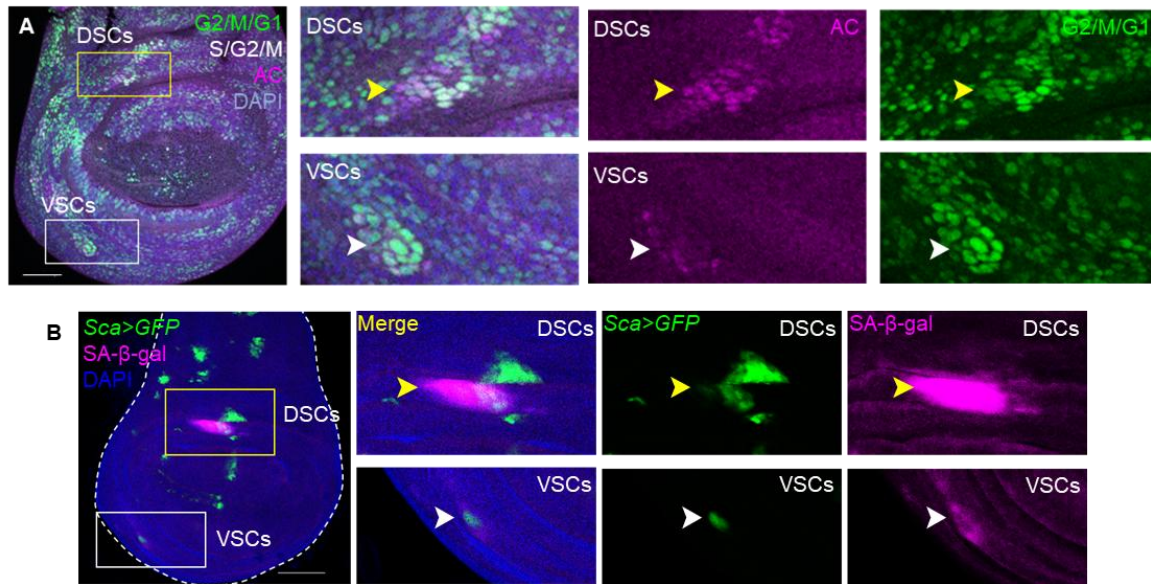


Figure 3-21. Proneural clusters are colocalized with senescent clusters. (A) Wing discs expressing fly-FUCCI were stained with AC antibody. GFP-E2F1 represents G2/M/G1 phase (green) and mRFP-NLS-CycB represents S/G2/M phase (white). Yellow arrowheads indicate DSCs, while white arrowheads indicate VSCs. (B) Wing discs expressing *Sca>GFP* were stained with SPiDER- β gal. Scale bars 50 μ m. (Adapted from Zang et al, 2025)

Notably, inhibiting programmed cell senescence by suppressing Ras signaling (*Ras^{N17}*) using the

ci-Gal4 driver led to a significant reduction in the number of campaniform sensilla on both the dorsal and ventral radii (Figure 3-22A, quantified in Figure 3-22B). These findings suggest that programmed cell senescence plays a crucial role in the proper development of this specific type of campaniform sensilla at the dorsal and ventral radii.

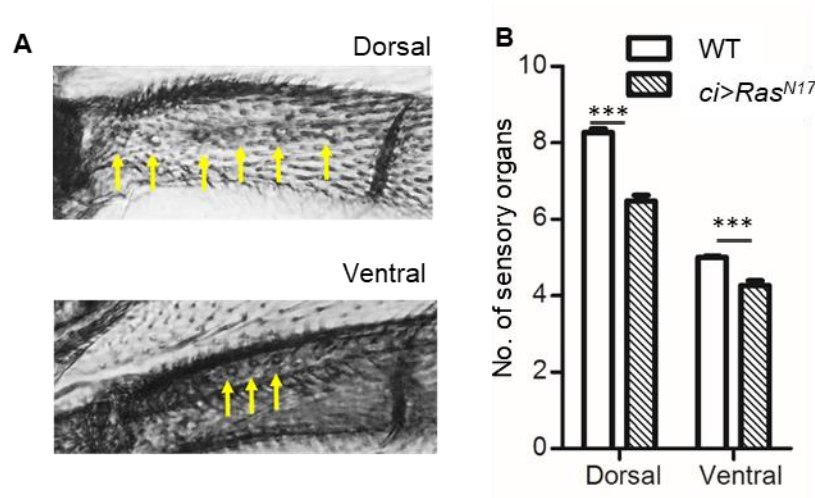


Figure 3-22. Campaniform sensilla are down regulated in senescence suppressed flies. (A) Campaniform sensilla (arrowheads) on dorsal and ventral radii of *ci>Ras^{N17}* flies. (B) The number of campaniform sensilla of wild-type and *ci>Ras^{N17}* flies was counted and quantified. Data were collected as mean $\pm$ s.e., $n=52$ (wild-type dorsal radius), $n=42$ (*ci>Ras^{N17}* dorsal radius), $n=29$ (wild-type ventral radius), and $n=37$ (*ci>Ras^{N17}* ventral radius). Student's *t*-test, *** $P < 0.001$. Scale bars 50 μ m. (Adapted from Zang et al, 2025)

3-7 The mechanism behind programmed cell senescence in *drosophila* wing

To investigate the mechanism underlying the induction of programmed cell senescence in the wing disc, I examined the role of Ras signaling. In mammals, Ras-induced cellular senescence is mediated by the *ETS1/2* transcription factor (Ohtani et al., 2001). *Drosophila* possesses an *ETS1/2* homologue, *pointed* (*pnt*), which encodes two isoforms: PntP1 and PntP2 (Klamt, 1993; Scholz et al., 1993). Notably, PntP1 is transcriptionally regulated by EGFR-Ras signaling and plays a key role in the development of photoreceptor neurons in the eye imaginal discs (Shwartz et al., 2013). Our previous work has demonstrated that PntP1 acts as a necessary and sufficient downstream effector of Ras-induced cellular senescence in imaginal discs (Ito and Igaki, 2021).

Interestingly, I observed that PntP1 expression was upregulated in both DSCs and VSCs (Figure 3-23A). This induction of PntP1 was abolished when Ras-MAPK signaling was inhibited either by overexpressing the dominant-negative Ras mutant (*Ras^{N17}*) or by knocking down the MAPK gene *rolled* (*rl*) (Figures 3-23B and C). These findings suggest that PntP1 functions as a downstream effector of Ras-MAPK signaling in DSCs and VSCs.

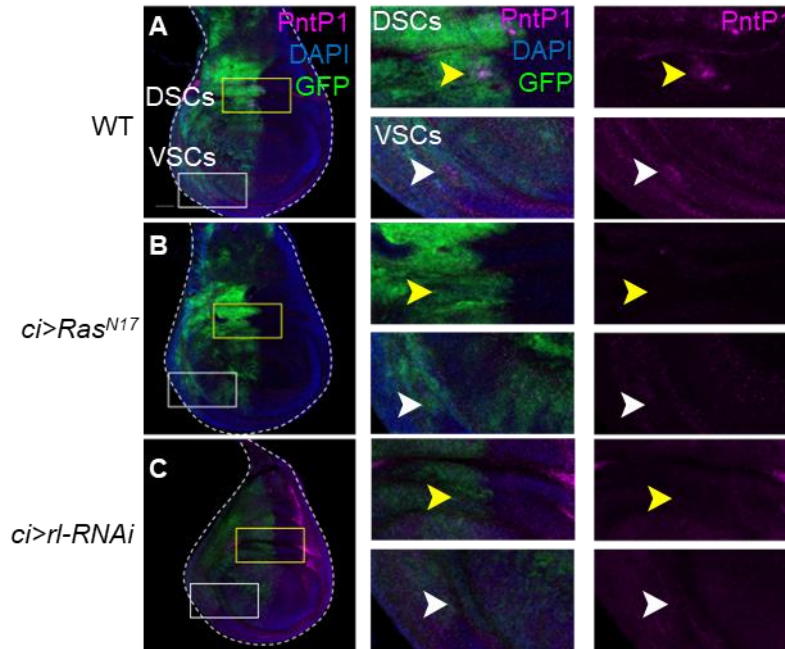


Figure 3-23. PntP1 expression exists in wing disc and regulated by Ras signaling. (A-C) *ci>GFP* (A), *ci>Ras^{N17}* (B), or *ci>rl-RNAi* (C) wing disc with Pnt-lacZ was stained with anti- β -gal antibody. Yellow and white arrowheads indicate senescent cells. Scale bars 50 μ m. (Adapted from Zang et al, 2025)

Importantly, knocking down Pnt using the *ci*-Gal4 driver led to a significant reduction in SA- β -Gal activity in these senescent cells (Figure 3-24A, quantified in Figure 3-24B). This suggests that Pnt plays a crucial role in regulating programmed cell senescence in DSCs and VSCs.

