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The role of anastasis in the regeneration of the *Drosophila melanogaster* wing disc

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by

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Abstract

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By

Rebecca Cheng

Apoptosis is the process of highly regulated programmed cell death that utilizes caspase-3 to initiate the executioner phase of apoptosis. However, under certain circumstances, cells are able to survive its activation through a process called “anastasis”. Anastasis has been documented to occur in several cell lines and in *Drosophila melanogaster*. However, the purpose of anastasis and how it is regulated are currently unknown. In Drosophila, anastasis can be visualized using a previously created biosensor, CasExpress, that permanently marks cells that have survived caspase-3 activation with GFP. CasExpress can detect anastasis in Drosophila wing disc during normal development. Since Drosophila wing discs have been used as a model for studying regeneration, it would be beneficial to use this model to investigate if anastasis occurs during tissue regeneration. Using the CasExpress system, we found that anastasis occurs during regeneration in response to reaper-induced tissue damage. To determine if anastasis is required for regeneration, overexpression of reaper in cells that have undergone caspase-3 activation results in a decrease in the number of CasExpress positive cells. We found that the removal of cells that survive caspase-3 activation during tissue ablation does not manifest in a significant change in wing size, possibly due to the incomplete killing of anastatic cells. To start unraveling how anastasis is regulated, CasExpress was utilized in screening the requirement of several genes known to function in wound healing.

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Chapter I: Introduction

1. Background on apoptosis

Programmed cell death is fundamental for embryogenesis, tissue homeostasis, and the immune response system. The most well studied form of programmed cell death is apoptosis. The word apoptosis originates from the Greek word απόπτωση, meaning “falling off”. It was first described in Kerr et al. as a mechanism for cell deletion that is a series of morphological changes that takes place in two stages [1]. The first stage is comprised of nuclear and cytoplasmic condensation and the fragmentation of the cell into multiple membrane bound compartments. Additional morphological characteristics have since then been defined, these include membrane blebbing, chromatin condensation, and chromosomal DNA fragmentation [2]. In the second stage, these apoptotic bodies are taken up by phagocytes and then degraded by lysosomal enzymes. This mode of elimination provides safe disposal of damaged DNA and prevents an inflammatory response [3]. Apoptosis has a key role in the proper development of a multicellular organism. The absence of apoptosis in the blastocyst results in the compromise of future maturation and can lead to early embryonic death or the formation of abnormalities in the fetus [57]. Apoptotic cell death is carried out by a family of cysteine-aspartate proteases known as caspases [4].

Biochemistry of apoptosis-caspases, extrinsic vs intrinsic apoptosis

Caspase zymogens are single chain proteins, with an N-terminal prodomain that precedes the conserved catalytic site. During activation, the catalytic domains are cleaved to form subunits that interact with each other. The activation of caspases occurs by a conserved mechanism that is subject to strict regulation, as once they are activated, they cleave a wide variety of cellular substrates, thus leading to rapid cell death [5]. Caspases with apoptotic roles are classified into two categories, initiators and executioners. Initiator caspases are apical caspases in apoptosis signaling cascades and are activated in response to death inducing stimuli. The pool of initiator caspase substrates is limited and includes self cleavage, BCL-2 homology 3 (BH3)-interacting domain death agonist (BID), and executioner caspases. Executioner caspases are the proteins that cleave hundreds of different substrates and are largely responsible for the phenotypic changes seen during apoptosis [6]. Caspases can be activated in two ways- an extrinsic pathway through external signals received from receptors on the cell's membrane or intrinsically within the cell [7].

Mitochondrial role in intrinsic apoptosis

The extrinsic pathway of apoptosis is activated by binding of death ligands to receptors on the surface of the cell. Once activated, these death receptors, such as tumor necrosis factor (TNF) related apoptosis inducing ligand receptor (TRAILR) and FAS, can activate initiator caspases through dimerization mediated by adaptor proteins [8]. On the other hand, the intrinsic pathway of apoptosis can be triggered by damage induced by heat shock, radiation, hypoxia, or exposure to cytotoxic

drugs. In addition to these stimuli, this pathway can also be induced when there is a lack of pro-survival factors such as nutrients or hormones [10]. The intrinsic apoptotic death is dependent on the permeabilization of the mitochondrial outer membrane [11]. Once the outer membrane is permeabilized, proteins found between the inner and outer membranes of the mitochondria are released. These proteins include cytochrome C, Smac/DIABLO, and endonuclease G. Once in the cytosol, these proteins trigger the execution of cell death by inducing apoptosome formation [12]. The apoptosome consists of cytochrome c and Apaf-1 (apoptotic protease activating factor 1). Once formed, it will activate initiator caspase-9 to trigger the activation of executioner caspases (3, 7, and 6) [58]. Both pathways of apoptosis are regulated by a family of proteins called inhibitor of apoptosis proteins (IAP). IAPs bind to both executioner and initiator caspases, preventing apoptosis from occurring [9].

Drosophila apoptosis

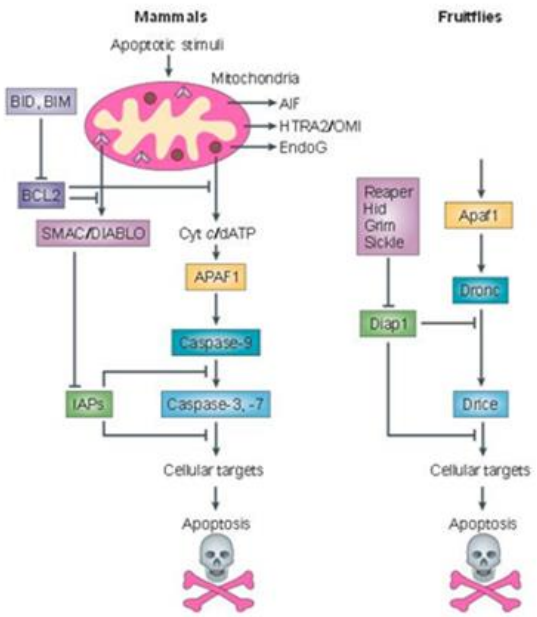
Many features of the regulation and execution of apoptosis are highly conserved in Drosophila melanogaster, making it a good model organism to study apoptosis (Figure1). Drosophila contain all of the canonical apoptosome proteins, including orthologs of caspase 9, Apaf-1, and cytochrome c. The caspases in Drosophila that are required for normal somatic apoptosis are the initiator caspase Dronc and the executioner caspases DrICE and Dcp-1. To negatively regulate apoptosis, the drosophila inhibitor of apoptosis protein, Diap1, is expressed and inhibits the activation of the caspase cascade by binding to caspase proteins. When

a cell receives an apoptotic stimulus, the pro-apoptotic proteins Reaper, Hid, and Grim, which function similarly as DIABLO/Smac, trigger the ubiquitin mediated degradation of Diap1. The degradation of Diap1 releases Dronc from inhibition, allowing it to proteolytically cleave and activate DrICE and Dcp-1, triggering the induction of apoptosis [13].

Biochemical markers of apoptosis

In Drosophila, deletion of the reaper, hid, and grim genes results in the blocking of apoptosis [14]. Apoptosis can be induced through the expression of the reaper, hid, and grim [15]. In particular, the reaper gene is transcriptionally activated in response to many different types of pro-apoptotic signals. These signals include steroid hormones, irradiation, developmental signals, and a variety of cellular stresses or injury [15-18]. Reaper, hid, and grim all contain an N-terminal peptide motif, IAP binding motif (IBM), which is required for IAP binding and cell killing [19]. Proteins with IBM domains, such as Smac/DIABLO and Omi/HtrA2, have also been discovered in mammals. Like in Drosophila, these IBM proteins bind to IAP, inhibiting them and allowing for apoptosis. However, unlike in Drosophila, the known mammalian IBM proteins are ubiquitously expressed in the mitochondria and are only released into the cytosol upon the onset of apoptosis [20].

Figure 1: Molecular mechanisms of apoptosis are conserved in mammals and fruit flies. Figure taken from Molecular mechanisms of caspase regulation during apoptosis [61].



Role of apoptosis in development and disease

Proper regulation of apoptosis is critical in development and tissue homeostasis. Uncontrolled or excessive apoptosis can lead to degenerative disease such as Alzheimer's, whereas lack of cell death is a hallmark of various cancers [21]. Understanding apoptosis in the context of diseases is important, as it gives insight into how diseases can be treated. The control of cell number in a mature organism is through the net result of cell proliferation and cell death signals. If a cell does not receive the appropriate signals, cell accumulation results from a failure to undergo apoptosis. Cancer is characterized by a lack of apoptosis; cancerous cells develop methods of ignoring the cellular signals regulating their growth, allowing them to be more proliferative than normal. The downregulation of caspase-9 was commonly found in patients with stage II colorectal cancer and it correlated with poor patient outcomes [22]. Another study found that in samples of breast, ovarian, and cervical

tumors, there was an overall low level of caspase-3 mRNA. These findings suggest that loss of caspase-3 activity contributes to breast cancer cell survival [23]. In addition to its effect in cancer survival, it is necessary to understand the role of apoptosis because there is increasing evidence of cellular survival from caspase activation.

