Establishing the *Drosophila* Larval Neuroblast as a System to Study Regulated Cell Death *in vivo*

by

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1478

To my wife Amanda,

whose patience made this work possible.

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PREFACE

The Ouroboros, a serpent consuming its own tail, is a mythological icon that has existed in various cultures throughout history. For many it symbolizes the endless, cyclical nature of the world. Every new thing comes from an old thing. To create, something must first be destroyed. I find that this theme applies quite well to my time spent as a graduate student.

My first project in this lab encountered unexpected difficulties. After having worked on this for almost an entire school year, I abandoned this project and switched completely to working on my eventual thesis project. The demise of my first project, though unfortunate, led to the start of my dissertation work.

The process of regulated cell death itself embodies the theme represented by Ouroboros. The cell corpses or debris do not simply vanish after the cells die. Its components will be broken down somehow and reused by the organism, which at some point will likely generate new cells. Thus, the death of old cells will lead to the birth of new ones.

Finally, my experience working on this project and writing this thesis has been a constant process of death and rebirth, whether it is ideas, experiments, or future directions. One of the greatest things about science is that there is always new knowledge to discover, even if it is not new to you, and the same knowledge can prompt different ideas or responses from different people. If nothing else, I hope that readers of this thesis will gain a better appreciation for the never-ending cycle of ideas, and how to test them, which makes this endeavor seem so futile and worthwhile at the same time.

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ABSTRACT

Programmed cell death is often thought of as a developmental process that is genetically hardwired to occur in the organism in a spatially and temporally specific manner. However, many forms of regulated cell death occur in response to pathological injury or environmental stress. Cell death in response to injury, in particular genotoxic stress, is well documented, but the pathways by which damage is translated to cell death have remained largely elusive. Furthermore, many cell death pathways function independently of the well-known process of apoptosis. These alternative regulated cell death pathways are likely controlled by novel molecular mechanisms.

While examining mutations that cause loss of neural stem cells (neuroblasts) in *Drosophila* larval brains, we discovered a novel mechanism elicited by Cdc20/Fizzy (Fzy) that maintains neuroblasts by promoting their survival. We identified two *fzy* loss-of-function mutations that specifically led to neuroblast loss without perturbing the proliferation of other cell types. Consistently, mutant Fzy or Cdc20 carrying the analogous mutation can substitute for wild-type Cdc20 and restore cell cycle progression in *fzy* mutant brains. Furthermore, these *fzy* mutant neuroblasts do not display characteristics indicative of known cell death pathways, such as apoptosis, autophagic cell death, or mitotic catastrophe. Instead, morphological and functional analyses strongly suggest that Fzy maintains neuroblasts by suppressing necrotic cell death. Inactivating the function of *apoptosis inducing factor* (*aif*) or c-Jun N-terminal kinase (JNK) signaling prolonged the survival of *fzy* mutant neuroblasts, while ectopically activating JNK signaling triggers premature necrotic cell death in neuroblasts. These results suggest that JNK signaling and *aif* play a role in neuroblast necrosis.

Loss of telomere capping proteins or chaperonin proteins required for spindle formation caused a similar necrosis phenotype as the *fzy* mutant neuroblasts. These mutations also caused DNA damage or cell cycle disruption. Furthermore, over-expression of the cell cycle checkpoint protein p53 also caused neuroblast necrosis. Importantly, Fzy expression was decreased in each of these mutations. We propose a novel necrotic cell death mechanism triggered by catastrophic damage of neuroblasts which leads to up-regulation of p53. Increased p53 activity results in loss of Fzy expression and Fzy-dependent necrotic cell death.

CHAPTER 1
INTRODUCTION

The ability of an organism to control the number of cells in its body during development and disease is absolutely critical to ensure proper growth, tissue homeostasis, body patterning, and survival of cellular insults from various sources. Stem cells play an important role in these processes because of their abilities to self-renew and differentiation, allowing them to produce new functional cells as well as replace old cells that are lost. Cell death complements the actions of stem cells by removing cells from the organism. Cell death can function to remove either differentiated cells or the stem cells themselves. An improper shift in the balance between the proliferation of stem cells and the death of stem cells or their progeny could lead to developmental defects or disease. The importance of stem cell death regulation is amplified compared to other cell types because the loss of a stem cell is typically irreversible whereas a lost differentiated cell can be replaced by a stem or progenitor cell. However, very little is known about the regulation of programmed cell death in stem cells.

Genetically regulated cell death can occur as a result of a developmental program or in response to injury or other environmental stimuli. There are situations where organisms find it advantageous to kill cells even if they are normally very important, such as when a stem cell has suffered irreparable DNA damage. The removal of these cells prevents the production of progeny with genetic alterations, which can predispose the cells to cancer. The most common cell death program, apoptosis, is thought to contribute to the majority of primary regulated cell death in response in injury. However, there are many situations in which apoptosis or the primary cell death program is blocked. Thus, the existence of secondary mechanisms, such as necrotic cell death, may be critical to prevent undesired consequences. While much work on cell death has focused on

mechanisms that trigger primary cell death, our understanding of secondary mechanisms of cell death is far less complete.

The *Drosophila* larval neuroblast is a well-established model for studying asymmetric cell division and stem cell biology. However, like all other stem cell models, very little is known about how neuroblasts regulate programmed cell death, particularly in response to irreversible damage. The goal of this introduction is to briefly review what is known about programmed cell death while giving special attention to the concept of primary versus secondary cell death mechanisms and the roles of apoptosis and necrosis. The current literature on programmed cell death in neuroblasts will also be reviewed.

Programmed Cell Death

The first published experiments on programmed cell death were performed in another insect: the silk moth pupa. In 1965, Lockshin and Williams demonstrated in a series of papers that in the first few days of adulthood, the silk moth intersegmental muscles undergo a programmed cytolysis[1,2]. This cytolysis consistently occurred a few days after the increase of circulating hormone and muscle protease levels. Furthermore, this process depended on the cessation of electric impulses from the nervous system. These studies represented a landmark in the nascent field of cell death. While the principle of a pre-programmed mechanism triggering developmental cell death may have been proposed before, this was the first demonstration of a mechanism correlated to cell death.

Several years later, Kerr, Wyllie, and Currie set the standard for detecting and distinguishing the different modes of cell death for decades[3]. Transmission electron microscopy was used to identify a distinct morphological pattern associated with each cell death pathway. Unique features of the newly coined "apoptosis" included nuclear condensation and fragmentation, shedding of membrane bound cell fragments and uptake of these fragments by phagocytic cells. Ultrastuctural evidence of apoptosis was found in both developmental as well as pathological contexts, in tissues ranging from embryonic mesenchyme to injured liver.

While very little molecular evidence was presented, these observations demonstrated a method for differentiating apoptosis from necrosis, which was defined as the breakdown and death of cells in an unregulated manner. Coincidentally, apoptosis was originally called shrinkage necrosis in reference to its morphological features. Even today, the term necrosis is sometimes used as an all-encompassing description for cell death that cannot be clearly delineated as apoptosis.

In the 1980s, Bob Horvitz and colleagues published arguably the most seminal studies to date on programmed cell death. They discovered the genes responsible for developmental apoptosis in the roundworm *Caenorhabditis elegans*[4,5]. In 2002, Sydney Brenner, Bob Horvitz, and John Sulston were awarded the Nobel Prize in medicine for their contributions to this discovery.

Apoptosis

Apoptosis has been found to be a critical and widespread mechanism for elimination of unneeded or unwanted cells during development and disease. It is generally thought of as a quiet cell 'suicide', as opposed to necrosis which is typically associated with the release of cellular contents and inflammation. All 131 somatic cells that are programmed to die in a developing *C. elegans* can survive if apoptosis is blocked[6]. *Drosophila* requires apoptosis for leg formation and proper morphogenesis of the head and male genitalia[7–9]. Mammalian digits would not form properly if apoptosis did not remove the interdigital webs present early in development[10]. During viral infections, mammalian immune systems are capable of detecting and eliminating infected cells by apoptosis to protect the host. Many cancers have been found to contain mutations that inhibit apoptosis, allowing the disease to escape a potential protective mechanism. In contrast, much of the damage caused by ischemia/reperfusion injuries of cardiomyocytes and neural tissues is due to cell death. Finally, some neurodegenerative diseases also lead to inappropriate apoptosis.

Kerr and colleagues were able to distinguish apoptosis from necrosis by morphology[3]. Apoptotic cells usually exhibit rounding up, nuclear condensation, overall cell shrinkage, shedding of apoptotic bodies containing intact plasma membrane and organelles, and engulfment by phagocytes. By contrast, necrotic cell death is typically associated with nuclear fragmentation, cellular swelling, rupture of the plasma membrane, and release of cellular contents into the surrounding environment which often triggers and inflammatory response[11].

The core machinery of apoptosis has been particularly well studied and is conserved among model organisms (Fig 1.1). While the known complexity varies between the different organisms, they all require the activation of caspases, a family of cysteine proteases. Caspases typically exist in an inactive form but cleavage at specific aspartic residues results in active subunits which heterotetramerize into active protease complexes[12]. The first caspases to be activated in the cascade are apical or initiator caspases and have quite specific targets. The last caspases to be activated, called executioner caspases, are more promiscuous and are largely responsible for the well

documented morphological changes associated with apoptosis[13]. Execution of cell death is the most famous function of caspases. However, not all caspases participate in apoptosis[14,15].

Apoptosis can be broadly characterized into two categories based on the source of the stimulus: intrinsic versus extrinsic (Fig 1.2). The extrinsic pathway begins with the binding of a death receptor on the cell surface to its ligand. The activation of the receptor then leads to activation of a pro-death caspase, Caspase-8. Caspase-8 is able to activate the downstream executioner caspase as well as trigger mitochondrial release of Cytochrome c. The mitochondria play a large role in the intrinsic apoptosis pathway and are also activated in some but not all instances of extrinsic death receptor signaling[13]. The intrinsic pathway begins with a cellular insult or stress resulting in a loss of mitochondrial membrane potential. The defective mitochondria are then triggered to release Cytochrome c, which subsequently binds to the adaptor subunit Apaf-1 to form an activated caspase complex which begins the caspase cascade.