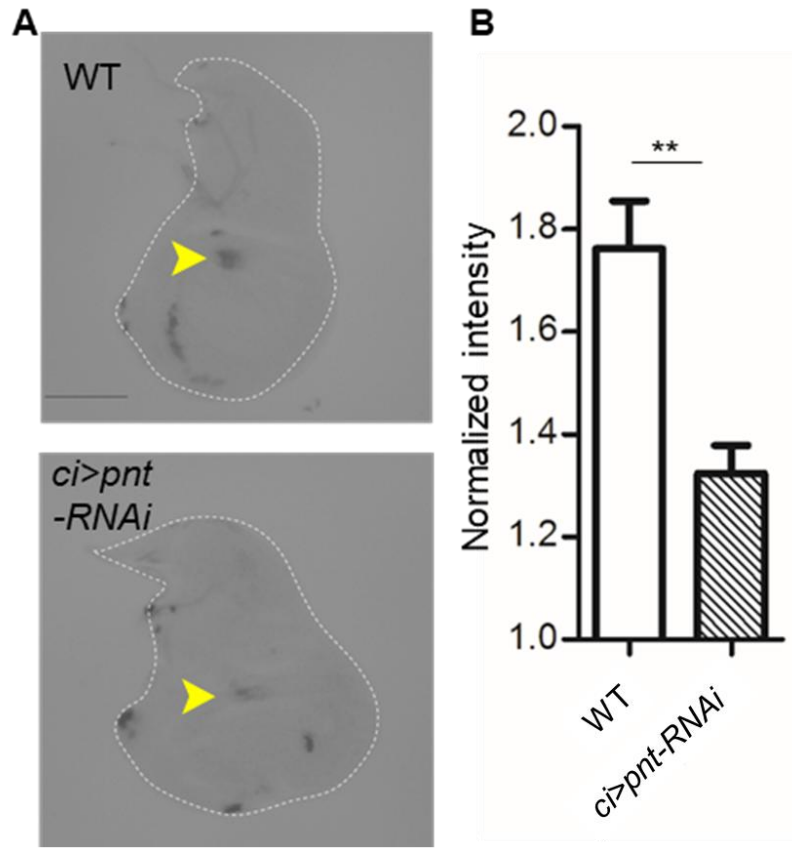


Figure 3-24. SA-β-gal activity is suppressed by *pnt-RNAi*. (A, B) Wild-type and *ci>pnt-RNAi* wing discs subjected to SA-β-gal assay. Arrowheads indicate SA-β-gal-activated clusters. Intensity of X-gal staining in senescent cells was measured with ImageJ and normalized with background (E). Data were collected as mean ± s.e., wild type (n=6), *ci>pnt-RNAi* (n=6) Student's *t*-test, ** $P < 0.01$. Scale bars 50 μm. (Adapted from Zang et al, 2025)

I further validated this finding by using the ap-GAL4 driver in combination with *pnt-RNAi*, which led to a significant reduction in SA-β-Gal activity in DSCs (Figure 3-25A, quantified in Figure 3-25B). This reinforces the role of Pnt in regulating programmed cell senescence in the wing disc.

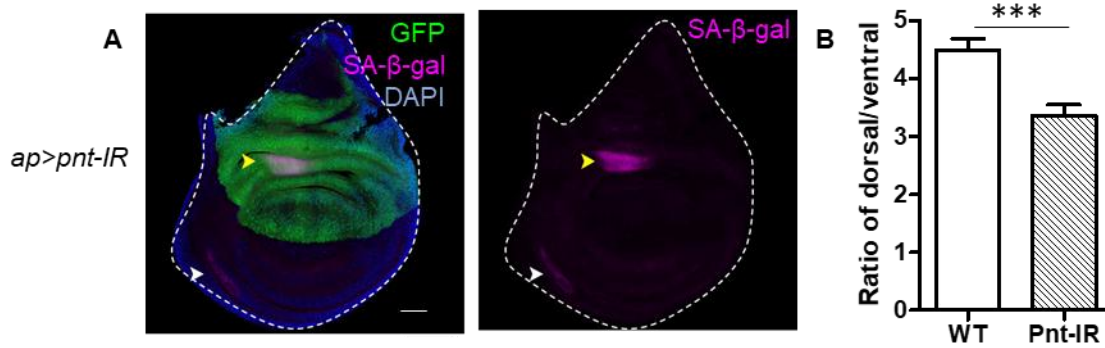


Figure 3-25. SA-β-gal activity of DVCs is suppressed by *pnt-RNAi*. (A) *ap>pnt-RNAi* wing disc was stained with SPiDER-βgal. Yellow arrowheads indicate DSCs and white arrowheads indicate VSCs. (B) Intensity of SA-β-gal in dorsal senescent cells was measured with ImageJ and normalized with ventral senescent cells. Data were collected as mean $\pm$ s.e., wild type (n=14), *ap > pnt-RNAi* (n=14) Student's t-test, ***P < 0.001. Scale bars 50 μ m. (Adapted from Zang et al, 2025)

Additionally, inhibition of Ras-MAPK signaling led to the appearance of cells in the S and M phases within DSCs and VSCs, as indicated by EdU incorporation and pH3 staining (Figures 3-26A-B and 3-26E-F, asterisks, quantified in Figures 3-26C, 3-26D, 3-26G, and 3-26H). This suggests that Ras-MAPK signaling is crucial for maintaining cell cycle arrest in these programmed senescent cells.