2. Evidence of survival from caspase

Although most instances of caspase activation result in cell death, caspases also have nonapoptotic roles in controlling cell migration, proliferation, differentiation, and cell shape. Spermatogenesis is a well studied instance in development where the cell survives caspase activation. The final stage of spermatid differentiation requires the removal of a bulk of the cytoplasm. This requires the activation of caspases and most notably, caspase inhibitors prevent the removal of the cytoplasm, blocked sperm maturation, and results in sterile males [24].

In addition to having nonapoptotic roles, in some instances, caspase activation is required for regeneration of tissues. Several animal tissues such as Drosophila larval imaginal discs, Xenopus tadpole tails, and zebrafish hearts have the ability to regenerate when damaged. When these tissues are damaged, apoptosis is a necessary step to induce regenerative proliferation [26,27]. Upon healing, the size and shape of the final tissue often match those of uninjured tissue due to compensatory proliferation. Without apoptosis, the cell proliferation regulators in Drosophila, Wingless and Decapentaplegic, are ectopically expressed and the tissue size is much larger than normal [28].

Conventionally it is thought that once caspases are activated, cell death is inevitable. However, there has been increasing evidence that cells can undergo caspase activation and exhibit other hallmarks of apoptosis and yet survive. In several instances, using various different sublethal insults to cells, survival from activation of caspase has been reported. Liu et al. showed that mammalian cells exposed to ionizing radiation are able to survive with persistent caspase-3 activation, promoting genetic instability and oncogenic transformation [29]. In addition, cell survival following executioner caspase cleavage has also been reported in neurons overexpressing Tau [42]. Ichim et al. demonstrated that a minority of mitochondria can undergo MOMP and trigger the activation of caspase without killing the cell [30]. Annexin V positive cells (a molecular hallmark of apoptosis) were found to survive transient myocardial ischemia in mice [59]. Cells that survived treatment with the death ligands TRAIL and FasL were found to have DNA damage [31]. These studies utilized sublethal insults to cells, however survival from the brink of apoptosis has been observed after transient exposure to potentially lethal doses of stimulants.

Anastasis

In 2012, several cell lines were observed to survive caspase activation following transient exposure to a lethal dose of an apoptotic stimulus. Once the apoptotic stimulus was washed away, a significant population of cells survived and proliferated, even after displaying traditional hallmarks of apoptosis, such as activation of caspase-3, the cleavage of its downstream target PARP1, mitochondrial fragmentation, and nuclear condensation. This phenomenon was named “anastasis”

[32]. Anastasis was observed to occur in several different cell lines including HeLa, NIH 3T3, macrophages, and primary mouse fibroblasts. In addition to occurring in different cell lines, this phenomenon has been observed in several different apoptotic inducers such as ethanol, DMSO, and staurosporine. It was reported that a small fraction of cells undergo an oncogenic transformation. It is hypothesized that anastasis may serve as a mechanism to preserve cells that are difficult to replace such as neurons and cardiomyocytes. On the other hand, anastasis could be a mechanism cancer cells use to evade death.

In order to understand the role of anastasis in the normal development of an animal, Ding, Sun et al. studied the process in Drosophila. Anastasis was observed in Drosophila through a reporter expression system called CasExpress, which marks the cells that survive caspase activation with fluorescent proteins. The system showed widespread survival from caspase activity. It was shown that in every animal, some cells activated caspase during their normal development without evidence of apoptosis, while in other tissues, expression was sporadic both spatially and temporally and overlapped with developmental apoptosis [33]. In adult flies, reporter expression was present in most tissues, but the precise pattern varied between individuals, suggesting that cells in a population vary in their sensitivity to apoptosis.

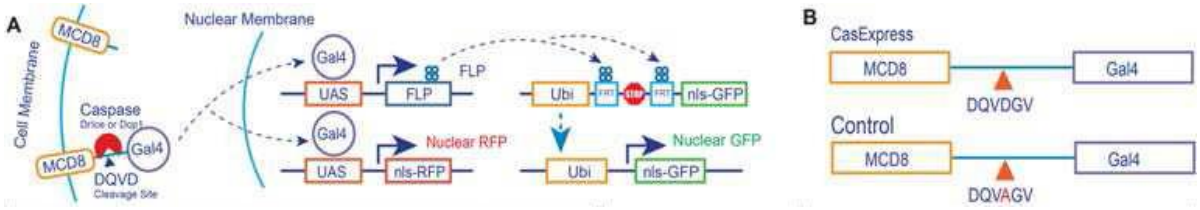
CasExpress system

Cells that have undergone anastasis look morphologically similar to cells that have not undergone anastasis. The CasExpress system utilizes the GAL4-UAS

system to temporarily and permanently mark cells that have undergone caspase activation (Figure 2). In CasExpress flies, a caspase-inducible Gal4 transcription factor was expressed under the control of an ubiquitin promoter to allow for the detection of caspase activation in as many cell types as possible. To keep Gal4 inactive in cells without caspase-3 activity, it was tethered to the plasma membrane by fusing it to mCD8, a transmembrane glycoprotein. To make the protein caspase-inducible, a caspase-3 binding and cleavage domain (DQVD) from the Drosophila Inhibitor of Apoptosis Protein 1 (DIAP1) was inserted in between the CD8 and Gal4. These sensor flies were then crossed to G-Trace flies, a fly line that expresses RFP and GFP under the control of UAS, as Gal4 responsive enhancer. G-Trace flies contain three transgenes: UAS-RFP, UAS-FLP, which encodes a yeast recombinase enzyme, and ubi-FRT-STOP-FRT-GFP, where FRT is a FLP Recombination Target sequence. By crossing the DQVD sensor flies to G-Trace flies, cells that have survived transient caspase activation will be marked with transient RFP expression and permanent GFP expression. Subsequently, their progeny will also have GFP [33].

The activity of CasExpress can be by temperature sensitive Gal80 ($Gal80^{ts}$). Gal80 binds to Gal4 and inhibits the activity of Gal4 and the transcription of downstream genes. The activity of $Gal80^{ts}$ is controlled by temperature. At 18C, the Gal80 activity is the highest, and Gal4 is suppressed. At 30C, Gal80 is degraded and Gal4 activity is restored.

Figure 2: Schematic of CasExpress. Figure taken from CasExpress reveals widespread and diverse patterns of cell survival of caspase-3 activation during development in vivo [33].



3. The wing disc as a model for regeneration

One set of tissues where anastasis was found to occur was the imaginal discs [33]. The imaginal discs in Drosophila larvae will develop into various adult structures, including the wings, legs, eyes, and antennae. The imaginal discs have long been used as a model for regeneration studies due to their capacity to replace lost tissue when damaged. In particular, the wing disc model for studying regeneration has been well characterized, with early experiments involving fragmentation of the imaginal discs and culture in host animals [38].

A series of experiments done by Ernst Hadorn and his colleagues from 1940-1970 laid the groundwork for the current understanding of regeneration in Drosophila imaginal discs. Disc fragments were implanted into third instar larvae prior to pupation. While little regeneration was observed when implanting disc fragments into older larvae, some regeneration was observed following implantation of disc fragments into younger larvae [40]. Later, it was demonstrated that imaginal discs will regenerate under physiological conditions when leg and wing imaginal discs were bisected by applying pressure to the disc through the cuticle [41]. However,

physically removing sections of the discs is not a viable method to ablate a large number of wing discs at once.

Later, genetic ablation was used in regeneration studies. Gal4/Gal80ts/UAS system was used to express a pro-apoptotic genes in a defined region at a specific developmental stage to ablate part of the wing discs. [34]. Cell death carried out by the expression of the pro-apoptotic gene *reaper* occurs rapidly within 10-24 hours of ablation [35]. The cellular debris from the apoptotic cells move basally, allowing wound healing to occur [36]. This system allows for the physical fragmentation of many wing discs at once by shifting temperature and for the recovery of the discs *in situ.*

Chapter II. The role of anastasis in tissue regeneration

Introduction

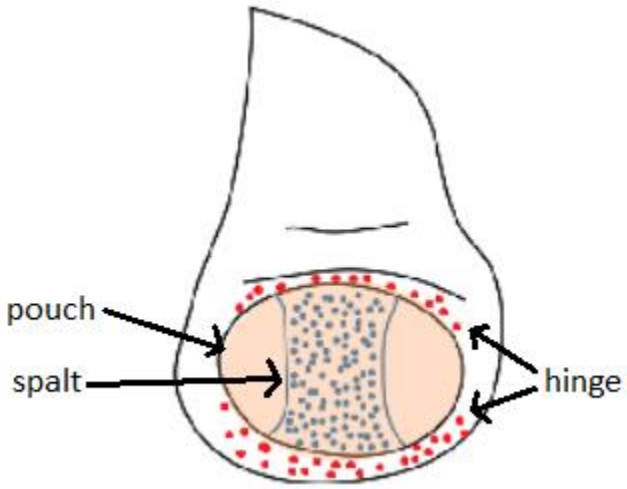
Caspase-3 cleavage activates apoptosis, however cell survival after incubation with a lethal stimulus has been found to occur under certain circumstances. The survival of caspase-3 activation was named “anastasis” and a system to mark cells that survive it was created in Drosophila. Widespread survival of caspase-3 activity was found during development and stress induced apoptosis. It is currently unknown what is the purpose of this process. One potential use for anastasis is to save cells under stress, so it would be beneficial to investigate if anastasis occurs during tissue regeneration.