All caspase-mediated cell deaths require the activation of downstream executioner or effector caspases (DrICE/Dcp-1 or Caspase-3/7) by upstream initiator caspases (CED-3, Dronc, or Caspase-9) (Fig 1.1). Furthermore, they all share the requirement for an adaptor subunit (CED-4, Ark, Apaf-1) to assemble the upstream caspase complexes. *Drosophila* and mammals also express inhibitors of apoptosis (DIAP or XIAP) which help regulate caspase activity in the absence of cell death activation. Upstream death signals which antagonize IAPs exist in both flies and mammals. The three IAP suppressing genes *reaper*, *hid*, and *grim* are thought to control almost all apoptosis in *Drosophila*[16]. Regulation of these factors acts as a nexus for integrating multiple signaling pathways to the core cell death machinery[17]. Analogous genes have been found in mammals as well[18,19].

Necrosis

Recent advances in cell death research have revealed a startling variety of programmed cell death pathways as well as complications in their regulation and crosstalk. These new findings warranted the formation of the Nomenclature Committee on Cell Death who have proposed formal definitions of cell death modalities[20]. Programmed necrosis is particularly intriguing because of the history of necrosis as an unregulated cell death pathway.

Necrosis was once defined as any cell death that is not regulated or genetically programmed. Necrosis became synonymous with uncontrolled cell death in response to injury or disease. Nonetheless, necrosis has always been associated with several key morphological features: swelling of the cells, breakdown of the nucleus and plasma membrane rupture. More recent additions to the signature of necrosis include increased cytoplasmic calcium, acidification of the cytoplasm, large scale DNA fragmentation, and increased reactive oxygen species.

In the late 1980s and 1990s, accumulating evidence suggested that some signaling molecules or cellular insults could trigger either apoptotic or necrotic cell death[21–23]. Numerous examples of regulated necrosis exist in the invertebrate literature, but the best characterized mechanism is mammalian receptor-interacting protein (RIP)-dependent necrosis or necroptosis[11,24]. The key feature of this mechanism is the requirement for activation of RIP1 and RIP3 kinases. Multiple upstream activators of RIP kinases have been found, including death signals such as TNF, Fas, genotoxic stress, and certain infections[25–27]. Once RIP1 is activated, it can signal in one of three fashions. Ubiquitination of RIP1 by cellular inhibitor of apoptosis 1 (cIAP1) leads to activation of nuclear-factor-κB (NF-κB) signaling, a pro-survival pathway. Alternately, RIP1 can also function in a complex with Caspase-8 and participate in the death receptor-dependent apoptosis pathway. Finally, in the absence of Caspase-8 activity or in the presence of certain stressors such as genotoxic damage or viral infections, RIP1 may associate with RIP3 as part of the ‘necrosome’ which ultimately triggers effectors of necrosis.

The developing fly oocyte is one of the few *Drosophila* tissues in which programmed necrosis has been established[28]. A mature fly ovary consists of multiple

ovarioles, each ovariole shaped like a chain of beads of increasing size. Each bead is a developing egg chamber comprising 16 germline cells surrounded by up to 1000 somatic follicle cells. Eventually, one of the germ cells of each egg chamber is specified to become the oocyte. The remaining germ cells become nurse cell which provide nutrients, organelles, and mRNA to the oocyte. Necrosis occurs in late stage fly oocytes, when nurse cells are programmed transfer their cytoplasmic contents to the oocyte and then die. This event appears to be a true programmed cell death, albeit with atypical course of action. The nurse cell remnants undergo transient autophagic activity followed by necrotic cell death based on autophagy markers and ultrastructure[29]. Nurse cell death displays morphological features and markers consistent with necrosis including nuclear fragmentation, lysosomal rupture, increased cytoplasmic calcium, increased reactive oxygen species production, and activation of non-caspase proteases such as cathepsins[24]. However, the exact molecular mechanisms leading to this process are still unclear.

The developing *Drosophila* eye has also shown promise as a model for novel cell death pathways, particularly in the elucidation of a TNF-mediated cell death pathway. The fly TNF homolog, Eiger, is able to kill at least partially through a caspase-mediated mechanism, although the exact contributions of caspase signaling and other pathways are still unclear. Initial studies suggested that classical upstream activators of fly apoptosis, Reaper, Hid and Grim, were only partially responsible for killing[30,31]. This result suggested that alternative cell death pathways were responsible for cell death. Mammalian TNF is capable of activating either apoptosis or necroptosis through diverging signaling pathways. There are no fly homologs of the RIP family kinases and therefore no formal necroptosis in flies, but TNF-induced cell death in the fly eye appears to partially act through c-Jun N-terminal kinase (JNK) signaling[32]. Furthermore, Reaper appears to specifically integrate into the TNF-JNK signaling axis by regulating *Drosophila* Inhibitor of Apoptosis 1 (DIAP1) which in turn regulates TNF receptor-associated factor 1 (TNRF1)[33]. Recently, Eiger mediated cell death has been found to act through energy production genes associated with the mitochondria[31]. Mutants in the glycolysis pathway, β-oxidation, or the TCA cycle all modulated Eiger-JNK dependent cell death, suggesting the maintenance of energy levels plays a crucial role in regulating this pathway. Importantly, this form of cell death cannot be blocked by loss of apoptosis

alone. The pathways controlling the alternative cell death mechanism have yet to be elucidated.

Primary and Secondary Cell Death

The current literature suggests that while necrosis may be genetically regulated, apoptosis is still the more common primary pathway by which cells are executed. However, there is evidence that other cell death pathways exist as backup mechanisms in case apoptosis or the primary cell death mechanism fails[20,34]. In a highly sensitive fibrosarcoma cell line, activation of the death receptor Fas caused death in a caspase-independent manner with necrotic morphology[35], suggesting that a non-apoptotic mechanism could be killing these cells. Stimulation of rat intestine epithelial cells with Tumor Necrosis Factor while blocking caspases pharmacologically resulted in necrotic cell death[36]. Furthermore, in caspase-deficient Jurkat cells, artificial activation of a Fas effector caused necrotic cell death[37]. In the aforementioned case of digit formation, apoptosis-deficient mice only exhibit a partial syndactyly phenotype, especially in comparison to severe mutations of developmental signaling pathways[38,39]. This suggests that even if apoptosis fails, an alternative cell death pathway can removed the interdigital tissues during development. These situations highlight the importance of backup or secondary mechanisms to trigger cell death if apoptosis is blocked.

The existence of secondary cell death pathways leads to the question of whether all programmed cell death mechanisms have secondary cell death pathways. In the *Drosophila* eye, TNF activated cell death appears to be only partially dependent on apoptosis[31]. Thus, it is possible that if apoptosis were eliminated, a similar degree of cell death would occur via the secondary mechanism.

Cell death in response to injury is another scenario in which having a secondary cell death mechanism would be valuable. In situations where a cell is damaged beyond its ability to repair itself, cell death may be the most beneficial outcome. A proliferating cell with DNA damage may eventually accumulate tumorigenic mutations. One of the primary mechanisms by which cancers promote tumorigenesis and resist therapeutic intervention is by antagonizing cell death pathways, in particular apoptosis[40,41]. It is likely that resistance to cell death is one of the clonally selected traits of cancers as they grow more aggressive. Interestingly, the proportion of cancer cells that undergo non-apoptotic cell death increases when apoptosis is blocked and the cells are treated with

conventional chemoradiation. Thus, there has been heightened interest in discovering novel secondary cell death pathways and how to target them with anti-cancer drugs

One of the most intensely studied areas of injury induced cell death is death in response to DNA damage or disruption of the cell cycle. This has been motivated primarily by the need for better cancer therapies. The p53 transcription factor is considered the guardian of the genome due to its roles in coordinating cell cycle arrest, DNA repair, and cell death. The primary response to DNA damage involves cell cycle arrest or delay and DNA repair[42,43]. Situations that require p53-mediated cell death are typically those involving such extreme cell damage or stress that normal repair mechanisms are no longer able to salvage the cell. Understanding how p53 regulates the decision to promote cell death and the mechanisms that execute p53- mediated cell death can provide valuable insight into targeted cancer therapies.

Drosophila p53 is similarly capable of inducing cell death in response to DNA damage. Ionizing radiation or loss of telomeres are both capable of triggering p53-mediated cell death[44]. In *Drosophila* larval imaginal discs, p53 appears to bind to and activate the canonical apoptosis regulator gene loci *reaper* and *hid*[43,45]. Over-expression of p53 leads to premature apoptosis. Curiously, while mid-oogenesis germ cell death can be induced by p53 over-expression, co-expression of caspase inhibitors is unable to block germ cell death[46]. This strongly suggests that an alternate mechanism exists to kill these germ cells. The necrotic cell death pathways previously described in the developing oocyte stands as strong candidates for this alternative mechanism. It is unclear why apoptosis would be suppressed or absent in p53-mediated cell death of the oocyte. Furthermore, the exact mechanism of cell death used in lieu of apoptosis has not been characterized. However, these results indicate that cells incapable of dying via apoptosis are able to trigger a secondary mechanism to execute themselves.

The *Drosophila* Neuroblast

Stem cells function in development and adult tissues, generating all the functional cells of an organism and replacing old or damaged cells in tissue homeostasis[47–49]. The stem cell field itself is multi-dimensional. Many researchers in the field are interested in embryonic and induced pluripotent stem cells, which are thought to hold the keys to limitless tissue regeneration after injury and even immortality. The similarities between cancer cells and stem cells have led to a whole new field based on the cancer stem cell hypothesis. Central to all of these offshoots are the basic mechanisms that regulate stem cell self-renewal and differentiation.

One of the cardinal properties of stem cells is self-renewal, the ability to maintain stem cell identity after cell division. The way stem cells maintain their viability and contend with cell stressors is critical to their ability to self-renew and maintain themselves, yet cell death in stem cells has received relatively little attention compared to the previously mentioned topics. A better understanding of cell death in stem cells, particularly in response to damage or stress, can inform our studies of other aspects of stem cell biology. Knowledge of cell death pathways could also allow us to modulate cell death and cell viability in various disease states such as cancer and neurodegeneration.

The *Drosophila* larval brain has been established as an excellent model system for studying stem cell biology[50–53]. Each hemisphere of the fly central brain lobe contains exactly 100 neural stem cells (neuroblasts) which give rise to the majority of adult neurons responsible for behavior and motor function. During larval development, each neuroblast continually divides asymmetrically to regenerate the neuroblast and produce a differentiating progeny. Each cell type in the neuroblast lineage is easily identifiable by staining with antibodies specific for unique molecular markers. The genetic tools built for fly research combined with the accessibility of this tissue have allowed us to gain a wealth of knowledge on the molecular mechanisms of asymmetric cell division, differentiation, and self-renewal. Furthermore, many parallels exist between the developing nervous system of the fly and that of mammals[51,54], suggesting that findings in the fly may be directly translatable to human biology.