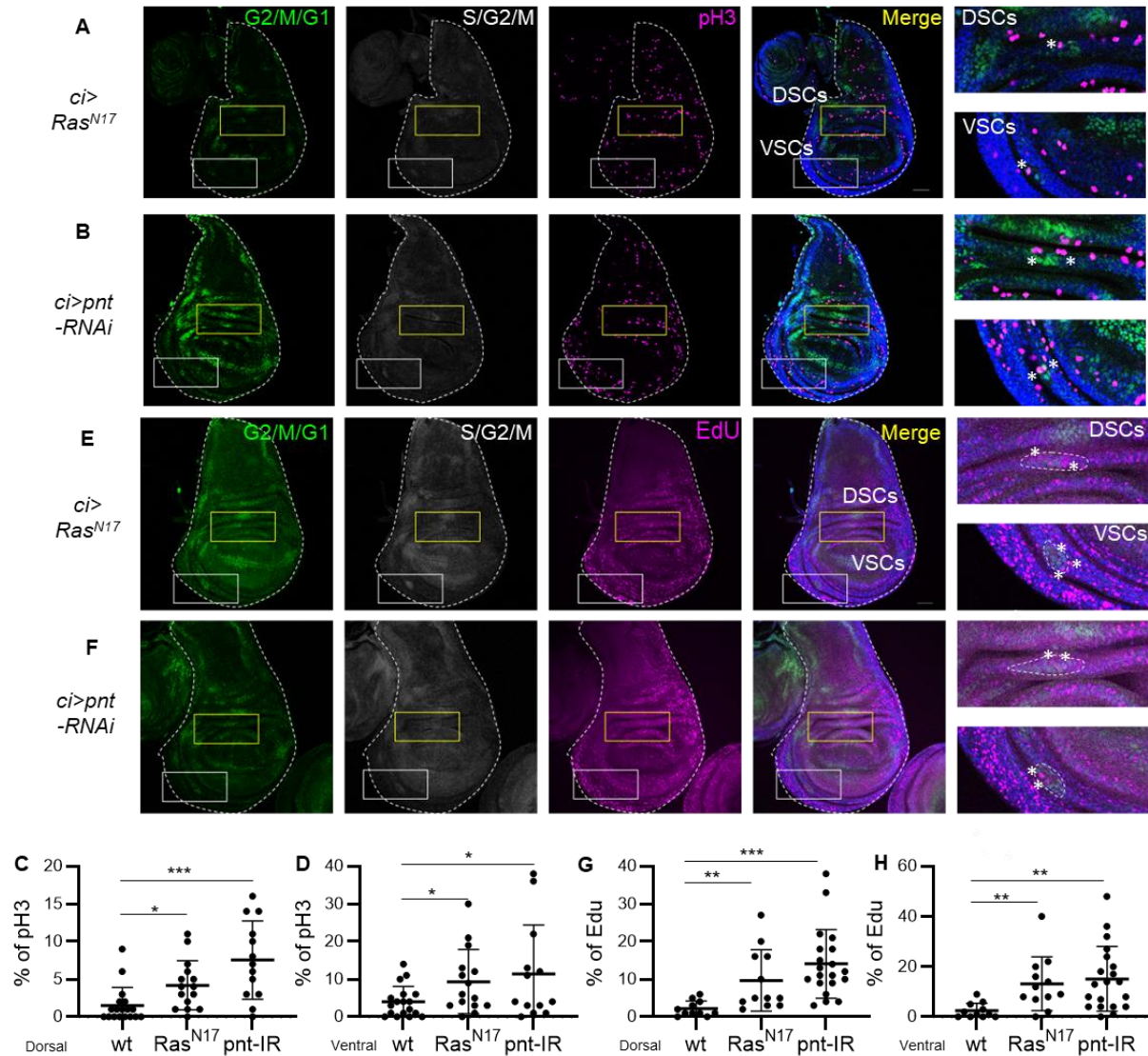


Figure 3-26. The number of cell cycle arrested cells is suppressed by blocking of Ras signaling. (A-D) Wing discs expressing fly-FUCCI with *ci> Ras^{N17}* (A), or *ci> pnt-RNAi* (B) were stained with anti-pH3 antibody. Asterisks represent pH3-positive cells. The percentage of pH3 positive area (magenta) in FUCCI clusters (green and white) was quantified in C, D). Data were collected as mean $\pm$ s.e., wild type dorsal (n=18), *ci> Ras^{N17}* dorsal (n=15), *ci> pnt-IR* dorsal (n=13), wild type ventral (n=18), *ci> Ras^{N17}* ventral (n=15), *ci> pnt-RNAi* ventral (n=13), Student's t-test, *P < 0.05, ***P < 0.001. (E-H) Wing discs expressing fly-FUCCI with *ci> Ras^{N17}* (E), or *ci> pnt-RNAi* (F) were stained with EdU incorporation. Asterisks represent EdU-positive cells. The percentage of EdU positive area (magenta) in FUCCI clusters (green and white) was quantified in (G, H). Data were collected as mean $\pm$ s.e., wild type dorsal (n=11), *ci> Ras^{N17}* dorsal (n=12), *ci> pnt-RNAi* dorsal (n=20), wild type ventral (n=11), *ci> Ras^{N17}* ventral (n=12), *ci> pnt-RNAi* ventral (n=20). Student's t-test, **P < 0.01, ***P < 0.001. Scale bars 50 μ m. (Adapted from Zang et al,

2025)

Moreover, pH3 and EdU staining further revealed that overexpression of String (Stg), a downstream regulator of the cell cycle in the Ras signaling pathway, significantly suppressed cell cycle arrest in DSCs and VSCs (Figure 3-27A and D, quantified in Figures 3-27B-C and 3-27E-F). These findings suggest that programmed cell senescence in the wing discs is driven by Ras-induced PntP1 expression.

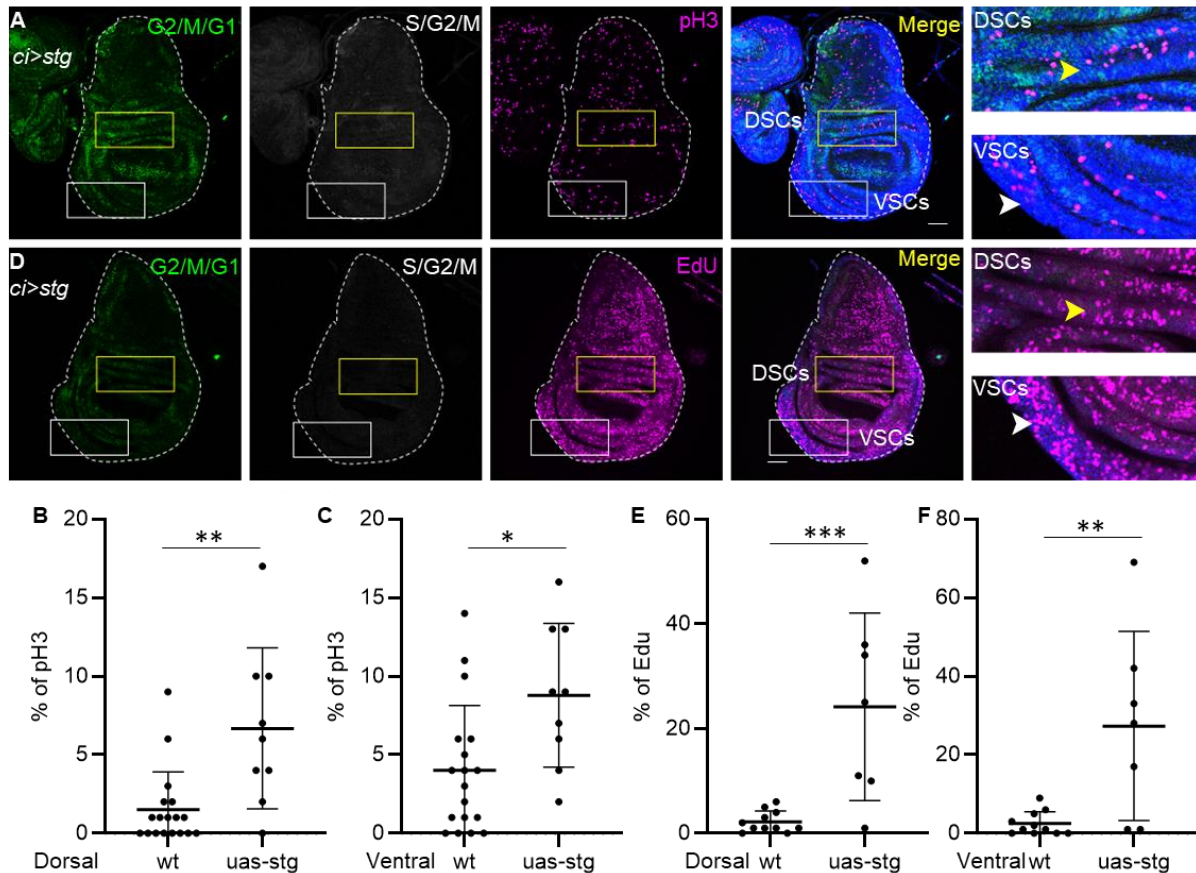


Figure 3-27. Cell cycle arrest dependent on Ras signaling is regulated by stg. (A-C) Wing disc expressing fly-FUCCI with *ci> stg* was stained with anti-pH3 antibody (A). Yellow arrowheads indicate pH-positive cells in DSCs and white arrowheads indicate VSCs. The percentage of pH3 positive area in FUCCI clusters (green and grey) were quantified in (B, C). Data were collected as mean $\pm$ s.e., wild type dorsal (n=18), *ci> stg* dorsal (n=9), wild type ventral (n=18), *ci> stg* ventral (n=9). Student's t-test, *P < 0.05, **P < 0.01. (D-F) Wing disc expressing fly-FUCCI with *ci> stg* was stained with EdU incorporation (D). Yellow arrowheads indicate EdU-positive cells in DSCs and white arrowheads indicate VSCs. Data were collected as mean $\pm$ s.e., wild type dorsal (n=11), *ci> stg* dorsal (n=7), wild type ventral (n=11), *ci> stg*

ventral (n=7). The percentage of EdU positive area in FUCCI clusters (green and grey) were quantified in (E, F). Student's t-test, **P<0.01, ***P < 0.001. Scale bars 50 μ m. (Adapted from Zang et al, 2025)

3-8 Programmed cell senescence regulates campaniform sensilla development through the ac-sc complex

Next, I examined the role of PntP1-mediated cellular senescence in the development of campaniform sensilla. Consistent with my findings that Pnt is essential for the induction of programmed cell senescence, knockdown of *pnt* led to the loss of campaniform sensilla in adult flies (Figure 3-28A, quantified in Figure 3-28B).

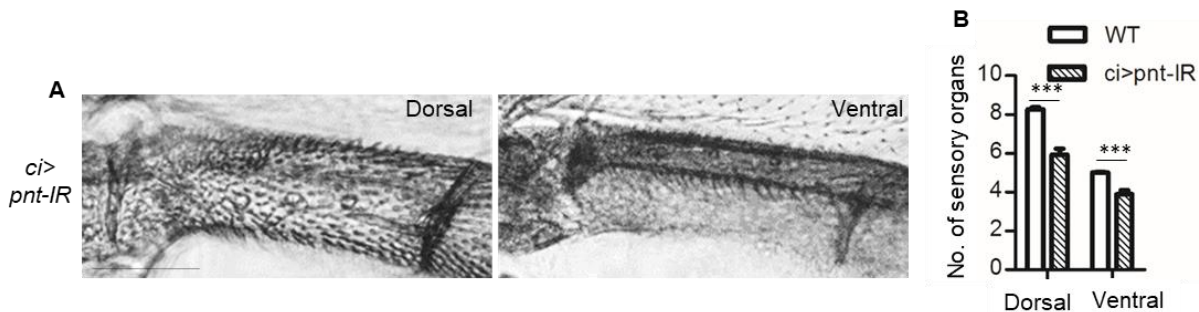


Figure 3-28. Development of campaniform sensilla is repressed by *pnt-RNAi*. (A, B) Campaniform sensilla on dorsal and ventral radii of *ci>pnt-RNAi* fly (A). The number of campaniform sensilla was counted and quantified (B). Data were collected as mean $\pm$ s.e., n=52 (wild-type dorsal radius), n=14 (*ci>pnt-RNAi* dorsal radius), n=29 (wild-type ventral radius), and n=19 (*ci>pnt-RNAi* ventral radius). Student's t-test, ***P < 0.001. Scale bars 50 μ m. (Adapted from Zang et al, 2025)

The basic helix-loop-helix transcription factor *achaete-scute* complex (*AS-C*) is known to be essential for sensory organ development in *Drosophila*. However, the role of Pnt in regulating proneural clusters specifically within campaniform sensilla remains unclear, as previous studies have primarily focused on sensory bristles and chordotonal organs (Culí et al., 2001; Lage et al., 1997). Notably, inhibiting programmed cell senescence through *pnt* knockdown using the *ci-Gal4* driver significantly suppressed *Achaete* (*Ac*) expression in DSC and VSC clusters (Figure 3-29A and B, quantified in Figure 3-29C and D). Importantly, *Ac* expression along the dorsoventral (DV) boundary, which gives rise to adult bristles, was unaffected by *pnt* knockdown (Figure 3-29A and B, asterisks).