The wing imaginal discs have long been used as a model for regeneration studies due to their capacity to replace lost tissue when damaged. The discs can replace large portions of lost tissue and dying cells. Even a loss of up to 60% of the cells in the wing disc can be compensated [39]. Cell loss in response to stress and damage can induce additional divisions of cells adjacent to the apoptotic cells and the additional proliferation results in adult wings of normal size. This is called compensatory proliferation and it has been observed after cell death induced by irradiation and genetic ablation in the Drosophila wing disc. Apoptotic cells release mitogenic signals to surrounding cells to promote tissue repair and compensatory proliferation. Initiator caspases have also been reported as required for compensatory proliferation in surrounding cells.

To test if anastasis occurs in ablated wing discs, we ablated part of the wing discs of CasExpress flies by expressing pro-apoptotic gene reaper (rpr) in the spalt-

expressing domain (Figure 3). The wing disc is composed of one layer of columnar epithelial cells and a squamous epithelium. The wing disc consists of the pouch, which will develop to the adult wing blade, the hinge, and the notum region. The spalt domain is a part of the pouch area (figure). Because CasExpress is controlled by Gal4-UAS, here we use LexA/LexO to express rpr. The lexA-lexO system works in the same way as the GAL4-UAS system, with lexA as the transcription factor and lexO as the operator for the gene of interest [53]. The LexA used in this study is a fusion protein contains the DNA binding domain of LexA and the regulatory domain of Gal4, so its activity can be regulated by Gal80ts. Using this system, we induce apoptosis in part of the wing disc at the mid-third instar, then detect the activity of CasExpress.

Figure 3: Morphology of a wing disc. The pouch domain is what develops into the adult wing of the fruitfly.



Methods and Materials

Fly strains

DQVD-Gal4/CyOGFP; lexO-rpr

G-trace/CyOGFP; $sal^{E/Pv}$-lexA $tubGal80^{ts}$/TM6B

DQVD-Gal4/G-trace; lexO-rpr/$sal^{E/Pv}$-lexA $tubGal80^{ts}$

DQVD-Gal4/UAS-rpr; lexO-rpr/$sal^{E/Pv}$-lexA $tubGal80^{ts}$

DQVD-Gal4/+; lexO-rpr/$sal^{E/Pv}$-lexA $tubGal80^{ts}$

DQVD UAS-Flp UAS-RFP/lexO-lacZFRTSTOPFRTGFP; $sal^{E/PV}$lexA$tubGal^{80ts}$/lexO-rpr

Induction of apoptosis in wing discs

DQVD-Gal4/CyOGFP; lexO-rpr female flies were crossed to G-trace/CyOGFP; $sal^{E/Pv}$-lexA $tubGal80^{ts}$/TM6B male flies and put into an 18°C incubator. After 3 days, the flies were transferred to embryo collection plates and embryos were collected every 4h at 25°C. After 4h of embryo collection, plates were transferred back to 18°C. After 2 days at 18°C, larvae were picked from the plate and 50 larvae were placed in each vial to avoid competition for nutrients. After 8 days at 18°C, the vials were transferred to a 29°C incubator, where 29°C is the permissive temperature for reaper expression, for 0, 8, 16, 24, or 48 hours to allow for reaper overexpression. DQVD-Gal4/G-trace; lexO-rpr/$sal^{E/Pv}$-lexA $tubGal80^{ts}$ larvae were then either dissected for their wing discs shortly afterwards or allowed to recover for 24 or 48 hours at 18°C and then dissected.

Immunostaining (imaging and quantification)

Larvae were sorted for size, TM6B and wild type. Wild type sized larvae were kept and then sorted for fluorescence under a Zeiss Axiozoom. RFP positive and GFP negative larvae were dissected in PBS. The larvae were inverted and fixed with 400ul of 4% PFA, washed twice with PBS, and incubated in 400ul of 0.2% PBST and 1ul Hoechst while covered with foil. Wing discs were dissected and mounted with 20ul of Vectashield.

Slides were imaged using a Zeiss LSM780 Confocal.

The ratio of RFP and GFP positive cells was measured using a macro in ImageJ.

Adult wing quantification

Adults flies were frozen at -20°C for at least 2 hours. Prior to dissection, flies were brought to room temperature. After equilibrating flies to the room temperature, flies were submerged in 500ul 100% ethanol to dehydrate them. Wings were dissected in the ethanol and then placed on a microscope slide to dry.

The wings were mounted with 15ul of 80% glycerol. After placing the coverslip on, a weight was placed onto the coverslip and left overnight to flatten.

The wings were imaged with a Zeiss Axiozoom at 30x magnification and the area of the wings was measured using ImageJ.

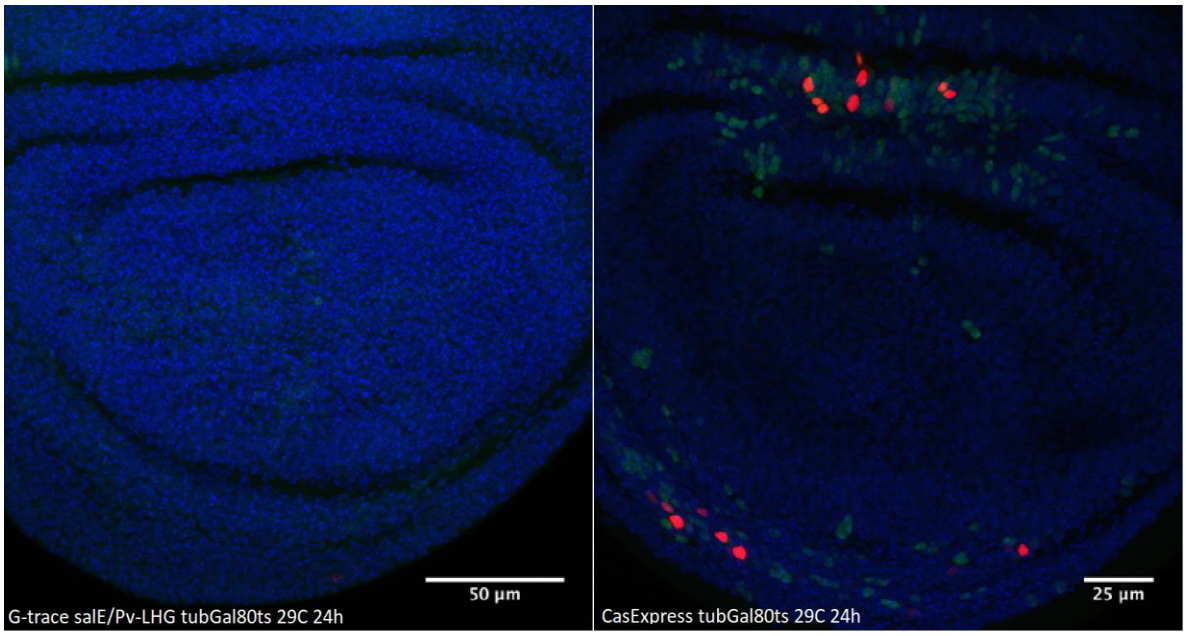
Results

Effect of reaper overexpression on CasExpress.

The hallmarks of apoptosis used to determine whether cell death had occurred were nuclear shrinkage that was visualized by staining with Hoechst and

activation of the biosensor. There was very little biosensor activation in the 0 hour time point and also very little cell death without ablation (Figure 4).

Figure 4: CasExpress and G-trace; sal$^{E/PV}$lexAtubGal80^{ts} wing discs after 24 hours at 29°C and stained with Hoechst show very little CasExpress or condensed nuclei.