An unexplored area of neuroblast biology is the way they respond to damage. The central brain of the *Drosophila* larva carries an unusual burden: it must generate a large number of post-mitotic progeny in three to four days but can only produce them with 100 asymmetrically dividing neuroblasts. This mode of division is limiting because the majority of neuroblasts can only produce one or two progeny with each division. The neurons produced by larval neuroblasts are absolutely critical for the viability of the adult. Therefore, the neuroblasts are biologically inclined to withstand as much cellular stress or damage as possible in order to maintain their proliferative rate. The type of damage required to force the neuroblasts to either stop dividing or die has never been characterized.

A common result of irreparable damage to cells is death. It is well established that catastrophic DNA damage can lead to p53-mediated apoptosis in certain cell types. Very little is known about its regulation of cell damage and cell death in central brain neuroblasts. The neuroblasts of the embryonic ventral nerve cord, the other half of the central nervous system of fly, have been shown to die by canonical apoptosis before entering the larval stages[55]. Most of the neuroblasts in the ventral nerve cord typically do not survive in the larvae and are programmed to die before the larval stages. The death of these ventral nerve cord neuroblasts is controlled by the pro-apoptotic genes *reaper*, *hid* and *grim*. These genes regulate cell death primarily by inhibiting the *Drosophila* inhibitor of apoptosis (DIAP). The central brain neuroblasts are also programmed to die by apoptosis at the end of larval development and before pupation[56]. Curiously, before these neuroblasts die, the apoptosis machinery is suppressed by Polycomb group proteins[57]. Thus, while central brain neuroblasts can be eliminated by apoptosis at the right time, they may not be competent to undergo apoptosis before that time. However, secondary cell death mechanisms may function to eliminate damaged cells even in the absence of apoptosis.

One of the main advances presented in this thesis are on how *Drosophila* larval central brain neuroblasts may be maintained through a pro-survival mechanism that is distinct from classical self-renewal pathways. Furthermore, this survival mechanism may represent a secondary, non-apoptotic, programmed cell death that lies downstream of the neuroblast response to catastrophic cellular damage.

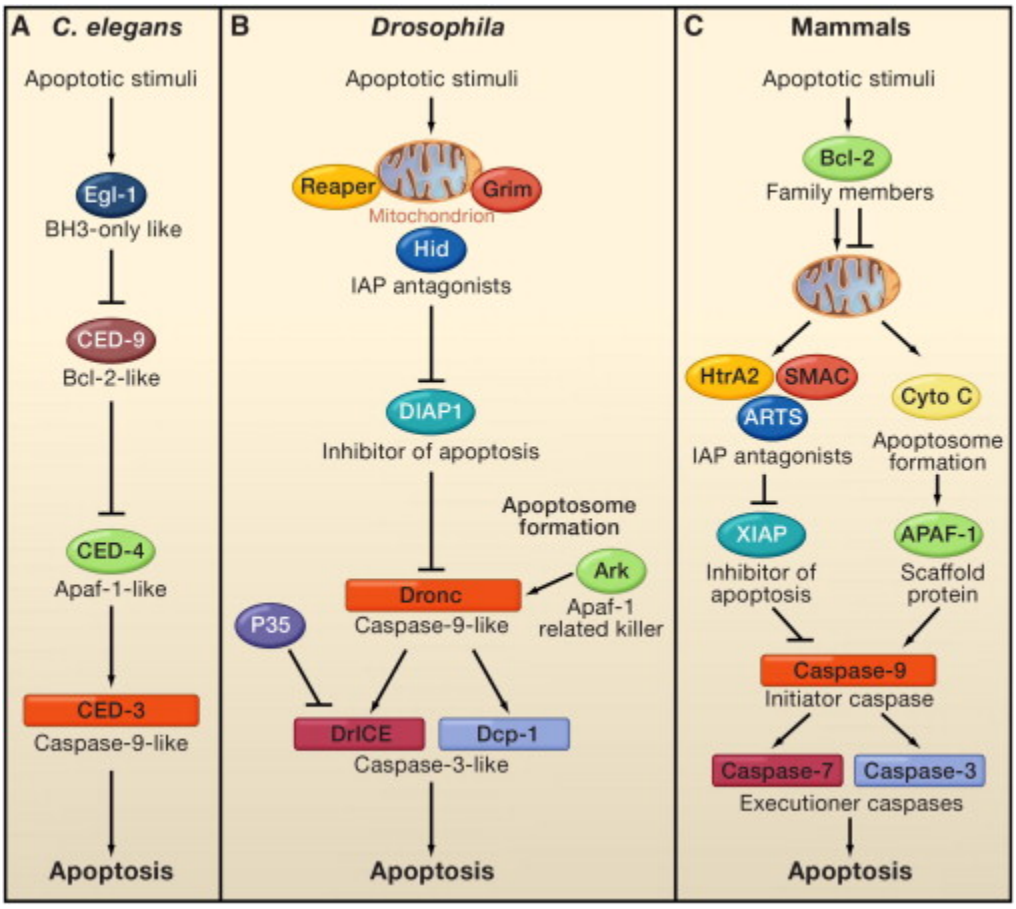

Figure 1.1. Evolutionary Conservation of the Core Apoptotic Machinery[58].

A comparison between the apoptotic pathways in *C. elegans*, *Drosophila*, and mammals reveals conservation and expansion of the apoptotic pathway during evolution. **(A)** In *C. elegans*, apoptotic signals regulate the interplay between Egl-1 (BH3-only homology) and CED-9 (Bcl-2 homolog), liberating CED-4 (Apaf-1 homolog) to activate CED-3 (caspase-9 homolog) for programmed death of 131 cells. **(B)** In *Drosophila*, many different signaling pathways regulate the IAP antagonists Reaper, Hid, and Grim (RHG) and the apoptosome proteins Ark (Apaf-1 homolog) and Dronc (caspase-9 homolog). On the one hand, this causes the ubiquitin-mediated degradation of DIAP1 and derepression of caspases, and on the other hand, it enables Dronc (caspase-9 homolog) to associate with Ark, creating active apoptosomes and activation of the effector caspases DrICE and Dcp1. Both pathways are required for efficient caspase activation and are coordinately regulated. The P35 baculovirus protein can specifically inhibit the activity of Dcp-1 and DrICE. **(C)** In mammals, the balance between proapoptotic and antiapoptotic Bcl-2 family members is a key factor in the commitment to apoptosis by regulating the release of cytochrome *c* and IAP antagonists from mitochondria. Binding of cytochrome *c* to Apaf-1 promotes apoptosome assembly, which recruits and activates caspase-9. IAP antagonists liberate caspases from the inhibition of IAPs, most notably X-linked inhibitor of apoptosis (XIAP), which targets both initiator and effector caspases. The XIAP

antagonist ARTS is localized to the mitochondrial outer membrane and acts prior to the release of cytochrome *c*, Smac, and other proteins released from the mitochondrial intermembrane space. Homologous proteins (by either function or sequence) are similarly illustrated.

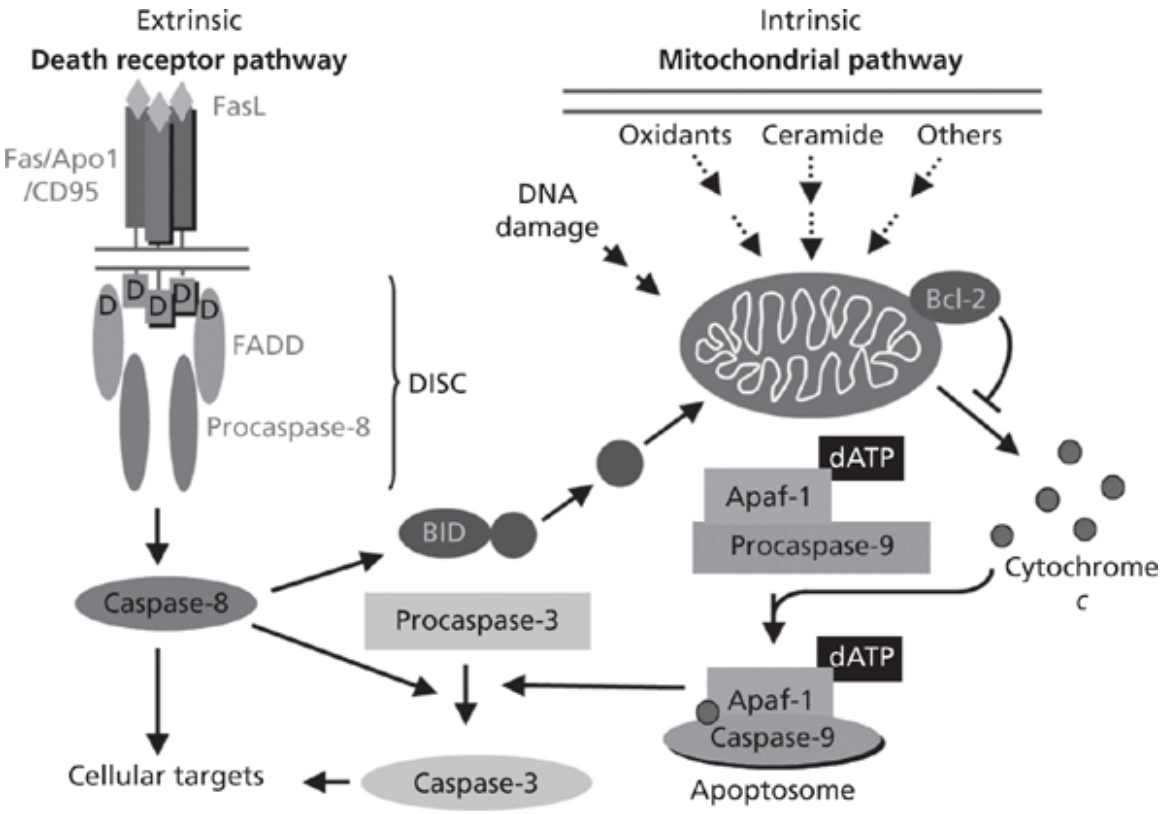

Figure 1.2. Major apoptotic pathways in mammalian cells[59].