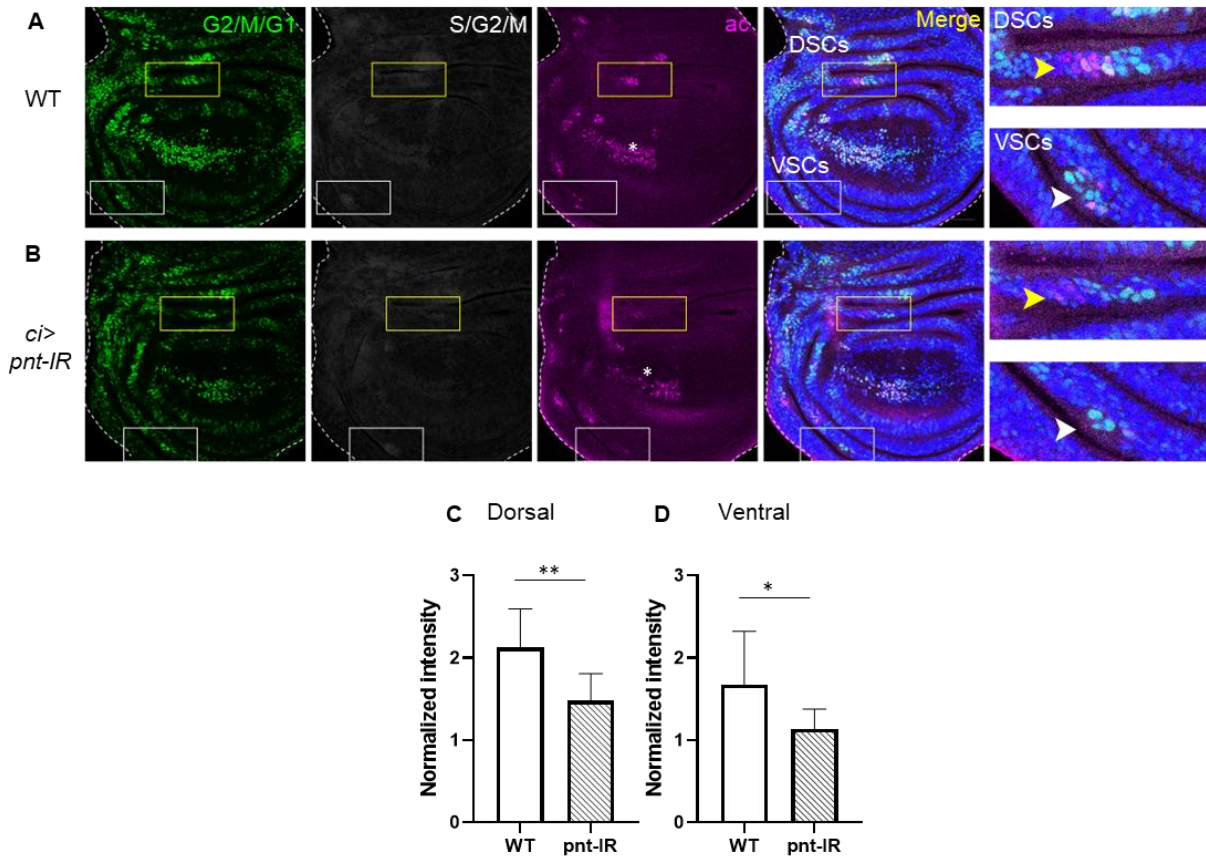


Figure 3-29. Development of proneural clusters is repressed by *pnt-RNAi*. (A, B) Wing discs expressing fly-FUCCI with *ci>+* (A) or *ci>pnt-RNAi* (B) were stained with anti-Ac antibody. Yellow arrowheads indicate DSCs and white arrowheads indicate VSCs. Intensity of Ac expression (magenta) in FUCCI clusters (green and white) of interest was measured and normalized with background intensity (C, D). Data were collected as mean $\pm$ s.e., wild type dorsal (n=16), *ci>pnt-IR* dorsal (n=9), wild type ventral (n=16), *ci>pnt-IR* ventral (n=9). Student's t-test, *P < 0.05, **P < 0.01. Scale bars 50 μ m. (Adapted from Zang et al, 2025)

Furthermore, knocking down *ac* and *sc* using the nub-Gal4 driver completely abolished the development of campaniform sensilla at the dorsal and ventral radiuses of the adult wing hinge (Figure 3-30A, quantified in Figure 3-30C). Notably, this genetic manipulation did not affect SA- β -gal activity in the programmed senescent cells (Figure 3-30B, quantified in Figure 3-30D), suggesting that while *ac* and *sc* are essential for campaniform sensilla formation, they do not influence the senescence process itself.

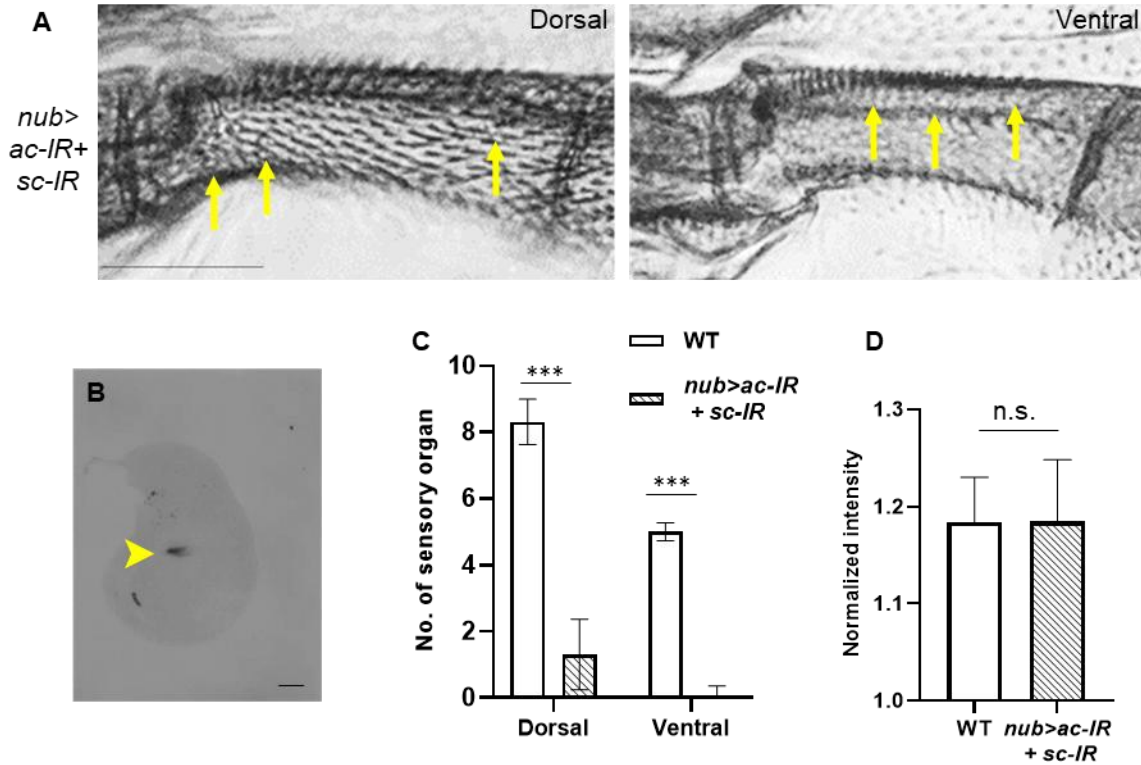


Figure 3-30. SA- β -gal activity is not repressed by ac-sc-RNAi. (A, B) Knock both *ac* and *sc* down lead to loss of campaniform sensilla in the adults (A), while SA- β -gal activity in the wing disc was not affected by *ac-sc* knockdown (B). Arrowheads indicate campaniform sensilla on dorsal or ventral radius and senescent cells in the wing disc. The number of campaniform sensilla was counted and quantified (C). Data were collected as mean $\pm$ s.e., $n=52$ (wild-type dorsal radius), $n=23$ (*nub>ac-RNAi + sc-RNAi* dorsal radius), $n=29$ (wild-type ventral radius), and $n=25$ (*nub>ac-RNAi + sc-RNAi* ventral radius). Student's *t*-test, *** $P < 0.001$. Intensity of X-gal staining in senescent cells was measured with ImageJ and normalized with background (D). Data were collected as mean $\pm$ s.e., wild type ($n=6$), *nub>ac-RNAi + sc-RNAi* ($n=4$) Student's *t*-test, $P > 0.5$. Scale bars, 50 μ m. (Adapted from Zang et al, 2025)

These findings indicate that the AS-C functions downstream of Pnt, supporting previous suggestions that activation of the EGFR pathway induces AS-C gene expression, leading to sensory organ formation within proneural clusters (Furman and Bukharina, 2008).