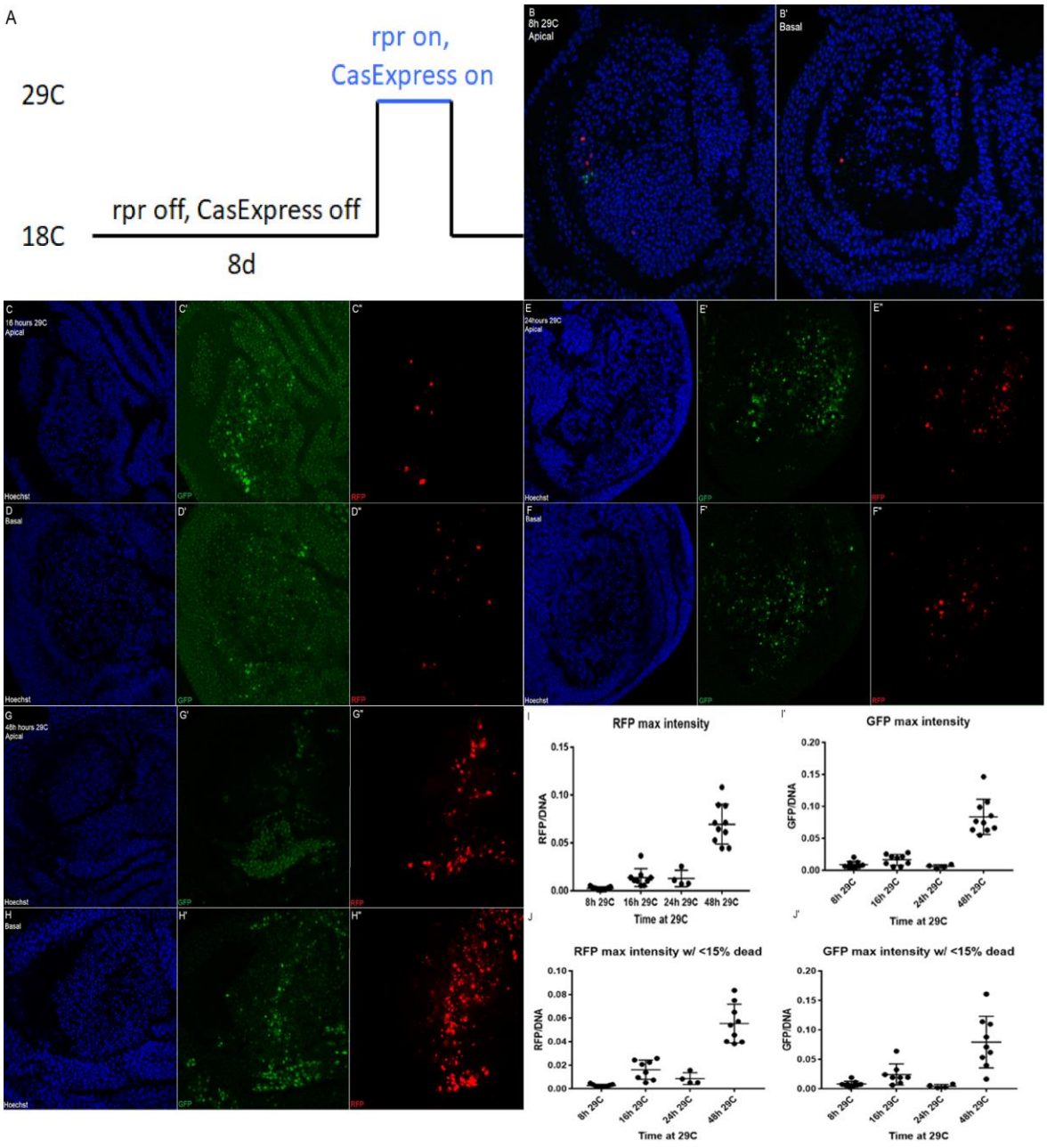


At 8 hours, there was very little CasExpress activation (Figure 5B). However, there was very obvious cell death, indicated by the nuclear shrinkage (Figure 5B'). As expected, as the amount of time allowed for reaper overexpression was increased, the number of dead cells and cells that were CasExpress positive also increased. Starting from 16 hours, the disc pouch became more folded as the dead cells drop off towards the basal layer (Figure 5C-D"). Most of the GFP at this time point colocalized with the condensed nuclei, suggesting that CasExpress is activated and then the cells die. At the 24 hour induction time point, RFP and GFP colocalized in the same cell and a majority of the cells that are only GFP positive had condensed

nuclei (Figure 5E-F”). Also, at 24 hours, a majority of the cells in the spalt region are dead. At 48 hours of ablation, there is massive cell death and a lot of folding in the pouch region (Figure 5G-H”).

Dead cells are extruded from the apical to the basal layer of the wing disc, resulting in the accumulation of dead cells in the basal layer. Therefore, to determine the ratio of GFP and RFP positive cells and quantify CasExpress expression, only the Z-stack images with less than 15% dead cells were counted. With the inclusion of this restriction, the same general trend of the ratio of GFP and RFP increasing as the length of induction increases was observed (Figure 5J,J’).

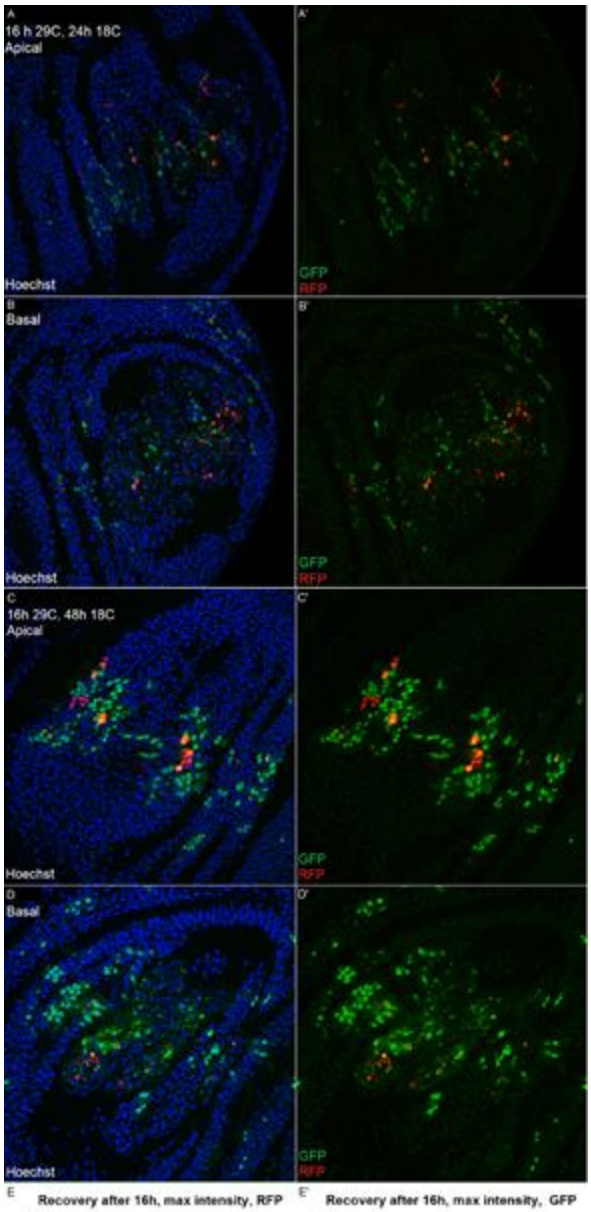
Figure 5: CasExpress activation from reaper expression for various time points. (A) Experimental design. (B-H’) Confocal images of DQVD-Gal4/G-trace; lexO-rpr/sal$^{E/Pv}$-lexA tubGal80^{ts} showing DAPI, RFP, and GFP expression in wing discs of flies incubated at 29°C for various time points. (I,I’) Ratio of RFP and GFP positive cells for B-H time points includes every Z stack. (J,J’) Ratio of RFP and GFP positive cells for B-H time points excluding Z stacks with >15% condensed nuclei.



To determine whether the GFP positive cells would eventually recover, larvae were moved back to 18°C for either 24 or 48 hours. For the samples that were allowed to recover, there was the expected decrease in RFP and an increase in GFP expression. After 24 hours of recovery from 16 hours of ablation, more than half of the GFP positive cells had normal sized nuclei (Figure 6A,A'). Furthermore, after 48

hours of recovery after 24 and 48 hours of ablation, there was a still some RFP expression, which was unusual, given that RFP would typically be gone at this point in the wild type (Figure 7C,C' and 8A,A'). A possibility for this explanation is that since the cells experience a prolonged caspase exposure, more RFP accumulated in the cells.

Figure 6: CasExpress activation from reaper expression for 16 hours and recovery for 24 or 48 hours. (A-D') Confocal images of DQVD-Gal4/G-trace; lexO-rpr/sal$^{E/Pv}$-lexA tubGal80^{ts} showing DAPI, RFP, and GFP expression in wing discs of flies incubated at 29°C for 16 hours and recovery after 24 or 48 hours at 18°C. (E,E') Ratio of RFP and GFP positive cells, includes every Z stack. (F,F') Ratio of RFP and GFP positive cells, excluding Z stacks with >15% condensed nuclei.


A
16 h 29C, 24h 18C
Apical
Hoechst
A'
GFP
RFP
B
Basal
Hoechst
B'
GFP
RFP
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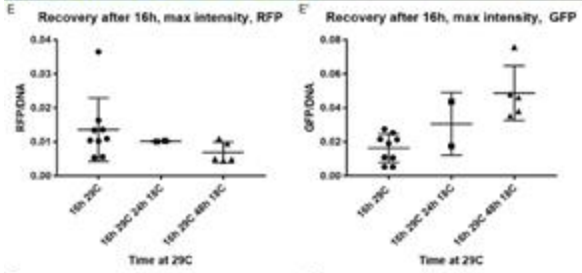
E
Recovery after 16h, max intensity, RFP
RFP/DNA
0.04
0.03
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0.00
16h 29C
16h 29C 24h 18C
16h 29C 48h 18C
Time at 29C