The extrinsic pathway and intrinsic pathway both act through mitochondrial signals, although the extrinsic pathway also has a major extra-mitochondrial component. The intrinsic pathway can be stimulated by numerous agents, all resulting in release of cytochrome c from the mitochondria by removing the repressive effect of B-cell lymphoma 2 protein (Bcl-2). Cytochrome c complexes with apoptotic protease activating factor 1 (Apaf-1) and recruits and activates caspase-9, forming the active apoptosome. The apoptosome activates the effector caspase-3 to initiate killing. The extrinsic pathway begins with signaling to a death receptor such as Fas to Fas ligand (FasL). Subsequent interaction of the death receptor intracellular domains with scaffolding subunits such as Fas-associated death domain (FADD) allows for recruitment of Procaspase-8 and formation of the death-inducing signaling complex (DISC). Fas is a member of the tumor necrosis factor superfamily. Activated caspase-8 promotes activation of caspase-3 directly. Caspase-8 also activates caspase-3 indirectly through the intrinsic pathway by activating the protein Bid, which then promotes release of cytochrome c.

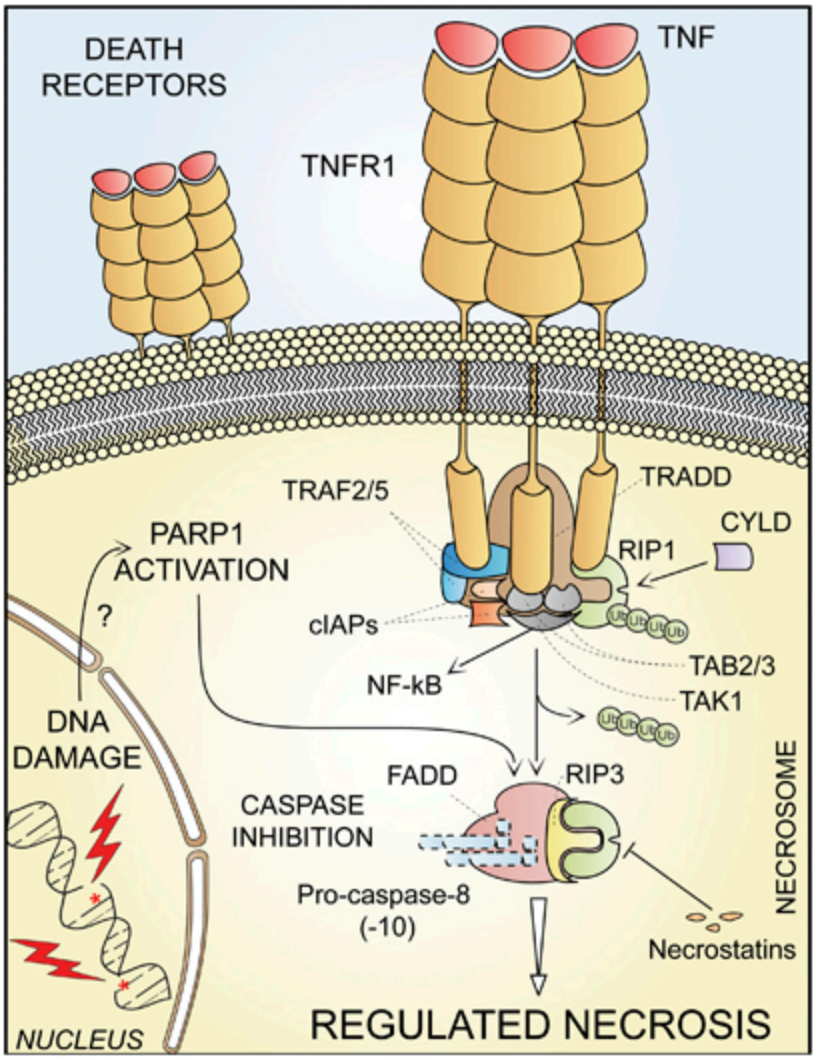

Figure 1.3. Regulated Necrosis[20].

Upon tumor necrosis factor α (TNFα) binding, the cytoplasmic tails of TNF receptor 1 (TNFR1, a prototypic death receptor) trimers recruit TNFR-associated death domain (TRADD), receptor-interacting protein kinase 1 (RIP1), cellular inhibitor of apoptosis 1 (cIAP1), cIAP2, TNFR-associated factor 2 (TRAF2) and TRAF5. Within the so-called complex I, RIP1 is polyubiquitinated by cIAPs, thereby providing a docking site for the recruitment of transforming growth factor β (TGFβ)-activated kinase 1 (TAK1), TAK1-binding protein 2 (TAB2) and TAB3 (which together deliver a pro-survival signal by activating the transcription factor NF-κB). In some pathophysiological and experimental settings, and in particular when caspase-8 is absent or when caspases are inhibited by pharmacological agents, cylindromatosis (CYLD)-deubiquitinated RIP1 engage in physical and functional interactions with its homolog RIP3, ultimately activating necrotic cell death. Regulated necrosis can also be induced by alkylating DNA damage (possibly by the overactivation of poly(ADP-ribose) polymerase 1 (PARP1). In some instances, regulated necrosis requires the kinase activity of RIP1, that is, it can be blocked by the RIP1-targeting compounds necrostatins.

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CHAPTER 2

CDC20/FIZZY –DEPENDENT SURVIVAL MECHANISM MAINTAINS NEURAL STEM CELLS BY SUPPRESSING NECROSIS

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Summary

Precise regulation of stem cell survival is essential to ensure continuous generation of differentiated progeny throughout the lifetime of an organism. However, cell viability is generally perceived as the consequence of the stem cell self-renewal program rather than the output of a dedicated cell survival mechanism. Here, we describe

a novel mechanism elicited by Cdc20/Fizzy (Fzy) that maintains the viability of neural stem cells (neuroblasts) in the fly larval brain. We identify two novel *fzy* mutations that specifically lead to premature loss of neuroblasts without affecting the viability and the proliferation of other diploid cell types. Unexpectedly, neuroblasts carrying either *fzy* mutation display morphological and functional characteristics indicative of necrotic cell death. Consistently, removing genes required for the activation of apoptosis or autophagy fail to suppress neuroblast loss in *fzy* mutant brains. Fzy maintains neuroblast viability via a novel anaphase promoting complex-dependent mechanism. Inactivating the function of *apoptosis inducing factor* (*aif*) or JNK signaling significantly prolong the survival of *fzy* mutant neuroblasts, indicating that Fzy maintains neuroblast viability by antagonizing these signaling mechanisms. Finally, neuroblasts exhibiting centrosomal defects or activated DNA damage response also undergo necrotic cell death, indistinguishable from *fzy* mutant neuroblasts. We propose that in response to catastrophic damage, the Fzy-dependent cell survival mechanism eliminates stem cells by necrotic cell death via multiple cell cycle checkpoints during fly larval brain neurogenesis.

Introduction

Stem cells need to maintain their identity and remain viable in order to generate sufficient numbers of differentiated progeny during development and homeostasis. The mechanisms that maintain the identity of stem cells allow them to retain extended developmental potential and proliferative capacity[1–3]. By contrast, maintenance of cell viability is generally perceived as the consequence of the mechanisms that maintain stem cell identity rather than a dedicated program that promotes stem cell survival. As such, specific mechanisms that preserve stem cell pools by promoting cell survival remain unknown. Mechanistic insight into the control of stem cell viability can potentially improve the efficiency of manipulating stem cells for tissue engineering and lead to the development of novel therapies for targeting tissue-specific tumor stem cells.

A *Drosophila* larval brain lobe maintains a steady 100 neuroblasts which can be unambiguously identified by the expression of various cell fate markers and provides a genetic model system for investigating the regulation of neural stem cells *in vivo*[4–6]. Neuroblasts in the larval brain undergo rapid and successive asymmetric divisions to give rise to adult-specific neurons and glia. A previous study shows that the Polycomb group genes are required to prevent larval brain neuroblasts from prematurely undergoing apoptosis[7]. However, the Polycomb group genes have pleiotropic functions and can regulate a wide array of downstream signaling mechanisms[8–10]. Specific downstream mechanisms by which the Polycomb group genes prevent premature onset of apoptosis in neuroblasts remain unknown. Furthermore, the roles of other forms of programmed cell death such in the maintenance of larval brain neuroblasts have never been established.

Cdc20/Fzy plays an evolutionarily conserved role in eliciting the functions of the Anaphase-Promoting Complex/Cyclosome (APC/C) complex[11–13]. The APC/C complex functions as an E3 ubiquitin ligase and targets its substrates for ubiquitin-dependent protein degradation. Cdc20/Fzy recognizes and recruits APC/C substrates through its carboxyl-terminal WD40 repeats[14,15]. A recent structural study on the substrate-bound WD40 repeats of yeast Cdc20 reveals that two of these repeats mediate its physical interaction with the canonical mitotic substrates of the APC/C complex[16]. Cdc20 elicits the function of APC/C in many cellular processes other than cell cycle progression

presumably by recognizing and recruiting various substrates[17–19]. How Cdc20 exerts substrate recognition and recruitment in non-cell cycle contexts remain unknown. For example, do the WD40 repeats that mediate the binding to mitotic regulatory proteins also function to recognize and recruit other APC/C substrates? Alternatively, could distinct WD40 repeats recognize and recruit unique APC/C substrates?

In this study, we describe a novel Fzy-dependent mechanism that preserves the neuroblast pool during larval brain neurogenesis by promoting cell viability. Through functional analyses of two novel *fzy* mutant alleles, we identified residues glycine291 and alanine444 as being specifically required for Fzy-dependent neuroblast maintenance. The analogous residue to glycine291 of fly Fzy is also dispensable for the function of vertebrate Cdc20 in promoting cell cycle progression. Furthermore, *fzy* mutant neuroblasts did not display diagnostic characteristics of mitotic catastrophe induced by defects in the degradation of the mitotic substrates of APC/C, Cyclin A or B. Thus, Fzy maintains neuroblasts via a mechanism independent of its role in regulating cell cycle progression. Mutant phenotypic analyses revealed that the nuclei of many *fzy* mutant larval brain neuroblasts lacked DNA content and prompted us to investigate how these neuroblasts die. Surprisingly, functional and morphological analyses indicated that Fzy maintains neuroblasts by suppressing necrotic cell death via an APC/C-dependent mechanism. Since the fly genome lacks functional orthologs of the canonical regulators of programmed necrosis, Fzy most likely suppresses necrotic cell death via a novel mechanism. Indeed, we found that removing *aif* function or inactivating JNK signaling prolongs the survival of *fzy* mutant neuroblasts whereas ectopically activating JNK signaling induces premature necrotic cell death in neuroblasts. Ectopic induction of DNA damage or cell cycle perturbation led to necrotic cell death of the neuroblasts with similar marker expression as the Fzy. Finally, these mutations also lead to loss of Fzy expression, suggesting that they induce neuroblast necrosis via loss of Fzy. Altogether, our results demonstrate that the novel Fzy-dependent mechanism maintains neuroblasts by promoting cell survival during larval brain neurogenesis. Furthermore, this mechanism may be a common pathway by which catastrophic cellular damage is translated into cell death.