Given that AS-C is required for the induction of sensory organs and Pnt is required for the formation of senescent cells and formation of AS-C mediated proneural cluster, programmed cell senescence plays

a role in the development of a specialized type of sensory organ in the wing hinge via Pnt-AS-C-mediated proneural cluster formation. This finding also raises the possibility of tracking senescent cells in adult flies. In Post-eclosion *Drosophila*, wings are populated by hemocytes, which strongly stain for SA- β -Gal, complicating the analysis of cellular senescence (Figure 3-31A). To address this, I used a Gal4 enhancer trap inserted into the *pnt* gene to track senescent cells in adults. My results show that Pnt-expressing cells persist in the adult hinge radius (Figure 3-31B). These findings suggest that while Pnt expression is essential for normal tissue development, the fate and regulatory mechanisms of programmed senescent cells may differ between *Drosophila* and vertebrates.

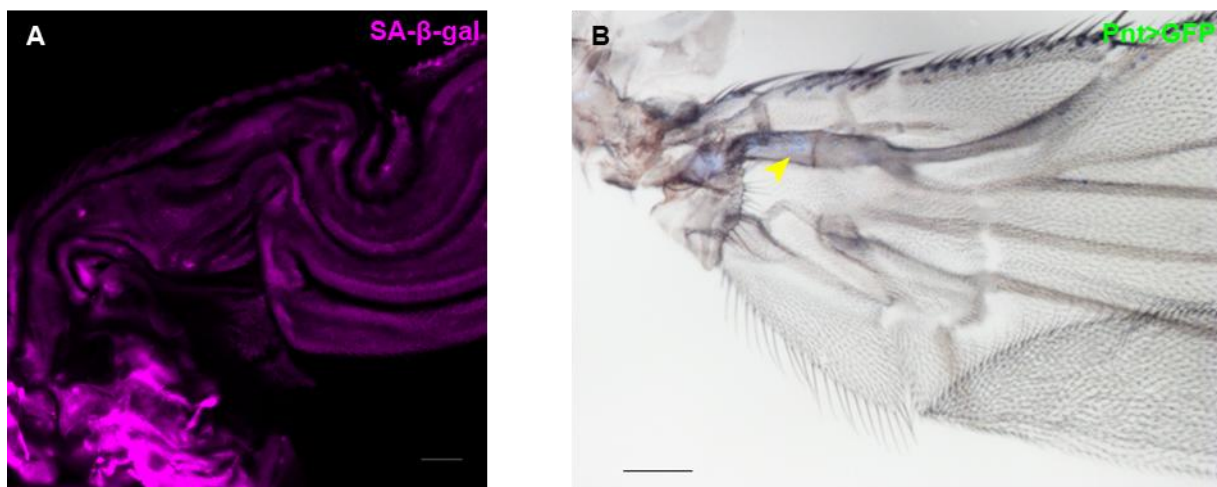


Figure 3-31. SA- β -gal activity still exists in adults. (A) SPiDER- β -gal staining in newly eclosed wild type fly. (B) *pnt*>GFP expression in newly eclosed fly. Yellow arrowheads indicate senescent cells. Scale bars, 100 μ m (B), 50 μ m (A). (Adapted from Zang et al, 2025)

3-9 The AS-C is promoted by Zfh2-mediated Ras-Pnt signaling

To further validate the role of programmed cell senescence in campaniform sensilla development,

I sought to identify additional genes involved in regulating this process in the wing disc. Using RNAi screening targeting genes highly expressed in the hinge region of the wing disc (Terriente et al., 2008), I identified the transcription factor *Zn finger homeodomain 2* (*zfh2*) as a key regulator. Notably, knockdown of *zfh2* significantly suppressed programmed cell senescence (Figure 3-32A, quantified in B).

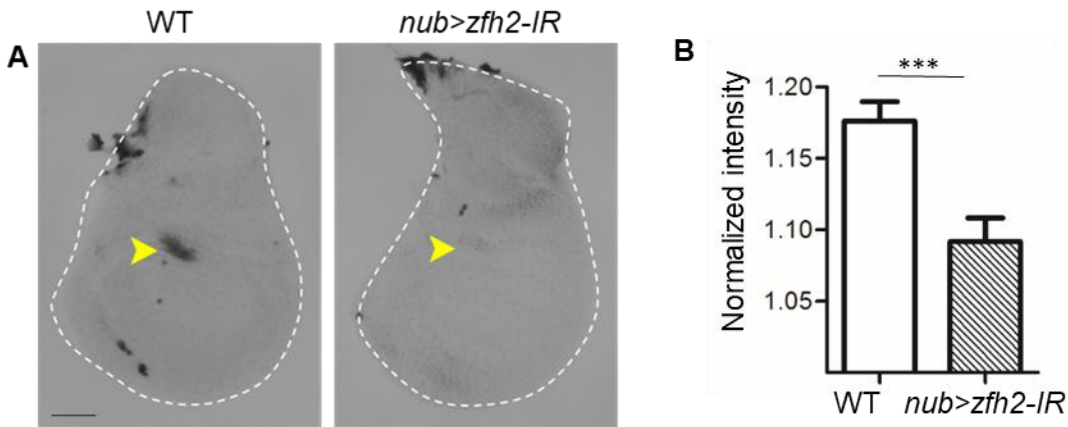


Figure 3-32. *zfh2*-RNAi represses SA-β-gal activity in wing disc. (A) Wing disc from wild-type (left) or *nub>zfh2-RNAi* (right) larva was subjected to SA-β-gal assay. Arrowheads indicate dorsal senescent cell cluster. Intensity of X-gal staining in senescent cells was measured with ImageJ and normalized with background (B). Data were collected as mean ± s.e., wild type (n=9), *nub>zfh2-RNAi* (n=12) Student's t-test, ***P < 0.001. Scale bars, 50μm. (Adapted from Zang et al, 2025)

I further validated this finding using *ap-GAL4*, which demonstrated a suppression of programmed cell senescence specifically in dorsal cells (Figure 3-33A).

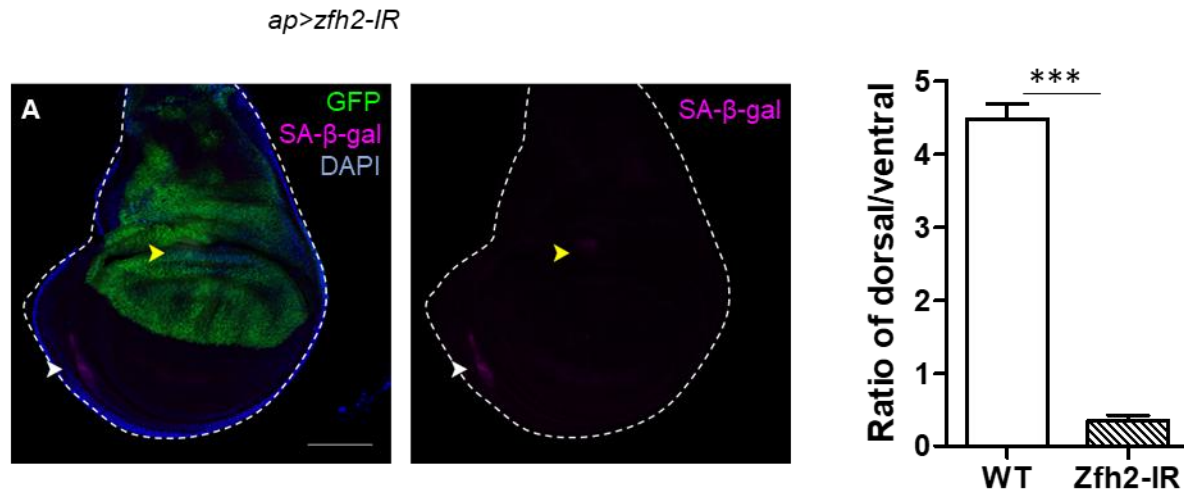


Figure 3-33. *zfh2*-RNAi represses SA-β-gal activity in DSCs. (A) *ap>zfh2*-RNAi wing disc was stained with SPiDER-β-gal. Yellow arrowheads indicate DSCs and white arrowheads indicate VSCs. Intensity of SA-β-gal in dorsal senescent cells was measured with ImageJ and normalized with ventral senescent cells. Data were collected as mean ± s.e., wild type (n=14), *ap > zfh2*-RNAi (n=9) Student's t-test, ***P < 0.001. Scale bars, 50μm. (Adapted from Zang et al, 2025)

Interestingly, *zfh2* is an ortholog of AT-motif binding factor 1 (*ATBF1*), a gene frequently mutated or deleted in various cancers and downregulated in *Ras^{ACT}*- and *Notch^{ACT}*-driven tumors in *Drosophila* (Doggett et al., 2015). This suggests that *zfh2* may function as a tumor suppressor by promoting cellular senescence. Supporting this idea, *zfh2* knockdown not only inhibited programmed cell senescence but also suppressed oncogene-induced senescence, as indicated by a significant reduction in *Ras^{V12}*-induced SA-β-gal activity (Nakamura et al., 2014) (Figure 3-34A, quantified in Figure 3-34B).