E'
Recovery after 16h, max intensity, GFP
GFP/DNA
0.08
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0.04
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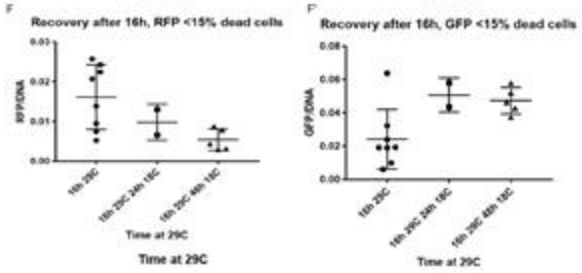
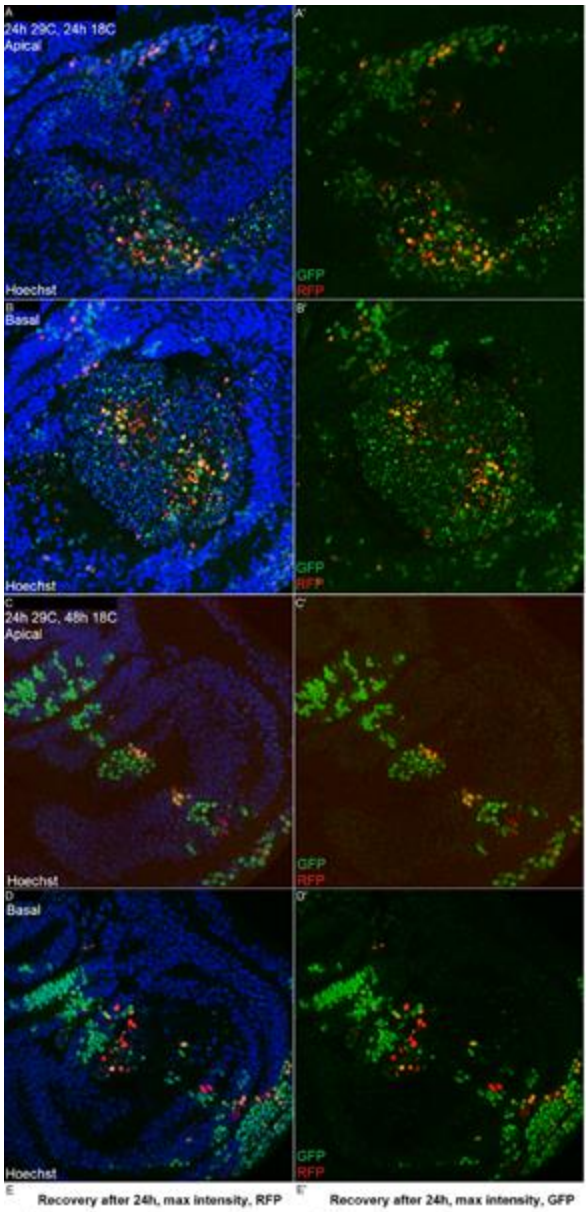
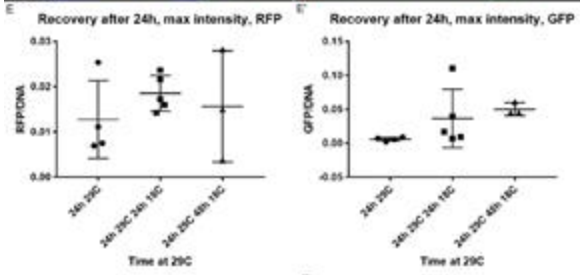
16h 29C
16h 29C 24h 18C
16h 29C 48h 18C
Time at 29C


F
Recovery after 16h, RFP <15% dead cells
RFP/DNA
0.03
0.02
0.01
0.00
16h 29C
16h 29C 24h 18C
16h 29C 48h 18C
Time at 29C
Time at 29C

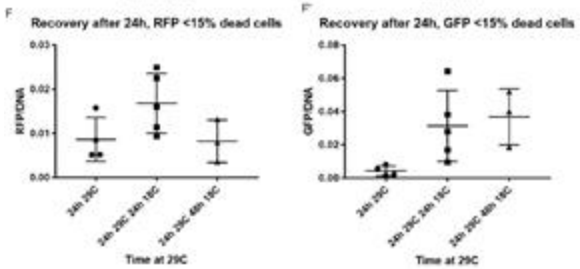
F'
Recovery after 16h, GFP <15% dead cells
GFP/DNA
0.08
0.06
0.04
0.02
0.00
16h 29C
16h 29C 24h 18C
16h 29C 48h 18C
Time at 29C

Figure 7: CasExpress activation from reaper expression for 24 hours and recovery for 24 or 48 hours. (A-D') Confocal images of DQVD-Gal4/G-trace; lexO-rpr/sal$^{E/Pv}$-lexA tubGal80^{ts} showing DAPI, RFP, and GFP expression in wing discs of flies incubated at 29°C for 24 hours and recovery after 24 or 48 hours at 18°C. (E,E') Ratio of RFP and GFP positive cells, includes every Z stack. (F,F') Ratio of RFP and GFP positive cells, excluding Z stacks with >15% condensed nuclei.


A
24h 29C, 24h 18C
Apical
Hoechst
A'
GFP
RFP
B
Basal
Hoechst
B'
GFP
RFP
C
24h 29C, 48h 18C
Apical
Hoechst
C'
GFP
RFP
D
Basal
Hoechst
D'
GFP
RFP

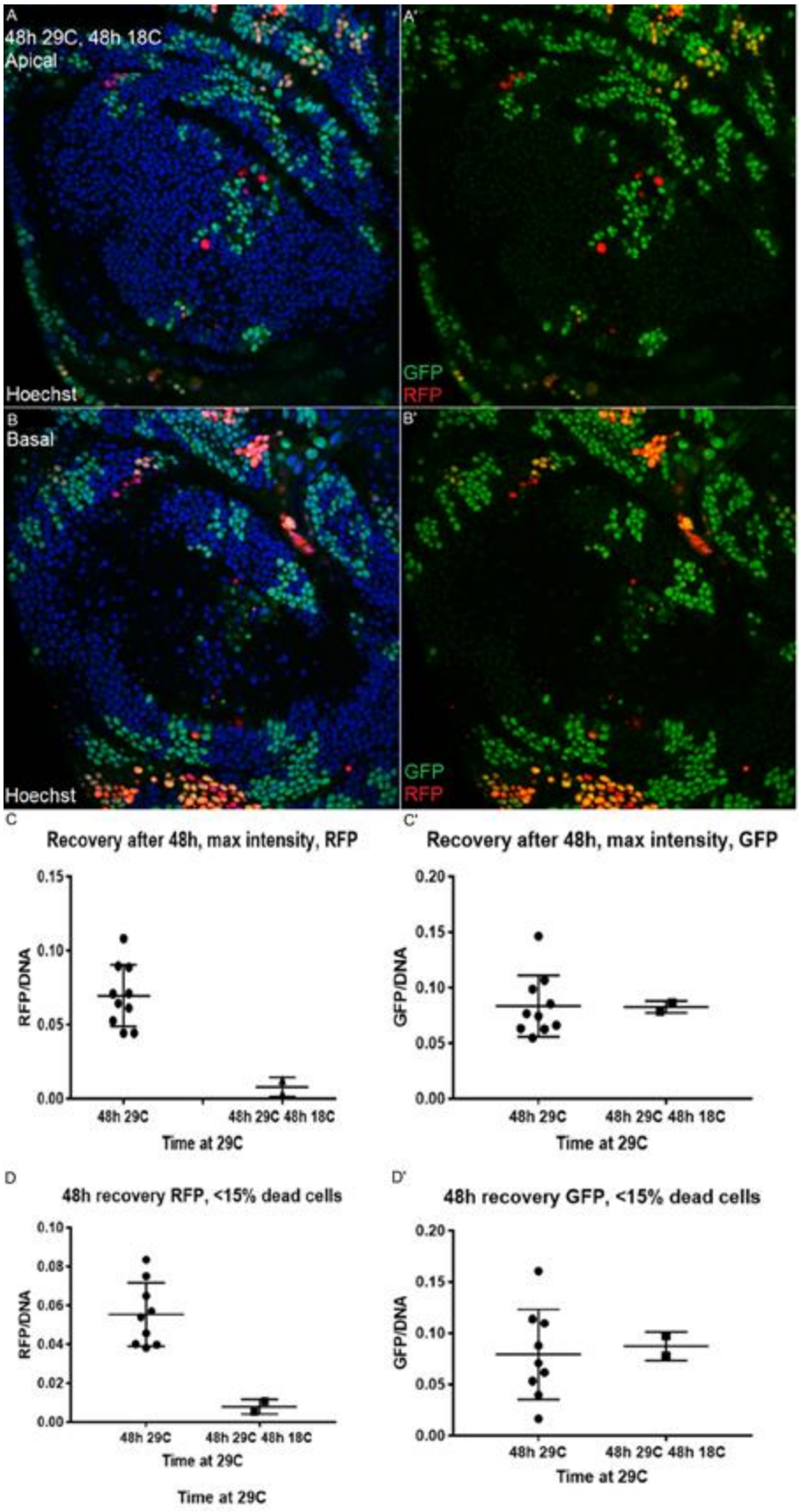

E
Recovery after 24h, max intensity, RFP
RFP/DNA
0.03
0.02
0.01
0.00
24h 29C
24h 29C 24h 18C
24h 29C 48h 18C
Time at 29C

E'
Recovery after 24h, max intensity, GFP
GFP/DNA
0.15
0.10
0.05
0.00
-0.05
24h 29C
24h 29C 24h 18C
24h 29C 48h 18C
Time at 29C


F
Recovery after 24h, RFP <15% dead cells
RFP/DNA
0.03
0.02
0.01
0.00
24h 29C
24h 29C 24h 18C
24h 29C 48h 18C
Time at 29C

F'
Recovery after 24h, GFP <15% dead cells
GFP/DNA
0.08
0.06
0.04
0.02
0.00
24h 29C
24h 29C 24h 18C
24h 29C 48h 18C
Time at 29C

Figure 8: CasExpress activation from reaper expression for 48 hours and recovery for 48 hours. (A-B') Confocal images of DQVD-Gal4/G-trace; lexO-rpr/sal$^{E/Pv}$-lexA tubGal80^{ts} showing DAPI, RFP, and GFP expression in wing discs of flies incubated at 29°C for 48 hours and recovery after 48 hours at 18°C. (C,C') Ratio of RFP and GFP positive cells, includes every Z stack. (D,D') Ratio of RFP and GFP positive cells, excluding Z stacks with >15% condensed nuclei.