Materials and Methods

Drosophila culture and genetics

All *Drosophila* stocks were maintained on yeast-glucose fly media. Embryos were collected on apple juice agar supplemented with yeast paste. Larvae were transferred to cornmeal agar at 0 ALH. In all experiments, heterozygous larvae with balancers were used as controls, since none of these animals had any detectable phenotypes at any stage. Mosaic clones were generated following previously published protocols[20]. The *fzy*^{5032} mutant allele was isolated from an unbiased EMS mutagenesis. The following mutations were used in this study: *fzy*^{1}, *fzy*^{4} and *fzy*$^{AC-10}$ [21,22], *dredd*^{B118} [23], *atg1* mutant alleles (*unc-51*^{3} and *unc-51*^{25}) [24], *apc2* mutant alleles (*mr*^{3} and *mr*^{4}) [25], *apc11* (*lmg*^{138}) [26], *aif* (*aif*^{KO})[27], *egr*^{1} [28], *bsk*^{1} [22], *dronc* (*Nc*^{51}) [29], *ark*^{82} [30], *p53*$^{5A-1-4}$ [31], *apc10*^{e01070} and *apc6* (*cdc16*^{MB09129})[32], *apc5* (*ida*^{B4} *and ida*^{D14})[33], *pink1*^{B8} [34]. The following transgenic fly lines were used in this study *UAS-cycA*$^{\Delta 170}$ and *hs-cycB*^{S} [35], *puc-lacZ* (*puc*^{E69}) [36], *gfp-atg8a* [37], *UAS-GCaMP3.0* [38], *UAS-apoliner* [39], *UAS-p35* [40], *UAS-dtak1*, *UAS-dtak1*^{DN}, *UAS-ask1* [41]. The following stocks were obtained from the Bloomington *Drosophila* Stock Center, *hs-flp*, *Elav-Gal4Tub-Gal4, UAS-mCD8-GFP*, *FRT40A*, *Tub-Gal80*, *Df(3L)BSC283*, *Df(2R)Exel6065*, *Df(2R)Exel7157*, *Df(2R)BSC355*, *Df(3L)ED4341*, *Df(3R)Exel9012*, *Df(2L)Exel7038*. The following stocks were obtained from the Vienna *Drosophila* RNAi Center, *UAS-ND42*$_{RNAi}$ (14444) and *UAS-ND75*$_{RNAi}$ (52047).

EdU Assay

EdU labeling of proliferating larval tissues were performed following a previously established protocol[42].

Protein structure modeling

All sequence alignments were performed using MegAlign version 8.1.4(7).414 by DNASTAR and Clustal W alignment method. *S. pombe* Slp-1 (CDC20) crystal structure PDB code: 4AEZ [43]. Structure analysis and modeling were performed using UCSF Chimera, alpha version 1.5 (build 31214).

Light Photography and Filming

Anterior pupal cases were removed from pharate adults to expose heads. The samples were examined using a Leica MZ95 stereomicroscope. Images and video were acquired using a Sony Handycam HDR-SR1. Images were processed using Microsoft Photoshop CS3.

Immunofluorescence staining and imaging

Larval brains were processed for immunofluorescent staining following a standard protocol[44]. The following primary antibodies were used: rat anti-Dpn (1:1000), rabbit anti-Mira (1:1000), mouse anti-Pros (1:100), chicken anti-GFP (1:2000, GFP-1020, Aves Labs), mouse anti-Fzy (1:20), mouse anti-Lamin (1:400, ADL67.10, DSHB), mouse anti-phospho-histone H3 (1:2000, 3H10, Upstate), mouse anti-CycB (1:50, F2F4, DSHB), mouse anti-Elav (1:100, 9F8A9, DSHB), mouse anti-ATP5A (1:500, MS507, Abcam), rabbit anti-Phospho-SAPK/JNK (1:100, 9251, Cell Signaling). Secondary antibodies were from Invitrogen and Jackson Labs (details are available upon request). We used AlexaFluor 488 phalloidin (1:100, A12379, Invitrogen), rhodamine phalloidin (1:100, R415, Invitrogen). The following small molecules were used: Hoechst 33342 (2 μg/mL, C10340 COMPONENT G, Invitrogen), Necrostatin-1 (various concentrations, BML-AP309, Enzo Life Sciences). For DHE staining, brains were dissected in PBS, incubated in DHE (1:1000, 30 μM, gift from Craig Byersdorfer) for 5 minutes at room temperature with rocking, washed in PBS for 5 minutes with rocking, and immediately mounted for imaging with PBS on glass slides. Samples were examined using a Leica TCS SP5 scanning confocal microscope system operated using LAS AF v.2.6.0.7266.

Quantitative PCR

RNA was isolated from 4 to 6 larvae per genotype aged for 96 hours after hatching using the Trizol (Gibco) reagent following manufacturer's suggested protocol. Primers used for qPCR: fzy1for (TCCACAAAGAGTGGCTCACGC), fzy1rev (CAAATAGACGCAACTGCCCAAGG), fzy2for (GCTGGAGGCGTCTCTAAATGGA), fzy2rev (GGGCGTGGTGTTGGACTT), rp49for (ATCGGTTACGGATCGAACAA), rp49rev (GACAATCTCCTTGCGCTTCT).

Transmission electron microscopy

Larval brains were fixed in 2.5% glutaraldehyde in 0.1 M Sorensen's buffer, pH 7.4, overnight at 4^{o}C. After several buffer rinses, they were post-fixed in 1% osmium tetroxide in the same buffer. They were then rinsed in double distilled water to remove phosphate salt and then *en bloc* stained with aqueous three percent uranyl acetate for 1 hour. They were dehydrated in ascending concentrations of ethanol, rinsed two times in propylene oxide, and embedded in epoxy resin. The samples were ultra-thin sectioned 70 nm in thickness and stained with uranyl acetate and lead citrate. The sections were examined using a Philips CM100 electron microscope at 60 kV. Images were recorded digitally using a Hamamatsu ORCA-HR digital camera system operated using AMT software (Advanced Microscopy Techniques Corp., Danvers, MA). Images were processed using Adobe Photoshop CS3.

Histology

Larvae were allowed to pupate and develop at 25°C. Anterior pupal cases were removed from pharate adults to expose heads and whole pupae were fixed in 1% glutaraldehyde, 4% formaldehyde, acid and alcohol, overnight at 4^{o}C. The samples were washed in 80% ethanol, dehydrated in ascending concentrations of ethanol, washed in xylenes, and embedded in paraffin. 5 μm sections were obtained and stained with Mayer's hematoxylin and eosin. The sections were examined using a Leica DM5000 B upright microscope equipped with a QImaging Retiga 2000R camera. Images were acquired using QCapture Pro v.5.1.1.14 software (Media Cybernetics, Inc.).

Results

Fzy maintains neuroblasts independently of its role in promoting cell cycle progression

From a genetic screen, we identified two novel *fzy* mutant alleles, fzy^{5032} and $fzy^{AC\text{-}10}$, that specifically lead to premature loss of larval brain neuroblasts. Larvae that were fzy^{5032} or $fzy^{AC\text{-}10}$ homozygous or *in trans* with the fzy^{1} null mutant allele showed similar patterns of brain neuroblast loss and developed into adults that exhibited poor motor coordination likely due to a dramatic reduction in neuron production (Fig. 2.1A-K; data not presented). The neuroblast loss phenotype in the fzy^{5032} or $fzy^{AC\text{-}10}$ mutant brains could be completely rescued by neuroblast-specific over-expression of a *UAS-fzy* transgene or a transgene containing the *fzy* genomic locus (Fig. 2.2A; data not presented). Thus, the fzy^{5032} and $fzy^{AC\text{-}10}$ mutations are strong loss-of-function mutant alleles. The fzy^{5032} mutation led to the substitution of glycine291 with arginine whereas the $fzy^{AC\text{-}10}$ mutation resulted in the substitution of alaine444 with valine (Fig. 2.2B). Most GFP-marked lineage clones derived from single neuroblasts mutant for fzy^{5032} or fzy^{4}, a *fzy* null mutant allele[45], lacked neuroblasts and contained far fewer cells than the similarly staged control clones (Fig. 2.1L-N,Z). Hence, the fzy^{5032} mutation acts as a genetic null allele in the context of neuroblast maintenance, and we focused the rest of this study on the fzy^{5032} allele. Consistent with the molecular lesion, fzy^{5032} homozygous mutant larvae displayed a similar level of *fzy* transcript and a similar expression pattern of Fzy protein as $fzy^{5032/+}$ control larvae (Fig. 2.2C-E). Thus, premature loss of neuroblasts in the fzy^{5032} mutant brain is most likely due to loss of Fzy protein function rather than a reduced *fzy* mRNA level or reduced Fzy protein stability.

Although the role of Fzy in regulating cell cycle progression is evolutionarily conserved, all adult-specific structures except the brain and bristles developed fully in the newly eclosed fzy^{5032} homozygous mutant flies (Fig. 2.1H-K; Fig. 2.2F-H). The lack of bristles may indicate a shared mechanism between neuroblasts and bristle progenitors, which are also of proneural developmental origin. Thus, we hypothesized that Fzy maintains central brain neuroblasts via a mechanism independent of its role in promoting cell cycle progression. Consistently, lineage clones derived from single fzy^{5032} mutant

optic lobe neuroepithelial cells contained similar numbers of optic lobe neuroblasts as wild-type clones (Fig. 2.1S-T). By contrast, fzy^4 null mutant neuroepithelial cell clones never exceeded a single cell in size, indicating that *fzy* function is essential for their expansion (Fig. 2.1U). These data indicate that the mutant Fzy protein carrying the G291R substitution function similarly to wild-type Fzy in promoting symmetric expansion of optic lobe neuroepithelial cells. In addition, fzy^{5032} mutant larval imaginal disc cells or adult intestinal stem cells underwent clonal expansion similarly to wild-type cells while fzy^4 mutant cells failed to expand (Fig. 2.2I-N). This result led us to conclude that the fzy^{5032} mutation specifically perturbs central brain neuroblast maintenance but does not abolish the proliferation of other cell types. We extended our analyses by testing whether the mutant vertebrate Cdc20 protein bearing an analogous amino acid substitution as the mutant Fzy protein carrying the G291R substitution can promote cell cycle progression (Fig. 2.2O). Indeed, expression of the mutant fly or mouse Cdc20 protein rescued the cell cycle arrest phenotype in fzy^4 optic lobe clones (Fig. 2.1U-Y, AA). However, consistent with earlier results, expression of mutant Cdc20 was unable to rescue the small clone size of fzy^4 central brain neuroblast clones (Fig. 2.1N-R, Z). Therefore, the G291R substitution induced by the fzy^{5032} mutation specifically abolishes the function of Fzy in maintaining central brain neuroblasts without removing its ability to promote cell proliferation.