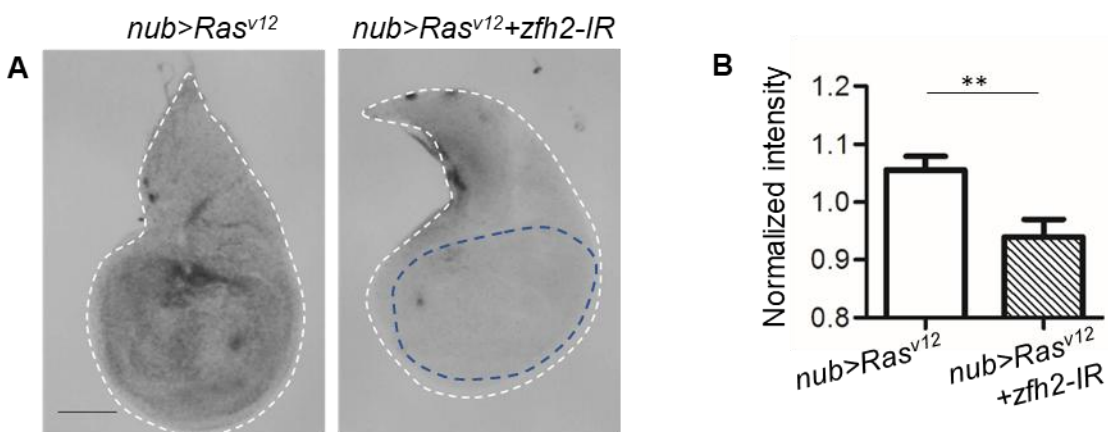


Figure 3-34. *zfh2-RNAi* represses *RAS^{V12}* induced SA- β -gal activity. (A) Wing disc from *nub>Ras^{V12}* (left) or *nub>Ras^{V12} + zfh2-RNAi* (right) larva was subjected to SA- β -gal assay. Intensity of X-gal staining in senescent cells was measured with ImageJ and normalized with background (B). Data were collected as mean $\pm$ s.e., *nub>Ras^{V12}* (n=6), *nub>Ras^{V12} + zfh2-RNAi* (n=5) Student's t-test, **P < 0.01. Scale bars, 50 μ m. (Adapted from Zang et al, 2025)

This indicates that the suppression of programmed cell senescence caused by *zfh2* knockdown is not due to abnormal hinge development but rather a direct blockage of senescence signaling.

Supporting this, *pnt-lacZ* expression in DSCs and VSCs was significantly downregulated upon *zfh2* knockdown (Figure 3-35A).

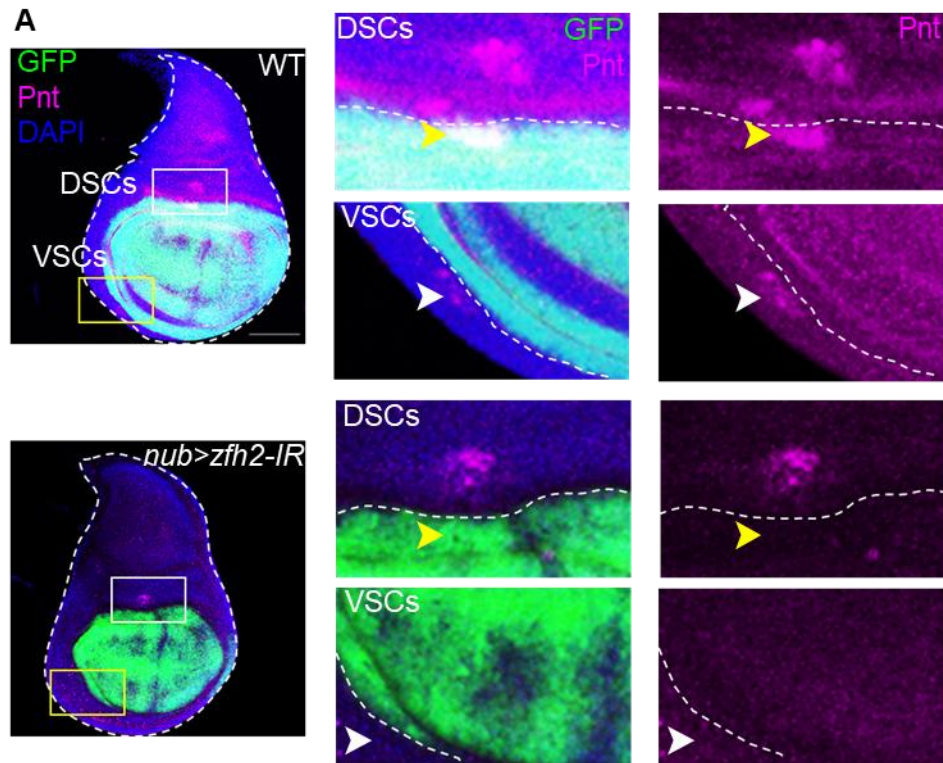


Figure 3-35. *zfh2-RNAi* represses expression of *pnt*. (A) Wild-type or *nub>zfh2-RNAi* wing disc with *pnt-lacZ* was stained with anti- β -gal antibody. Yellow arrowheads indicate DSCs and VSCs. Scale bars, 50 μ m. (Adapted from Zang et al, 2025)

Additionally, the loss of *zfh2* led to severe impairment in the development of the campaniform sensilla (Figure 3-36A, quantified in B).

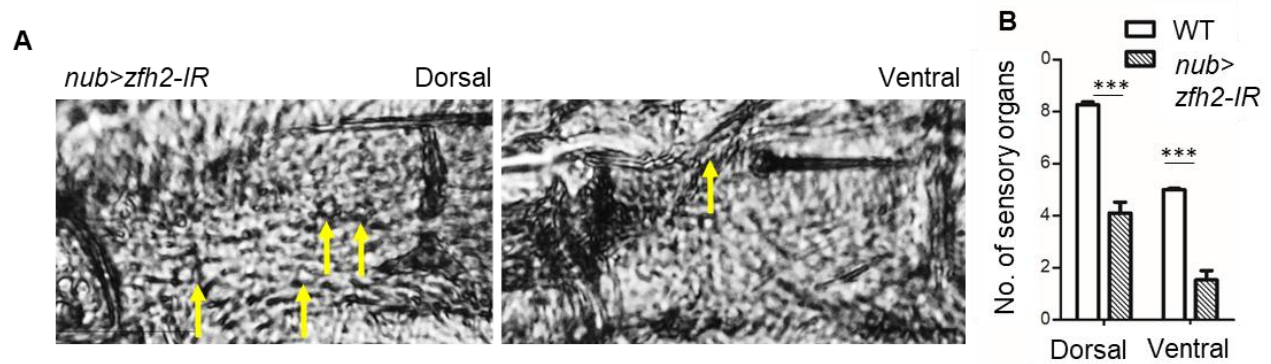


Figure 3-36. The number of campaniform sensilla is reduced by *zfh2*-RNAi. (A, B) Campaniform sensilla (arrowheads) on dorsal and ventral radii of *nub>zfh2-RNAi* fly. The number of campaniform sensilla was counted and quantified (B). Data were collected as mean $\pm$ s.e., $n=52$ (wild-type dorsal radius), $n=19$ (*nub>zfh2-RNAi* dorsal radius), $n=29$ (wild-type ventral radius), and $n=19$ (*nub>zfh2-RNAi* ventral radius). Student's *t*-test, *** $P < 0.001$. Scale bars, 50 μ m. (Adapted from Zang et al, 2025)

Overall, these findings indicate that *zfh2* mediates *pnt*-dependent programmed cell senescence, which is triggered by Ras signaling. Moreover, programmed cell senescence is crucial for the proper development of campaniform sensilla (Figure 3-37A).