A
48h 29C, 48h 18C
Apical
Hoechst
A'
GFP
RFP
B
Basal
Hoechst
B'
GFP
RFP
C
Recovery after 48h, max intensity, RFP
RFP/DNA
0.15
0.10
0.05
0.00
48h 29C
48h 29C 48h 18C
Time at 29C
C'
Recovery after 48h, max intensity, GFP
GFP/DNA
0.20
0.15
0.10
0.05
0.00
48h 29C
48h 29C 48h 18C
Time at 29C
D
48h recovery RFP, <15% dead cells
RFP/DNA
0.10
0.08
0.06
0.04
0.02
0.00
48h 29C
48h 29C 48h 18C
Time at 29C
Time at 29C
D'
48h recovery GFP, <15% dead cells
GFP/DNA
0.20
0.15
0.10
0.05
0.00
48h 29C
48h 29C 48h 18C
Time at 29C

Stress induced anastasis is nonautonomous

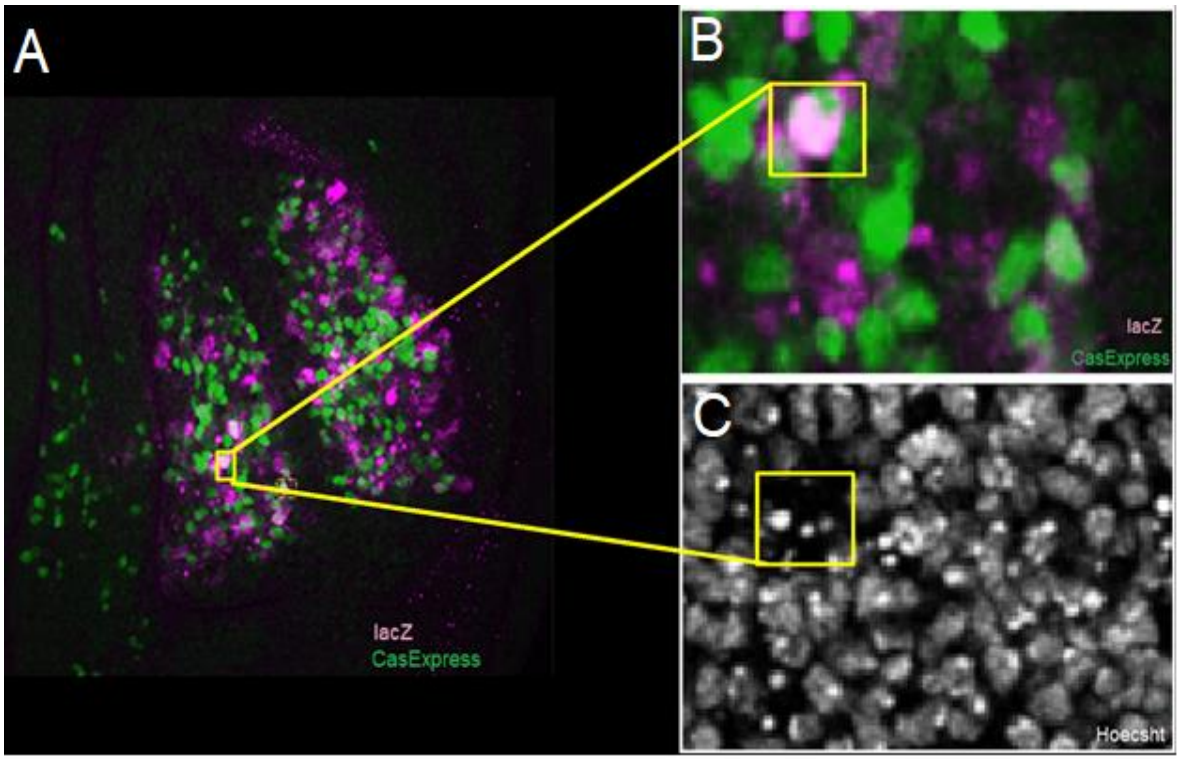
Since we observed CasExpress positive cells after rpr expression, we wondered if the cells that turn on the sensor are the cells that express rpr. To mark the rpr-expressing cells, lacZ was co-expressed together with rpr in the spalt domain. This way, all rpr-expressing cells expressed lacZ. Larvae with the following genotype were collected:

DQVD UAS-Flp UAS-RFP/lexO-lacZFRTSTOPFRTGFP; $sal^{E/PV}$lexAtubGal^{80ts}/lexO-rpr

and checked whether lacZ expression overlapped with GFP and/or RFP positive cells.

If stress induced anastasis is autonomous, there should be overlap between lacZ expression and reaper expression in the spalt domain. However, if there are CasExpress positive cells that do not stain positive for lacZ, this means that stress induced anastasis is nonautonomous and that the CasExpress cells migrated in towards the spalt domain to compensate for the dying cells.

Figure 9: Stress induced anastasis is non autonomous. (A) Entire spalt region of wing disc stained with β-Galactosidase antibody and Hoechst. (B) Overlap of CasExpress GFP and β-Galactosidase. (C) Fragmented nuclei stained with Hoechst corresponds to cell outlined in (A) and (B).



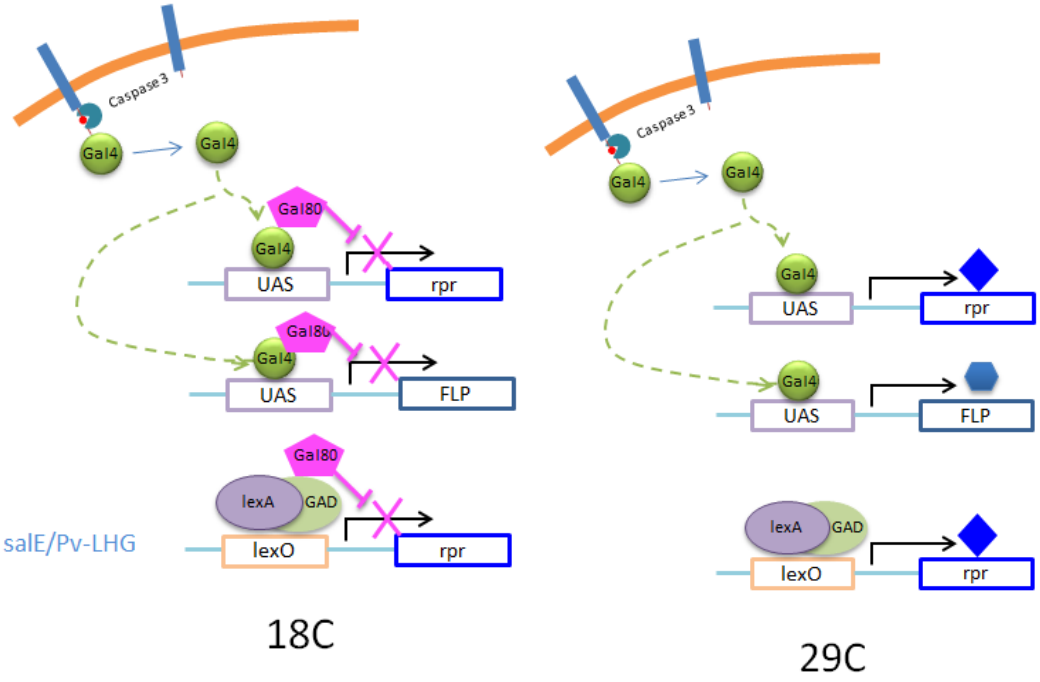
As shown in figure 9, some cells were both CasExpress positive and lacz positive, but most of these cells had shrunken nuclei, indicating that these cells were dead. Most of the CasExpress positive living cells were not lacZ positive, suggesting stress-induced anastasis is nonautonomous. The living CasExpress positive cells localized in the spalt domain, although they did not express lacZ and rpr, suggesting these cells originated from outside the spalt domain and migrated to the spalt domain to compensate for the loss of cells.

Anastatic cells do not contribute to regeneration after ablation by reaper overexpression in the wing disc.