To more closely examine the kinetics of neuroblast loss due to the *fzy* mutants, we examined fzy^{5032} and fzy^4 mutant neuroblast mosaic clones 24 hours after clone induction. At this time point, the frequency of central brain clones had already been reduced drastically in mutant clones compared to wild-type (Fig. 2.2P). Since the vast majority of central brain clones are derived from neuroblasts, this result suggests that a significant number of *fzy* mutant neuroblasts disappeared within 24 hours of clone induction. Furthermore, a larger percentage of the remaining mutant clones lacked a neuroblast as compared to wild-type, arguing that a large number of *fzy* mutant neuroblasts which remained long enough to produce progeny disappeared within 24 hours as well (Fig. 2.2Q). These results are consistent with the observation that neuroblasts are lost within the first 24 hours of larval development in fzy^{5032} and $fzy^{AC\text{-}10}$ mutant brains. Taken

together, these data demonstrate that *fzy* mutant neuroblasts disappear rapidly within the first 24 hours that *fzy* function is lost, and many disappear before producing progeny.

Neuroblast loss in the *fzy*^{5032} mutant brain unlikely occurs due to mitotic arrest

Fzy elicits the function of APC/C in promoting cell cycle progression by recruiting mitotic regulatory proteins for ubiquitin-dependent protein degradation[46]. Thus, we tested if loss of neuroblasts in the *fzy*^{5032} mutant brain occurs through mitotic catastrophe triggered by aberrant accumulation of APC/C substrates[47,48]. Since Fzy shares greater than 50% amino acid identity with yeast CDC20, we first modeled the location of glycine291 relatively to the surfaces that mediate the interaction between yeast Cdc20 and the mitotic substrates of APC/C. A recent structural study reveals that the binding between yeast Cdc20 and the mitotic substrates of APC/C mainly occurs through residues located amino terminal to the first WD40 repeat as well as in the first WD40 repeat itself[49]. By contrast, the analogous residue for glycine291 is located in the third WD40 repeat and is not involved in binding to the mitotic substrates of APC/C (Fig. 2.3A). Furthermore, glycine291 is located in a loop between two β-sheets, so the G291R substitution induced by the *fzy*^{5032} mutation is predicted not to perturb the overall tertiary structure of the WD40 repeat. Thus, the Fzy mutant protein induced by the *fzy*^{5032} mutation should fulfill its role in the recruitment of mitotic substrates to the APC/C complex.

The structural modeling of the G291R substitution strongly supported our hypothesis that neuroblast loss in the *fzy*^{5032} mutant brain unlikely occurs due to aberrant accumulation of known APC/C substrates. We directly tested this hypothesis by over-expressing a non-degradable form of Cyclin A or B, the canonical substrates of APC/C during mitosis, in neuroblasts. All neuroblasts in the *fzy*$^{5032/+}$ control brain expressed Dpn and lacked the expression of neuronal marker Elav (Fig. 2.3B). Furthermore, every neuroblast in the control brain maintained 10-12 μm in diameter and possessed a single nucleus containing DNA (Fig. 2.3C-D). Additionally, the level of Cyclin A and B expression varied between neuroblasts due to their asynchronous cell cycle stages (Fig. 2.3B-D; data not presented). Neuroblasts mis-expressing non-degradable Cyclin A or B showed an identical combination of cell identity marker expression as control neuroblasts

but displayed hallmarks of mitotic catastrophe. Specifically, these mutant neuroblasts became drastically enlarged, possessed multiple nuclei per neuroblast that each contained constitutively condensed chromosomes and showed aberrant accumulation of Cyclin A and B (Fig. 2.3E-G; data not presented). By contrast, neuroblasts in the *fzy*^{5032} mutant brain never became enlarged and multi-nucleated and never showed aberrant Cyclin A and B accumulation (Fig. 2.3H-J; data not presented). Most strikingly, neuroblasts in the *fzy*^{5032} mutant brain lacked detectable nuclear DNA, and *fzy*^{5032} mutant brains contained far fewer neuroblasts than larval brains expressing non-degradable Cyclin A or B (Fig. 2.3H-K; data not presented). Thus, we conclude that the neuroblast loss phenotype in the *fzy*^{5032} mutant brain unlikely occurs due to mitotic arrest or an inability to degrade mitotic APC/C substrates.

Fzy maintains neuroblasts by suppressing necrotic cell death

Thus far, our data showing loss of DNA in *fzy*^{5032} mutant neuroblasts strongly suggested they undergo premature cell death and prompted us to investigate the cell death mechanisms underlying neuroblast loss in the *fzy*^{5032} mutant brain. We first tested if activation of apoptosis contributes to neuroblast loss in the *fzy*^{5032} mutant brain. However, we did not detect activation of caspases in *fzy*^{5032} mutant neuroblasts by using an *in vivo* caspase reporter protein[50], and over-expressing the caspase inhibitor p35 protein[40] did not prevent neuroblast loss in the *fzy*^{5032} mutant brain (data not presented). Furthermore, removing the genes encoding the core component of the apoptosome[51] or the apical activator caspase[29,52] also did not suppress neuroblast loss in the *fzy*^{5032} mutant brains (Fig. 2.4A). Thus, *fzy*^{5032} mutant neuroblasts did not undergo premature apoptosis. We next tested if Fzy maintains neuroblasts by suppressing autophagy. Despite increased expression of the autophagic vacuole marker GFP-Atg8a[37] in some neuroblasts in the *fzy*^{5032} mutant brain compared to neuroblasts in control brains, we never detected GFP-Atg8a localized in discrete puncta indicative of autophagosome formation (data not presented). Additionally, removing the function of the *atg1* or *atg7* gene required for autophagy[53,54] did not prevent neuroblast loss in the *fzy*^{5032} mutant brains (Fig. 2.4A). Therefore, *fzy*^{5032} mutant neuroblasts did not undergo premature autophagy. Altogether,

these data led us to conclude that Fzy likely maintains neuroblasts by suppressing a novel form of programmed cell death.

To investigate how *fzy*^{5032} mutant neuroblasts die, we characterized their morphological changes by transmission electron microscopy. Neuroblasts in the *fzy*$^{5032/+}$ control brain always contained single large nuclei, and their cytoplasm were filled with organelles including mitochondria, ribosomes and endoplasmic reticulum (Fig. 2.4B-B'). By contrast, neuroblasts in the *fzy*^{5032} mutant brain often did not have identifiable nuclei, and their cytoplasm appeared largely devoid of organelles and vacuoles (Fig. 2.4C-C'). Remnants of the endoplasmic reticulum aggregated with remaining mitochondria into electron dense masses (Fig. 2.4C'). Mitochondria in *fzy*^{5032} mutant neuroblasts appeared swollen and lost their integrity, and their membranes became ruptured (Fig. 2.4C'). These morphological changes strongly resembled those displayed by cells undergoing necrotic cell death[48,55]. Consistent with their morphological changes, mitochondria became aggregated in 25% of the remaining *fzy*^{5032} mutant neuroblasts but are evenly interspersed in *fzy*$^{5032/+}$ control neuroblasts (Fig. 2.4D-F). In addition, we detected a high level of reactive oxygen species (ROS) in the cytoplasm of 65% of *fzy*^{5032} mutant neuroblasts while only 3% of *fzy*$^{5032/+}$ control neuroblasts showed similar cytoplasmic ROS (Fig. 2.4G-I). Greater than 50% of *fzy*^{5032} mutant neuroblasts displayed a high level of cytoplasmic calcium ions whereas less than 1% of *fzy*$^{5032/+}$ control neuroblasts showed similarly elevated cytoplasmic calcium ions (Fig. 2.4J-L). Finally, almost 40% of *fzy*^{5032} mutant neuroblasts contained ubiquitin-conjugated protein aggregates which were virtually nonexistent in *fzy*$^{5032/+}$ control neuroblasts (Fig. 2.4M-O). The combination of morphological features and marker expression led us to conclude that *fzy*^{5032} mutant neuroblasts undergo necrotic cell death.

Fzy maintains neuroblasts by suppressing necrotic cell death via an APC/C-dependent mechanism

The role of Fzy in substrate recognition and recruitment for the APC/C complex is evolutionarily conserved[11–13]. Thus, we tested if Fzy suppresses necrotic cell death in neuroblasts via an APC/C-dependent mechanism. Larval brains mutant for either the scaffolding, catalytic, or substrate recruitment subunits of the APC/C complex all showed

a reduction in central brain neuroblasts (Fig. 2.5B-G, Z)[25,26,56–58]. Distinct from the *fzy*^{5032} mutant, however, the expansion of neuroepithelial cells in the *apc* mutant optic lobes became prematurely arrested (Fig. 2.5B-G). Thus, APC/C function is essential for both neuroblast maintenance as well as the expansion of neuroepithelial cells. To confirm that the APC/C complex indeed maintains neuroblasts by suppressing necrosis, we examined whether APC/C mutant neuroblasts displayed similar marker expression as *fzy*^{5032} mutant neuroblasts. Indeed, neuroblasts carrying an APC/C mutation exhibited aggregation of mitochondria, increased cytoplasmic ROS and increased cytoplasmic calcium ions (Fig. 2.5H-Y). Thus, the function of APC/C is essential for maintaining neuroblasts as well as promoting the proliferation of neuroepithelial cells. These data are consistent with *fzy* mutant phenotypic analyses and strongly suggest that the *fzy*^{5032} mutation specifically disrupts the role of Fzy in eliciting the function of APC/C in neuroblast maintenance.