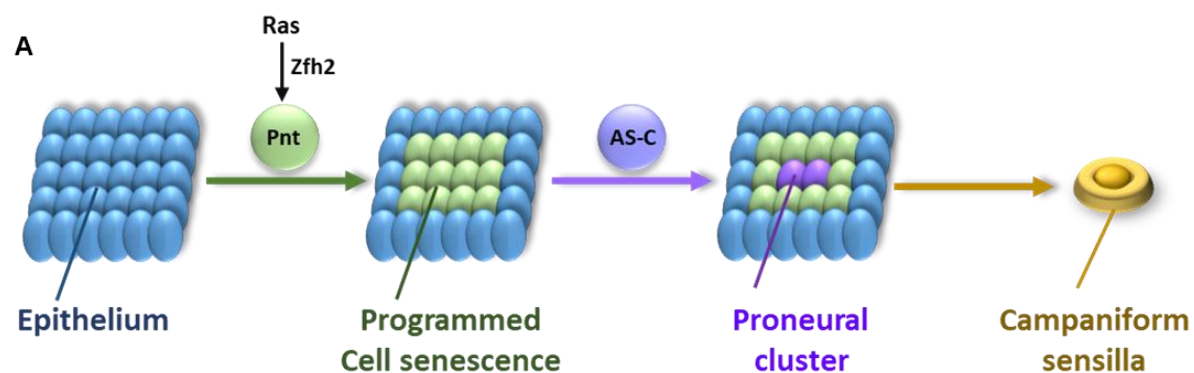


Figure 3-37. Schematic representation of campaniform sensilla development via programmed cell senescence. *Zfh2*-mediated Ras-*Pnt* signaling induces programmed cell senescence, which promotes development of campaniform sensilla on dorsal and ventral radii via AS-C.

Chapter 4 Discussion

4-1 Programmed cell senescence is conserved from vertebrate to invertebrate

In this study, I report that two distinct clusters of cells within *Drosophila* wing discs, which later give rise to campaniform sensilla on the adult hinge radius, undergo programmed cell senescence during larval and pupal development. My findings indicate that programmed cell senescence is a conserved developmental process conserved from invertebrates to vertebrates. Genetic analyses suggest that in the wing discs, programmed cell senescence is closely integrated with the development of a specialized type of campaniform sensilla through the Ras-Zfh2-Pnt signaling pathway. This parallels the role of programmed cell senescence in mammals, where it is similarly essential for normal tissue development (Muñoz-Espín et al., 2013; Storer et al., 2013).

Programmed senescent cells in *Drosophila* exhibit several key features of cellular senescence, including increased SA- β -gal activity, cell cycle arrest, heterochromatinization, upregulation of the p21 homolog *dacapo*, oxidative stress, cellular hypertrophy, *upd3*, and Ras-ERK signaling activation. However, they do not show a detectable DNA damage response (data not shown), which is consistent with findings in vertebrate programmed cell senescence (Muñoz-Espín et al., 2013; Storer et al., 2013). In mouse embryos, ERK signaling plays a crucial role in regulating programmed cell senescence and AER patterning via paracrine mechanisms (Storer et al., 2013). In contrast, in amphibians, programmed cell senescence occurs independently of ERK signaling (Davaapil et al., 2017). In *Drosophila* imaginal epithelium, pERK is detected not only in senescent cells but also in non-senescent cells, suggesting that while pERK may be important in certain contexts, it is neither necessary nor sufficient for inducing cellular senescence.

A key distinction between *Drosophila* and mammalian programmed senescence lies in cell fate. In mammals, senescent cells in the AER are removed through apoptosis and macrophage-mediated clearance (Storer et al., 2013). However, in *Drosophila* wing discs, programmed senescent cells persist at least until the pupal stage. After eclosion, *Drosophila* wings contain hemocytes (Kiger et al., 2001) that strongly stain positive for SA- β -gal, and the adult wings exhibit autofluorescence

similar to SPiDER, complicating senescence analysis in adult tissues using SPiDER or X-gal staining. Interestingly, I found that Pnt-expressing cell clusters remain in the adult hinge radii. These findings suggest that although programmed cell senescence is essential for proper tissue development, its fate and regulatory mechanisms differ between *Drosophila* and vertebrates.

4-2 Programmed cell senescence contribute to campaniform sensilla development in *Drosophila*

Inhibiting programmed cell senescence resulted in the loss of campaniform sensilla in both dorsal and ventral radius. Interestingly, campaniform sensilla are also present in adult legs and halteres (Cole and Palka, 1982; Smith and Shepherd, 1996), where I also observed SA- β -gal-positive cells in the leg and haltere discs. Structurally, campaniform sensilla consists of a dome-shaped cuticular structure with an underlying sensory neuron. When flies move or experience mechanical forces, the dome deforms, activating the sensory neuron and enabling the detection of mechanical changes. This function is essential for maintaining balance, posture, and coordinated wing movement, allowing flies to walk and fly effectively.

A distinct morphological feature of campaniform sensilla, compared to other mechanoreceptors like macrochaetes or microchaetes, is the formation of caps rather than hair-like structures. This unique structural adaptation may arise through programmed cell senescence-mediated tissue remodeling, similar to processes observed in developing mice. Notably, my findings indicate that senescent cells in *Drosophila* upregulate the SASP factor Upd3, which may contribute to the specialized structure of campaniform sensilla. Future studies using senolytic drug treatments in flies could provide further insights into the role of programmed cell senescence across different tissues and organs. Although my attempt to eliminate DSCs and VSCs in wing discs using the senolytic drug Navitoclax was unsuccessful, alternative senolytic treatments or modified experimental conditions may offer deeper insights into the developmental roles of programmed cell senescence.

In mammals, programmed cell senescence and oncogene-induced senescence share common signaling pathways for their induction (Muñoz-Espín and Serrano, 2014). Interestingly, my

analysis in *Drosophila* revealed that programmed cell senescence is also triggered by Ras signaling, a key regulator of oncogene-induced senescence. Furthermore, I identified Zfh2 as a novel mediator that regulates both programmed and oncogene-induced senescence in *Drosophila*. These findings not only highlight the evolutionary conservation of programmed cell senescence but also reveal mechanistic parallels between programmed and oncogene-induced senescence. This expanded understanding may contribute to insights into various senescence-related diseases in humans.

4-3 Future investigation

My findings show that programmed cell senescence in *Drosophila* wing discs is triggered by the Ras-Pnt-Zfh2 pathway and plays an important role in forming the adult sensory organ, campaniform sensilla. When I observed JNK activity in these senescent cells, I didn't detect any signs of activation. Similarly, my analysis of JAK-STAT signaling in the wing disc revealed that, rather than being active in the senescent cells themselves, JAK-STAT activity was only seen in the surrounding cells. This suggests that Zfh2 in senescent cells may not be controlled by JAK-STAT signaling as previously thought. However, the roles of both JNK and JAK-STAT in programmed senescence are still unclear.

In the future, it will be important to track senescent cells from the larval wing disc stage to their role in adult sensory organ development, though current technical challenges make this difficult. Another key question is how exactly programmed cell senescence helps shape campaniform sensilla. Additionally, JNK signaling could still be involved in senescence through other mechanisms, and JAK-STAT activity in surrounding cells might have indirect effects on the process. Addressing these questions will help us better understand the link between programmed senescence and organ development.

Bibliography

- Aldaz, S., Escudero, L.M., and Freeman, M. (2010). Live imaging of *Drosophila* imaginal disc development. *Proc. Natl. Acad. Sci.* *107*, 14217–14222.
- Brandl, A., Meyer, M., Bechmann, V., Nerlich, M., and Angele, P. (2011). Oxidative stress induces senescence in human mesenchymal stem cells. *Exp. Cell Res.* *317*, 1541–1547.
- Capparelli, C., Chiavarina, B., Whitaker-Menezes, D., Pestell, T.G., Pestell, R.G., Hult, J., Andò, S., Howell, A., Martinez-Outschoorn, U.E., Sotgia, F., et al. (2012). CDK inhibitors (p16/p19/p21) induce senescence and autophagy in cancer-associated fibroblasts, “fueling” tumor growth via paracrine interactions, without an increase in neo-angiogenesis. *Cell Cycle* *11*, 3599–3610.
- De Cecco, M., Jeyapalan, J., Zhao, X., Tamamori-Adachi, M., and Sedivy, J.M. (2011). Nuclear protein accumulation in cellular senescence and organismal aging revealed with a novel single-cell resolution fluorescence microscopy assay. *Aging (Albany, NY)* *3*, 955–967.
- Classen, A., Aigouy, B., Giangrande, A., and Eaton, S. (2008). Imaging *Drosophila* Pupal Wing Morphogenesis. In *Methods in Molecular Biology: Drosophila: Methods and Protocols*, pp. 265–275.
- Cole, B.E.S., and Palka, J. (1982). The pattern of campaniform sensilla on the wing and haltere of *Drosophila melanogaster* and several of its homeotic mutants. *J. Embryol. Exp. Morphol.* *71*, 41–61.
- Collado, M., Blasco, M.A., and Serrano, M. (2007). Cellular Senescence in Cancer and Aging. *Cell* *130*, 223–233.
- Culí, J., Martín-Blanco, E., and Modolell, J. (2001). The EGF receptor and N signalling pathways act antagonistically in *Drosophila* mesothorax bristle patterning. *Development* *128*, 299–308.
- Davaapil, H., Brockes, J.P., and Yun, M.H. (2017). Conserved and novel functions of programmed cellular senescence during vertebrate development. *Development* *144*, 106–114.
- Davies, D.M., van den Handel, K., Bharadwaj, S., and Lengefeld, J. (2022). Cellular enlargement - A new hallmark of aging? *Front. Cell Dev. Biol.* *10*, 1–17.
- Debacq-Chainiaux, F., Erusalimsky, J.D., Campisi, J., and Toussaint, O. (2009). Protocols to detect senescence-associated beta-galactosidase (SA- β gal) activity, a biomarker of senescent cells in culture and in vivo. *Nat. Protoc.* *4*, 1798–1806.
- Demaria, M., Ohtani, N., Youssef, S.A., Rodier, F., Toussaint, W., Mitchell, J.R., Laberge, R.M., Vijg, J., VanSteeg, H., Dollé, M.E.T., et al. (2014). An essential role for senescent cells in optimal wound healing through secretion of PDGF-AA. *Dev. Cell* *31*, 722–733.
- Doggett, K., Turkel, N., Willoughby, L.F., Ellul, J., Murray, M.J., Richardson, H.E., and Brumby, A.M. (2015). BTB-zinc finger oncogenes are required for ras and notch-driven tumorigenesis in *drosophila*. *PLoS One*.