It is of interest to determine whether anastatic cells have a physiological function in regenerating tissue. To determine whether anastasis contributes to

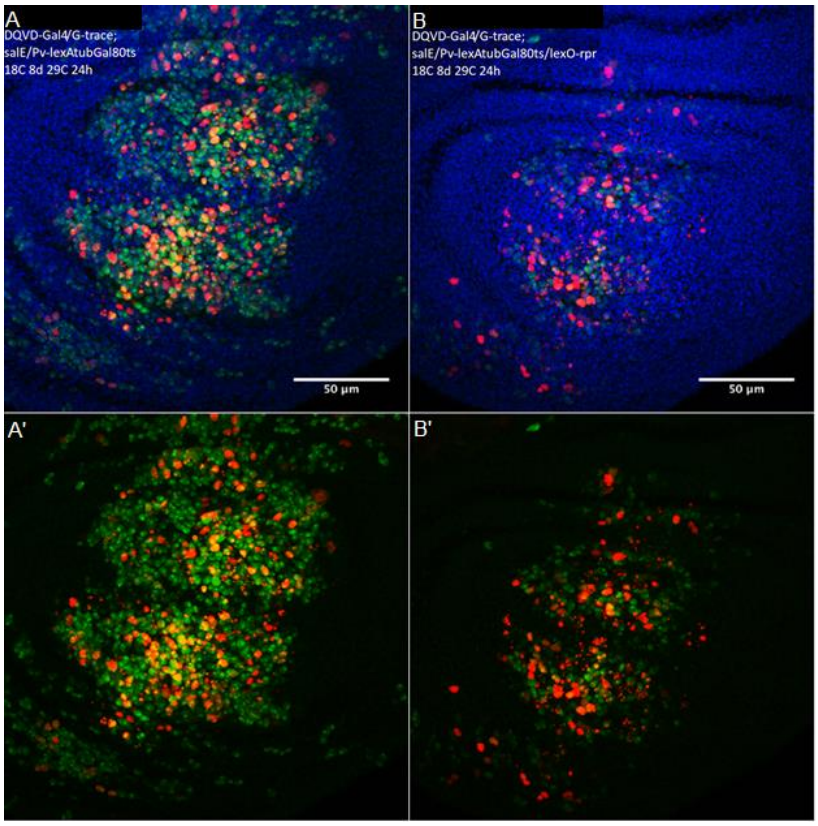
regeneration after genetic ablation, we took advantage of the fact that CasExpress is a caspase-inducible Gal4. In addition to overexpressing rpr under salPE-lexA to induce apoptosis in wing discs, a UAS-rpr was expressed under CasExpress to kill all cells that experienced caspase-3 activation (Figure 10). Wing disc ablation and killing of possibly anastatic cells was induced by transferring larvae from 18°C to 29°C.

Figure 10: Overexpression of reaper using the CasExpress and lexA systems.



As shown in figure 11, 24h induction at 29°C strongly reduced the number of CasExpress positive cells. 48h induction at 29°C killed almost all of the CasExpress positive cells.

Figure 11: Overexpression of reaper strongly reduces the number of CasExpress positive cells. (A,A') DQVD-Gal4/G-trace; sal$^{E/Pv}$-lexAtubGal80^{ts} dissected after 8 days at 18°C and 24h at 29°C. (B,B') DQVD-Gal4/G-trace; sal$^{E/Pv}$-lexAtubGal80^{ts}/lexO-rpr dissected after 8 days at 18°C and 24h at 29°C.

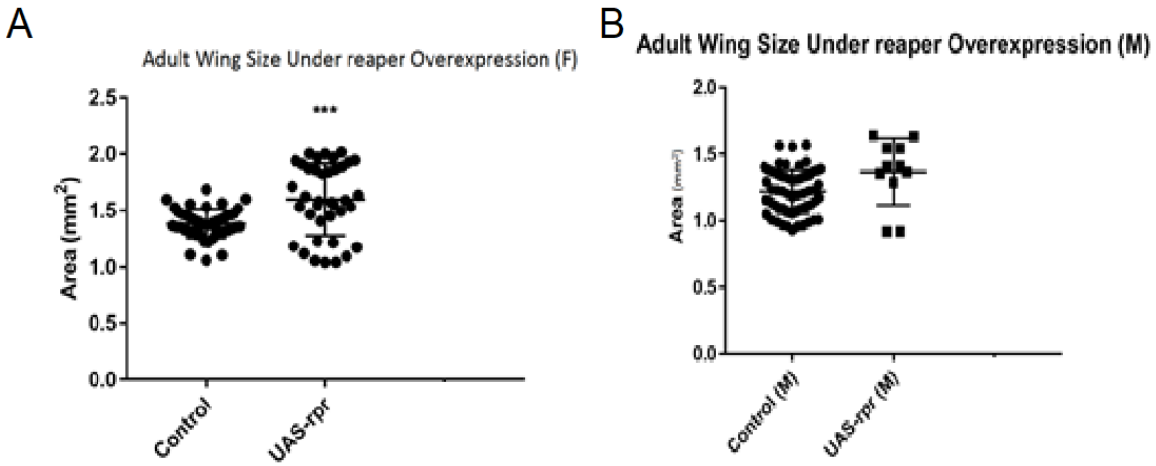


To test the requirement of anastasis in regeneration, after 24 hours of induction of the transgene, larvae were shifted back to 18°C until they became adults. Wing sizes were measured to evaluate regeneration capacity. The crosses:

1) DQVD-Gal4; lexO-rpr/TM6B (Female) X UAS-rpr/If; sal$^{E/Pv}$-lexA tubGal80^{ts}/TM6B (Male)
2) DQVD-Gal4; lexO-rpr/TM6B (Female) X sal$^{E/Pv}$-lexA tubGal80^{ts}/TM6C (Male)

There was no statistically significant difference between the control groups and the UAS-rpr flies. This suggests that the population of anastatic cells in the spalt region did not have an effect on regeneration. However, there was a large variation in wing size in the UAS-rpr female flies.

Figure 12: Adult wings after 24 hours of reaper overexpression at 29°C. (A) Female flies. (B) Male flies.



Future directions

There is evidence [52] that cells in the hinge region migrate into the spalt region to compensate for the ablation of cells in the pouch region. To determine whether cells that experienced caspase-3 activation migrated from the hinge into the spalt region to make up for the dead cells and whether caspase-3 activity is required for migration, we can use p35 expression to block caspase in the hinge region using a hinge specific driver. If cells in the pouch region are unable to turn on the biosensor, this means the CasExpress cells in the pouch originated from the hinge

region. If the discs can still regenerate, that means the migration does not require caspase-3 activity. If the discs fail to regenerate, that means the migration requires caspase-3 activity.

The observation of anatasis in stressed wing discs raises an interesting question. Wing discs are a fast proliferating tissue. Previous studies showed when wing discs are damaged, damaged cells are removed from the epithelium and surrounding healthy cells overproliferate to compensate for the loss. Then why do cells with caspase-3 activity try to survive? One possibility is that anastasis is an intrinsic self salvage program that can be activated in response to all lethal stress, no matter whether it is necessary or not. Then, the other possibility is when apoptosis is induced in a large group of cells, the tissue tries to save some of them to serve as a basis for faster regeneration. To test which one is true, we can induce cell death in a very small region and see if anastasis still occurs.

Although there was not a significant difference in the size of the wings when anastasis is inhibited, there was a large variation in wing size for the UAS-rpr flies. There is the possibility that this variation affects their ability to fly. The ability to fly can be tested using a flight assay. This can be done by placing a plastic sheet coated with tangle trap inside a large tube and dropping flies down the tube. The flies will reorient themselves and fly upwards, eventually landing on the side of the tube. The higher the flies land is an indication of how well they fly [60].

Chapter III: Search for genes involved in regulation of anastasis

Introduction

One interesting question about anastasis is which genes regulate anastasis. Drosophila are a powerful genetic tool and our previous results showed that the CasExpress sensor can detect anastasis in wing discs during development and under stress. Therefore, we used CasExpress to test the requirement of some candidate genes in the regulation of anastasis.

Our previous results in mammalian cells showed similarity between cells undergoing anastasis and wound healing. And our results in Chapter II also showed that anastasis occurs in response to tissue damage. Thus, the first set of candidates are genes known to function in wound healing and regeneration.

The genes that were screened were Hemipterous (hep^{r75}), Myc, Snail (Sco), STAT92E, Wingless (wg), and Yorkie (yki). Yorkie (yki) is an oncogene and the downstream transcription factor of the Hippo pathway. Yki is upregulated in cells surrounding the wound and is required for regeneration [51]. Myc has a role in regenerative proliferation, as ablation of the wing disc with the proapoptotic gene eiger results in the upregulation of Myc [35]. Sco is mutant allele of Snail, which has been found to control proliferation in Drosophila follicle stem cells in females. Disruption of Snail resulted in follicle stem cells with compromised proliferation, while overexpression of Snail resulted in an increase in proliferation and lifespan [56]. Wingless (wg) is required in regeneration, without it, fragmented imaginal discs fail to transdetermine and regenerate missing structures. However, when provided with

exogenous Wg, fragments are able to regenerate the missing structures [62]. STAT92 is in the JAK/STAT pathway and it has been observed that STAT is upregulated in cells that have an intrinsic resistance to apoptosis after irradiation. Through lineage tracing, it was shown that apoptosis resistant cells will lose their identity and migrate to areas of the wing disc that have suffered abundant cell death. This suggests that there is a subpopulation of cells that are more resistant to cell death and following damage, are required to help regenerate the tissue [55]. Hemipterous (hep) is a jun kinase kinase. Hep^{r75} mutants disrupt the JNK pathway, resulting in impaired wound healing [54].

Methods

Fly genetics

Since the biosensor utilizes the UAS-Gal4 system, it was not possible to use UAS-RNAi lines to screen for whether a gene has an effect or role in anastasis. However, there were some mutant lines for these genes available. These seven lines were screened: Hep^{r75}, Myc^{p0}, Sco, STAT92, Wg^{l12}, and yki^{B5}.

Strains:

DQVD/G-trace

DQVD/G-trace; eSTAT92E/$STAT92E^{06343}$

DQVD sco/G-trace

DQVD yki^{B5}/G-trace

hep^{r75}/Y; DQVD/G-trace

Myc^{PO}/Y; DQVD/G-trace

wg ^{l12} DQVD/G-trace

Immunostaining and imaging

Larvae were sorted for fluorescence under a Zeiss Axiozoom. CasExpress positive larvae were dissected in PBS. Male hep^{r75} and Myc^{PO} larvae were selected. The larvae were inverted and fixed with 400ul of 4% PFA, washed twice with PBS, and incubated in 400ul of 0.2% PBST and 1ul Hoechst while covered with foil. Wing discs were dissected and mounted with 20ul of Vectashield.