Necrosis markers are not sufficient for cell death execution

The presence of necrosis markers led us to examine these markers' relationship to neuroblast necrosis in detail. In order to investigate whether the markers are functionally important for neuroblast necrosis, we examined mutations that elicit each of the necrosis markers individually and determine if the presence of the marker was sufficient for cell death. The *Pten-induced putative kinase 1* (*pink1*) gene regulates mitochondrial fission and fusion[34,59,60]. However, larval brains mutant for the *pink1* contained a similar number of neuroblasts as control brains (Fig. 2.6A-B, D). Moreover, knocking down the function of *ND75* or *ND42* genes, which encode components of the mitochondrial respiratory chain[61], triggered a massive increase in the level of cytosolic ROS but failed to induce premature neuroblast loss in the larval brain (Fig. 2.6C-D). Thus, neither mitochondrial dysfunction nor increased ROS production alone appears sufficient to induce premature neuroblast loss. Next, we examined the contribution of ubiquitin-conjugated aggregates. Mutations in transactive response DNA-binding protein 43 (TDP-43) and valosin containing protein (VCP) mutations have been associated with neurodegenerative diseases such as amyotrophic lateral sclerosis and frontotemporal lobar degeneration in which ubiquitinated inclusion bodies are a signature pathological feature. Over-

expression of either a pathologic form of TDP-43 or VCP caused increased frequency of ubiquitin-conjugated aggregates (Fig. 2.6E-G, I). However, neither mutation was sufficient to cause loss of neuroblasts (Fig. 2.6D). Finally, over-expression of dominant negative components of the proteosome complex also caused the presence of ubiquitin-conjugated aggregates, presumably by blocking the ability of proteasome complex to degrade ubiquitin-conjugated proteins (Fig. 2.6H-I). This mutation also did not kill neuroblasts (Fig. 2.6D). Thus, despite the presence of individual necrosis markers in these mutants, none of them caused necrosis of neuroblasts. Taken together, these data suggest that mitochondrial dysfunction, oxidative stress, and protein stress are not individually sufficient to induce necrosis of neuroblasts.

Fzy maintains brain neuroblasts by suppressing a non-canonical necrotic cell death mechanism

Lack of detectable DNA in *fzy*^{5032} mutant neuroblast nuclei strongly suggested that a robust endonuclease activity might contribute to neuroblast loss in the *fzy*^{5032} mutant brain. The *aif* gene encodes a mitochondrial oxidoreductase protein and upon activation, can recruit an endonuclease complex to trigger large-scale DNA fragmentation[62,63]. An *aif* mutant mosaic clone contained a single neuroblast and possessed many neuronal progeny, indistinguishable from a wild-type neuroblast clone (Fig. 2.7A, C). This result indicates that *aif* is dispensable for the maintenance and the proliferation of neuroblasts. By contrast, removing *aif* function significantly prolonged the survival of *fzy*^{5032} mutant neuroblasts allowing them to generate more neuronal progeny compared identically staged *fzy*^{5032} mutant neuroblasts alone (Fig. 2.7B-E). Thus, necrotic cell death of *fzy*^{5032} mutant neuroblasts requires *aif* function. Over-expression of a truncated Aif transgenic protein lacking the amino terminal mitochondrial localization domain but not the wild-type Aif transgenic protein can trigger caspase activation and cell death in larval eye imaginal disc cells[27,63]. However, over-expression of neither the truncated nor the wild-type Aif transgenic protein was sufficient to induce premature loss of neuroblasts (data not presented). This result is consistent with our results indicating that *fzy*^{5032} mutant neuroblasts undergo necrotic cell death rather than apoptotic cell death. Together, these data led us to conclude that *aif* function is required

for necrotic cell death of *fzy*^{5032} mutant neuroblasts but additional caspase-independent downstream mechanisms must exist.

Activation of c-Jun N-terminal kinase (JNK) signaling was previously shown to coincide with increased ROS production in necrotic cells in vertebrates and in non-apoptotic cell death in the fly[64–67]. To test whether aberrant activation of JNK signaling might contribute to neuroblast loss in the *fzy*^{5032} mutant brain, we examined the expression of *puc-lacZ* and the phosphorylated form of JNK. The expression of *puc-lacZ* and phosphorylated JNK was undetectable in neuroblasts in the *fzy*$^{5032/+}$ mutant brain, indicating that JNK signaling is not activated in control neuroblasts (Fig. 2.7F; data not presented). By contrast, we consistently detected the expression of both *puc-lacZ* and phosphorylated JNK in many neuroblasts in the *fzy*^{5032} mutant brain (Fig. 2.7G; data not presented). Thus, JNK signaling becomes aberrantly activated in *fzy*^{5032} mutant neuroblasts. We next tested if neuroblast loss in the *fzy*^{5032} mutant brain required the function of JNK encoded by the *basket* (*bsk*) gene. A *bsk* mutant mosaic clone maintained a single neuroblast and possessed as many neuronal progeny as a wild-type neuroblast clone (Fig. 2.7A, I). This result indicates that *bsk* is dispensable for the maintenance and the proliferation of neuroblasts. By contrast, removing *bsk* function from *fzy*^{5032} mutant neuroblasts allowed them to generate more neuronal progeny compared to identically staged *fzy*^{5032} mutant neuroblasts alone (Fig. 2.7I-K). This suggests that loss of *bsk* prolonged the survival of *fzy*^{5032} mutant neuroblasts which allowed them to produce significantly more progeny. Thus, necrotic cell death of *fzy*^{5032} mutant neuroblasts requires *bsk* function. To complement the loss-of-function analyses, we examined whether aberrant activation of JNK signaling is sufficient to induce premature necrotic cell death of neuroblasts. Previous studies showed that the JNK kinase kinase (JNKKK) encoded by the *transforming growth factor beta-activate kinase* (*tak1*) gene plays a critical role in JNK signaling during development and over-expression of *tak1* can induce premature cell death[41,68]. Indeed, over-expression of a high level of *tak1* or its direct target, the JNK kinase (JNKK) *hemipterous* (*hep*), efficiently induced premature neuroblast loss in the larval brain (Fig. 2.7L-O). Similar to *fzy*^{5032} mutant neuroblasts, neuroblasts with aberrant JNK activation often showed diminished expression of Dpn (Fig. 2.7M). Co-expression of P35 failed to prevent premature loss of

neuroblasts induced by *tak1* over-expression (Fig. 2.7O), suggesting that this death is caspase-independent. Finally, expression of either a kinase dead form of Tak1 or an analogous JNKKK, Apoptotic signal-regulating kinase 1 (Ask1), were not able to induce premature neuroblast loss, arguing that the kinase signaling property is required for neuroblast death in aberrant JNK activation and that this process is specific to Tak1 activation (Fig. 2.7O). Together, these results led us to conclude that aberrant activation of JNK signaling through Tak1 contributes to necrotic cell death of neuroblasts in the fzy^{5032} mutant brain.

Catastrophic genotoxic stress leads to necrosis in neuroblasts

Since Fzy, which is normally expressed in neuroblasts, is required to suppress necrosis in neuroblasts, we hypothesized that necrosis may be used to eliminate cells in pathological situations. However, thus far we have demonstrated that energetic, oxidative, and protein stress are insufficient to induce neuroblast necrosis. This led us to examine another major target of cellular damage: genotoxic stress. Knockdown of subunits in the chaperonin containing Tcp-1 complex (CCT), CCT2 and CCT4, caused a significant loss of neuroblasts as well as the appearance of abnormal mitochondrial morphology, ROS, and ubiquitin-conjugated aggregates (Fig. 2.8A-C, G-I, M-O, Y-AA). Importantly, loss of CCT function led to loss of Fzy expression in neuroblasts, suggesting this complex functions upstream of Fzy in promoting neuroblast survival (Fig. 2.8S-X, BB). The CCT complex plays a major role in folding structural proteins such as tubulin, which is required for proper centriole and spindle formation[69,70]. Indeed, loss of CCT function caused decreased tubulin content in mitotic spindles (Fig. 2.9F-H). This result suggests that perturbation of cell cycle processes by loss of CCT may be promoting neuroblast necrosis.

To test the impact of genotoxic stress further, we examined mutations in the core telomere capping complex, *caravaggio* (*cav*) and *suppressor of variegated 205* (*Su(var)205*), and of *flap endonuclease-1* (*fen1*)[71,72]. Each of these mutations faithfully recapitulated the loss of neuroblast phenotype as well as the appearance of necrosis markers (Fig. 2.8D-F, J-L, P-R, Y-AA). Furthermore, they also caused loss of Fzy expression in the neuroblasts (Fig. 2.8V-X, BB). Loss of telomere capping exposes

chromosomal ends which are treated as double strand breaks by DNA repair machinery. Similarly, loss of Fen1 function allows unresolved DNA flaps which are treated as lesions by the cell. Accordingly, DNA damage as detected by the presence of phosphorylated histone H2A variant (pH2Av) was completely undetectable in wild-type neuroblasts but appeared in both telomere capping mutant neuroblasts and *fen1* mutant neuroblasts (Fig. 2.9A-E). Together, these data suggest that catastrophic damage to DNA or cell cycle machinery causes loss of Fzy expression and neuroblast necrosis.

p53 up-regulation induces necrosis in neuroblasts

Both the disruption of spindle formation and DNA damage can cause dividing cells to trigger cell cycle arrest and possible death. p53 transcription factor is a key regulator of cell cycle arrest and cell death in response to genotoxic stress in many dividing cells[73,74]. Thus, we examined whether p53 plays a role in neuroblast death. Over-expression of wild-type p53 specifically in neuroblasts led to a significant decrease in neuroblast number as well as appearance of ROS and ubiquitin-conjugated aggregates (Fig. 2.10E-F, I-J, Q, S). p53 up-regulation also caused loss of Fzy expression, suggesting that Fzy acts downstream of p53 in regulating necrosis (Fig. 2.10M-N, T). A dominant-negative form of p53 did not present with loss of neuroblasts, necrosis marker expression, or loss of Fzy expression (Fig. 2.10C, G, K, O, Q-T). Importantly, co-expression of dominant-negative p53 with wild-type p53 resulted in a partial rescue of the loss of neuroblasts, ubiquitin-conjugated aggregates, appearance of ROS, and loss of Fzy expression phenotypes caused by wild-type p53 (Fig. 2.10H, L, P, Q, S-T). Curiously, mitochondrial morphology was not detectably altered by over-expression of wild-type p53 (Fig. 2.10A-B, R). This may indicate that change in mitochondrial morphology depends on a stronger loss of Fzy function than that caused by p53 over-expression. These results suggest that increased p53 transcriptional regulatory activity induces loss of Fzy expression and neuroblast necrosis.