- Faraonio, R. (2022). Oxidative Stress and Cell Senescence Process. *Antioxidants* *11*, 13–15.
- Furman, D., and Bukharina, T. (2008). How *Drosophila melanogaster* Forms its Mechanoreceptors. *Curr. Genomics* *9*, 312–323.
- Hayflick, L., and Moorhead, P.S. (1961). The serial cultivation of human diploid cell strains. *Exp. Cell Res.* *25*, 585–621.
- Ito, T., and Igaki, T. (2021). Yorkie drives Ras-induced tumor progression by microRNA-mediated inhibition of cellular senescence. *Sci. Signal.* *14*, 1–14.
- Kang, T.W., Yevsa, T., Woller, N., Hoenicke, L., Wuestefeld, T., Dauch, D., Hohmeyer, A., Gereke, M., Rudalska, R., Potapova, A., et al. (2011). Senescence surveillance of pre-malignant hepatocytes limits liver cancer development. *Nature* *479*, 547–551.
- Kiger, J.A., Natzle, J.E., and Green, M.M. (2001). Hemocytes are essential for wing maturation in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci.* *98*, 10190–10195.
- Klambt, C. (1993). The *Drosophila* gene pointed encodes two ETS-like proteins which are involved in the development of the midline glial cells. *Development* *117*, 163–176.
- Krtolica, A., Parrinello, S., Lockett, S., Desprez, P.Y., and Campisi, J. (2001). Senescent fibroblasts promote epithelial cell growth and tumorigenesis: A link between cancer and aging. *Proc. Natl. Acad. Sci. U. S. A.* *98*, 12072–12077.
- Kuilman, T., Michaloglou, C., Mooi, W.J., and Peeper, D.S. (2010). The essence of senescence. *Genes Dev.* *24*, 2463–2479.
- Lage, P., Jan, Y.N., and Jarman, A.P. (1997). Requirement for EGF receptor signalling in neural recruitment during formation of *Drosophila* chordotonal sense organ clusters. *Curr. Biol.* *7*, 166–175.
- Liu, X. ling, Ding, J., and Meng, L. hua (2018). Oncogene-induced senescence: a double edged sword in cancer. *Acta Pharmacol. Sin.* *39*, 1553–1558.
- Meier, A., Fiegler, H., Muñoz, P., Ellis, P., Rigler, D., Langford, C., Blasco, M.A., Carter, N., and Jackson, S.P. (2007). Spreading of mammalian DNA-damage response factors studied by ChIP-chip at damaged telomeres. *EMBO J.* *26*, 2707–2718.
- Moujaber, O., Fishbein, F., Omran, N., Liang, Y., Colmegna, I., Presley, J.F., and Stochaj, U. (2019). Cellular senescence is associated with reorganization of the microtubule cytoskeleton. *Cell. Mol. Life Sci.* *76*, 1169–1183.
- Muñoz-Espín, D., and Serrano, M. (2014). Cellular senescence: From physiology to pathology. *Nat. Rev. Mol. Cell Biol.* *15*, 482–496.
- Muñoz-Espín, D., Cañamero, M., Maraver, A., Gómez-López, G., Contreras, J., Murillo-Cuesta, S.,

- Rodríguez-Baeza, A., Varela-Nieto, I., Ruberte, J., Collado, M., et al. (2013). Programmed cell senescence during mammalian embryonic development. *Cell* *155*.
- Nacher, V., Carretero, A., Navarro, M., Armengol, C., Llombart, C., Rodríguez, A., Herrero-Fresneda, I., Ayuso, E., and Ruberte, J. (2006). The quail mesonephros: A new model for renal senescence? *J. Vasc. Res.* *43*, 581–586.
- Nakamura, M., Ohsawa, S., and Igaki, T. (2014). Mitochondrial defects trigger proliferation of neighbouring cells via a senescence-associated secretory phenotype in *Drosophila*. *Nat. Commun.* *5*, 1–11.
- Narita, M., Núñez, S., Heard, E., Narita, M., Lin, A.W., Hearn, S.A., Spector, D.L., Hannon, G.J., and Lowe, S.W. (2003). Rb-mediated heterochromatin formation and silencing of E2F target genes during cellular senescence. *Cell* *113*, 703–716.
- Ohtani, N. (2022). The roles and mechanisms of senescence-associated secretory phenotype (SASP): can it be controlled by senolysis? *Inflamm. Regen.* *42*.
- Ohtani, N., Zebedee, Z., Huot, T.J.G., Stinson, J.A., Sugimoto, M., Ohashi, Y., Sharrocks, A.D., Peters, G., and Hara, E. (2001). Opposing effects of Ets and Id proteins on p16INK4a expression during cellular senescence. *Nature* *409*, 1067–1070.
- Roch, F., Jiménez, G., and Casanova, J. (2002). EGFR signalling inhibits Cic-dependent repression.
- Scholz, H., Deatrick, J., Klaes, A., and Klambt, C. (1993). Genetic dissection of pointed, a *Drosophila* gene encoding two ETS-related proteins. *Genetics* *135*, 455–468.
- Schumacher, B., Pothof, J., Vijg, J., and Hoeijmakers, J.H.J. (2021). The central role of DNA damage in the ageing process. *Nature* *592*, 695–703.
- Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D., and Lowe, S.W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* *88*, 593–602.
- Shay, J., Wright, W. (2000). Hayflick, his limit, and cellular ageing. *Nat. Rev. Mol. Cell Biol.* *1*, 72–76.
- Shreeya, T., Ansari, M.S., Kumar, P., Saifi, M., Shati, A.A., Alfaifi, M.Y., and Elbehairi, S.E.I. (2023). Senescence: A DNA damage response and its role in aging and Neurodegenerative Diseases. *Front. Aging* *4*, 1–12.
- Shwartz, A., Yogev, S., Schejter, E.D., and Shilo, B.Z. (2013). Sequential activation of ETS proteins provides a sustained transcriptional response to EGFR signaling. *Development* *140*, 2746–2754.
- Smith, S.A., and Shepherd, D. (1996). Central afferent projections of proprioceptive sensory neurons in *Drosophila* revealed with the enhancer-trap technique. *J. Comp. Neurol.* *364*, 311–323.
- Storer, M., Mas, A., Robert-Moreno, A., Pecoraro, M., Ortells, M.C., Di Giacomo, V., Yosef, R., Pilpel, N., Krizhanovsky, V., Sharpe, J., et al. (2013). Senescence is a developmental mechanism that

contributes to embryonic growth and patterning. *Cell* *155*, 1119–1130.

Terriente, J., Perea, D., Suzanne, M., and Díaz-Benjumea, F.J. (2008). The *Drosophila* gene *zfh2* is required to establish proximal-distal domains in the wing disc. *Dev. Biol.* *320*, 102–112.

Varela-Eirín, M., and Demaria, M. (2022). Cellular senescence. *Curr. Biol.* *32*, R448–R452.

Xue, W., Zender, L., Miething, C., Dickins, R.A., Hernando, E., Krizhanovsky, V., Cordon-Cardo, C., and Lowe, S.W. (2007). Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature* *445*, 656–660.

Yoshimoto, S., Loo, T.M., Atarashi, K., Kanda, H., Sato, S., Oyadomari, S., Iwakura, Y., Oshima, K., Morita, H., Hattori, M., et al. (2013). Obesity-induced gut microbial metabolite promotes liver cancer through senescence secretome. *Nature* *499*, 97–101.

Zhao, Y., Tyshkovskiy, A., Muñoz-Espín, D., Tian, X., Serrano, M., De Magalhaes, J.P., Nevo, E., Gladyshev, V.N., Seluanov, A., and Gorbunova, V. (2018). Naked mole rats can undergo developmental, oncogene-induced and DNA damage-induced cellular senescence. *Proc. Natl. Acad. Sci. U. S. A.* *115*, 1801–1806.

Zielke, N., Korzelius, J., vanStraaten, M., Bender, K., Schuhknecht, G.F.P., Dutta, D., Xiang, J., and Edgar, B.A. (2014). Fly-FUCCI: A Versatile Tool for Studying Cell Proliferation in Complex Tissues. *Cell Rep.* *7*, 588–598.

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