Slides were imaged using a Zeiss LSM780 Confocal.

Quantification

The ratio of RFP and GFP positive cells was measured using a macro in ImageJ.

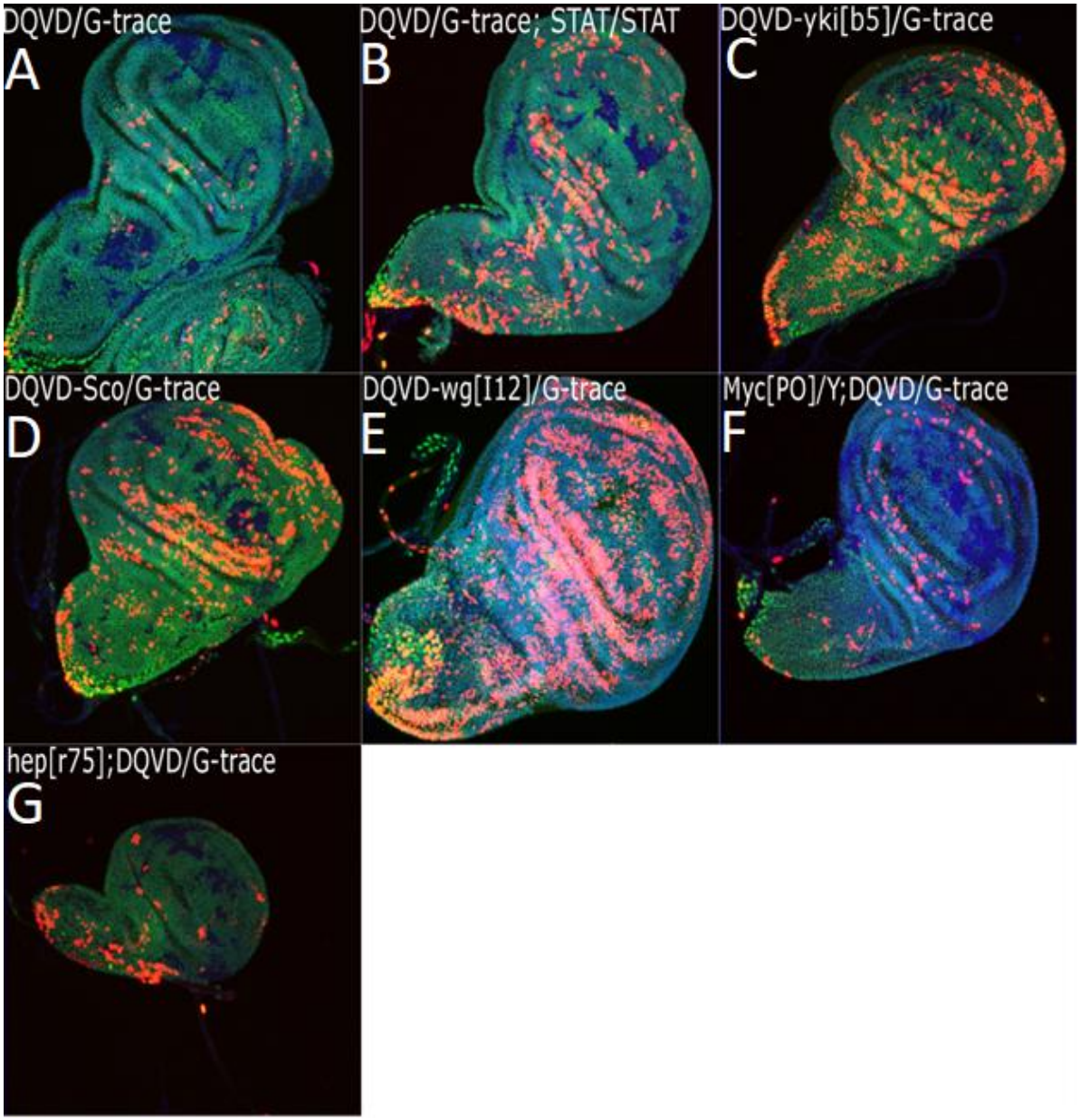
Results

Table 1: Quantification of RFP+ cells percentage and GFP+ cells percentage in wing discs with wound healing mutant genotypes.

Genotype	Average RFP/DNA	Standard Deviation RFP/DNA	Average GFP/DNA	Standard Deviation GFP/DNA
DQVD/G-trace	0.030165	0.013282	0.648638	0.150516
DQVD/G-trace; eSTAT92E/STAT92E^{06343}	0.028736	0.026164	0.505009	0.067702
DQVD yki^{B5}/G-trace	0.051155	0.029805	0.532317	0.095844
DQVD sco/G-trace	0.059620	0.040926	0.573612	0.099734
wg ^{l12} DQVD/G-trace	0.126561	0.073705	0.624464	0.059537
Myc^{PO}/Y; DQVD/G-trace	0.034323	0.018118	0.249674	0.093284
hep^{r75}/Y; DQVD/G-trace	0.044872	0.025349	0.477853	0.110417

Figure 13: Confocal images of wound healing mutants expressing CasExpress showing DAPI, RFP, and GFP expression in wing discs. (A) DQVD/G-trace (B) DQVD/G-trace; eSTAT92E/STAT92E^{06343} (C) DQVD yki^{B5}/G-trace (D) DQVD sco/G-

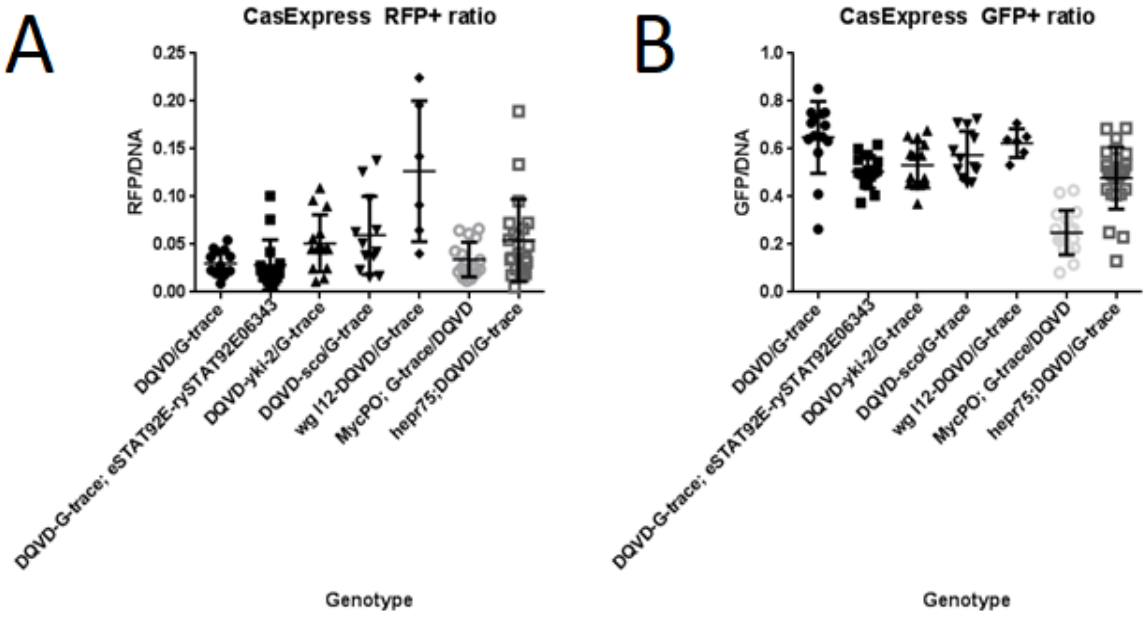
trace (E) wg ^{I12} DQVD/G-trace (F) Myc^{PO}/Y; DQVD/G-trace (G) hep^{r75}/Y; DQVD/G-trace.



Wg, yki, and sco mutants had higher RFP expression, indicating that these mutants were most likely more susceptible to caspase activation than just the CasExpress control. However, since their GFP expression was about the same as the CasExpress control, indicating about the same percentage of cells survive. While

the Myc mutants had approximately the same percentage of RFP positive cells as the CasExpress control, they had a lower percentage of GFP positive cells. This most likely means that the cells without Myc were unable to recover as well from caspase activation.

Figure 14: Ratio of CasExpress positive cells in wound healing mutants. (A) Ratio of RFP positive cells. (B) Ratio of GFP positive cells.



Conclusions

Our results suggest that anastasis occurs in response to tissue ablation in wing discs. While the effect of inhibiting anastasis was not evident in a measurable difference in wing size, this does not mean that anastasis has no effect on

regeneration and compensatory proliferation. The effect of anastasis on regeneration could manifest in the fly's ability to fly or anastasis may be more important in postmitotic cells, such as mature cardiac myocytes or neurons. The initial screen of genes involved in the regeneration and compensatory proliferation pathways resulted in uncovering several genes that have an effect on the activation of CasExpress. The identification of genes that are essential to anastasis will lead to a better understanding of the pathways underlying anastasis.

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