When activated by genotoxic stress, p53 can trigger cell cycle arrest in a context specific manner[75]. We tested if p53-triggered cell cycle arrest caused neuroblast death. Wee1 is normally suppressed by Cdk1 (encoded by *cdc2*). Activation of Wee1 causes cell cycle arrest at the G2 to M transition downstream of p53 activation. Consistently,

either over-expression of *wee1* or knockdown of *cdc2* caused a decreased frequency of neuroblasts with cytoplasmic Dpn localization (Fig. 2.11A-D). Since Dpn is a nuclear transcription factor, the percentage of cells with cytoplasmic Dpn indicates the percentage of cells with nuclear envelope breakdown. This served as a proxy measurement for the percentage of cells in mitosis, suggesting that over-expression of *wee1* or loss of *cdc2* did indeed cause cell cycle arrest. Furthermore, both mutations caused enlargement of the neuroblasts, consistent with cells that are growing in preparation for cell division but unable to actually divide (Fig. 2.11A-C). However, neither mutation was sufficient to cause loss of neuroblasts (Fig. 2.10Q). These results demonstrate that cell cycle arrest downstream of p53 activation is not sufficient to induce neuroblast necrosis. p53 over-expression likely acts through a parallel pathway to trigger neuroblast necrosis.

p53 activation of apoptosis is believed to be a potent tumor suppressing mechanism[76]. Cancer depends on conditions which favor frequent mutagenesis. Removing cells that have acquired too many random mutations by programmed cell death is an efficient way to eliminate the threat posed by these cells. To test if Fzy-dependent neuroblast necrosis is capable of removing tumorigenic cells, we examined fzy^{5032} mutants in the *brain tumor* (*brat*) mutant genetic background. *brat* mutant larvae are able to produce a large number of supernumerary neuroblasts and have served as a good model for studying tumorigenesis (Fig. 2.12A)[77,78]. The fzy^{5032} mutation was able to completely suppress the neuroblast overproliferation phenotype in a strong *brat* mutant background (Fig. 2.12B-C). These results suggest that the Fzy-dependent cell death mechanism functions downstream of the *brat* phenotype and that a p53-dependent necrotic cell death would be capable of killing *brat* mutant neuroblasts.

Discussion

We report here the discovery of a novel mechanism that is dedicated to maintaining stem cell survival and not involved in determining stem cell identity. We have demonstrated that the APC/C-Fzy complex mediates the survival of *Drosophila* larval neuroblasts independently of its role in promoting cell proliferation. Mutations that occur in the surfaces of the Fzy/Cdc20 WD40 repeats not required for the binding of Fzy to mitotic regulatory proteins, specifically lead to premature neuroblast cell death. Furthermore, we have found that catastrophic genotoxic stress on the neuroblasts causes them to undergo necrosis and display the same morphological markers as Fzy loss of function mutants. Meanwhile, p53 up-regulation produced the same effect, arguing that cellular damage and the coping mechanisms for damage may utilize this necrosis mechanism to mediate cell survival.

We propose that APC/C-Fzy promotes neuroblast proliferation while also surveilling the neuroblast pool by selectively targeting distinct substrates for ubiquitin-dependent protein degradation (Fig. 2.12D). In wild type neuroblasts, an uncharacterized necrosis activator(s) is recruited to the APC/C-Fzy complex to be ubiquitinated. Routine degradation of this factor ensures survival of neuroblasts. In fzy^{5032} mutant neuroblasts, this activator is unable to be recruited by Fzy because of the specific lesion in the WD40 repeats, despite Fzy^{5032} still being able to activate APC/C ubiquitantion activity and promote cell cycle progression. The perdurance of this factor leads to activation of JNK signaling and Aif pathways, which ultimately contribute to neuroblast necrosis. Discovering the necrosis activator directly targeted by APC/C-Fzy will be crucial to substantiate this model. It is possible that multiple factors which can promote cell death are suppressed by APC/C-Fzy in this manner.

Accumulating evidence indicates that Fzy/Cdc20 functions to regulate many cellular processes in addition to their well-established role in promoting cell proliferation via an APC/C complex-dependent mechanism[17,18]. For example, loss of Cdc20 in cerebellar slices has been shown to reduce synaptic cluster density by stabilizing NeuroD2 in a mechanism similar to the one we propose[18]. Cdc20 also regulates the degradation of Id1 during dendrite growth[17]. Most importantly, Cdc20/Fzy executes these functions by recruiting novel substrates to the APC/C complex for ubiquitin-

dependent protein degradation. The best-characterized degradation motifs that bind to APC/C-Cdc20 are the KEN- and D-box motifs[49]. Indeed, both the NeuroD2 and Id1 proteins contain motifs that are highly similar to the canonical D-box, but it is currently unknown whether the degradation motif from NeuroD2 and Id1 binds to Cdc20/Fzy through the same surfaces on the WD40 repeats that mitotic cyclins bind to, or different surfaces. Since this diversity in substrate binding appears unlikely to be caused by allosteric changes in APC/C conformation upon co-activator binding[13], one attractive possibility is that distinct surfaces on the WD40 repeats of Cdc20/Fzy might recognize distinct categories of substrates. Our work indicates that the WD40 repeats containing the fzy^{5032} and fzy^{AC-10} mutations are required for binding novel substrates of APC/C-Fzy which mediate neuroblast survival. More thorough characterization of the Fzy binding surfaces that are required to maintain neuroblast survival will provide critical insight toward understanding how the APC/C complex regulates diverse biological processes.

Since the fly genome appears to lack orthologs of RIP kinases, this raises important questions about the regulation of necrotic cell death in *Drosophila*. One possible mechanism is that downstream TNF signaling triggers a form of non-apoptotic cell death with similar properties to mammalian programmed necrosis[67]. Another possibility might be that JNK signaling leads to increased oxidative stress upon activation[64,65]. Removing the function of TNF had no effect on premature loss of neuroblasts in *fzy* mutant brains whereas removal of JNK significantly prolonged the survival of *fzy* mutant neuroblasts. Furthermore, ectopic JNK signaling in neuroblasts caused by over-expression of a JNKKK or JNKK killed them in a manner similar to fzy^{5032} mutation. Thus, we propose that APC/C-Fzy prevents neuroblast necrotic cell death in part by antagonizing the activation of JNK signaling. Curiously, loss of *tak1* did not rescue the neuroblast death phenotype in fzy^{5032} mutants (data not presented). However, there are at least 5 fly orthologs of JNKKK with some partially overlapping functions[79]. While over-expression of one of these orthologs, Ask1, did not lead to loss of neuroblasts, it is possible that other JNKKK orthologs can contribute. Therefore, it is likely that loss of a single JNKKK would have no phenotype. Indeed, the *tak1* null mutant animals are perfectly viable and fertile. Consistent with this hypothesis, loss of another JNKKK, Wallenda, also did not rescue neuroblasts from death in fzy^{5032} mutants

(data not presented). Additional experiments will be necessary to determine the mechanism by which APC/C-Fzy suppresses activation of JNK signaling.

Aif has emerged as a tantalizing candidate for executing non-apoptotic cell death[63,80]. Aif is a mitochondrial protein and becomes released following an increase in ROS and calcium ions. The soluble Aif can then translocate to the nucleus where it may activate DNA fragmentation and cell death. While *fzy*^{5032} mutant neuroblasts displayed an increase in ROS production and cytosolic calcium ion, increased ROS production alone seemed to be insufficient for triggering neuroblast necrosis. These data are consistent with ROS simply facilitating the release of Aif following onset of necrotic cell death. Similarly, mitochondria defects alone were insufficient to induce neuroblast death. In contrast, cytosolic calcium ion activates Calpain I to cleave the full-length Aif and release it from mitochondria. Since the fly proteins CalpA and CalpB share homology with Calpain I, these two proteases are excellent candidate proteins that likely are involved in the activation of neuroblast necrotic cell death in *fzy* mutant brains. Functional assessment of the roles of CalpA and CalpB in neuroblast necrotic cell death will be critical in the future to test this hypothesis.

The biological significance of a Fzy-mediated necrosis mechanism appears to be linked to the health of the cell. Thus, this mechanism may present a novel cell checkpoint which monitors the neuroblast for damage, and translates irreparable damage into cell death. Neuroblasts appear to be able to sustain certain damage, such as increased ROS production or ubiquitin-conjugated aggregates, without triggering death. However, catastrophic genotoxic stress was able to induce neuroblast necrosis. Interestingly, p53 over-expression produced a similar phenotype, suggesting that it may function to translate the cell damage into a cell death response. This would be consistent with its known role as a genomic stability checkpoint master regulator. The ability of p53 to induce necrosis seems to be distinct from its ability to arrest the cell cycle, since over-expression of factors that induce cell cycle arrest downstream of p53 activation did not cause loss of neuroblasts. In the literature, p53 is most commonly described as a regulator of apoptosis. However, the exact mode of cell death caused by p53 is frequently not characterized in detail and overlapping death mechanisms may exist. The apoptosis pathway in fly larval neuroblasts is epigenetically repressed before pupation.

Thus, even if pro-apoptotic signals exist in neuroblasts, they would unlikely be able to trigger apoptosis. This study suggests that a novel programmed necrosis mechanism may exist is neuroblasts as a backup to trigger cell death in the absence of apoptosis. This pathway is responsive to cell damage and p53 activation, very similarly to how apoptosis may act in such a context.

Our study discovered a previously unknown function of Fzy in maintaining stem cell viability by suppressing necrotic cell death exclusively in the brain. Mechanistic insight into how Fzy maintains the viability of neural stem cells by antagonizing necrotic cell death might lead to novel strategies to selectively attenuate the pro-survival function of Fzy in cells possessing stem cell characteristics in the brain. Brain tumor stem cells possess similar functional properties as normal neural stem cells, except they proliferate more rapidly[81–83]. Thus, brain tumor stem cells are likely more dependent on Fzy and thus more sensitive to manipulations that perturb Fzy function. As such, selectively inactivating the pro-survival function of Fzy might preferentially target brain tumor stem cells while sparing normal neural stem cells and stem cells in other organs. Further investigation into whether Cdc20 is required for maintaining specific stem cell populations in mammals is required. These strategies will likely improve the efficiency of cancer therapies while reducing unwanted side effects.

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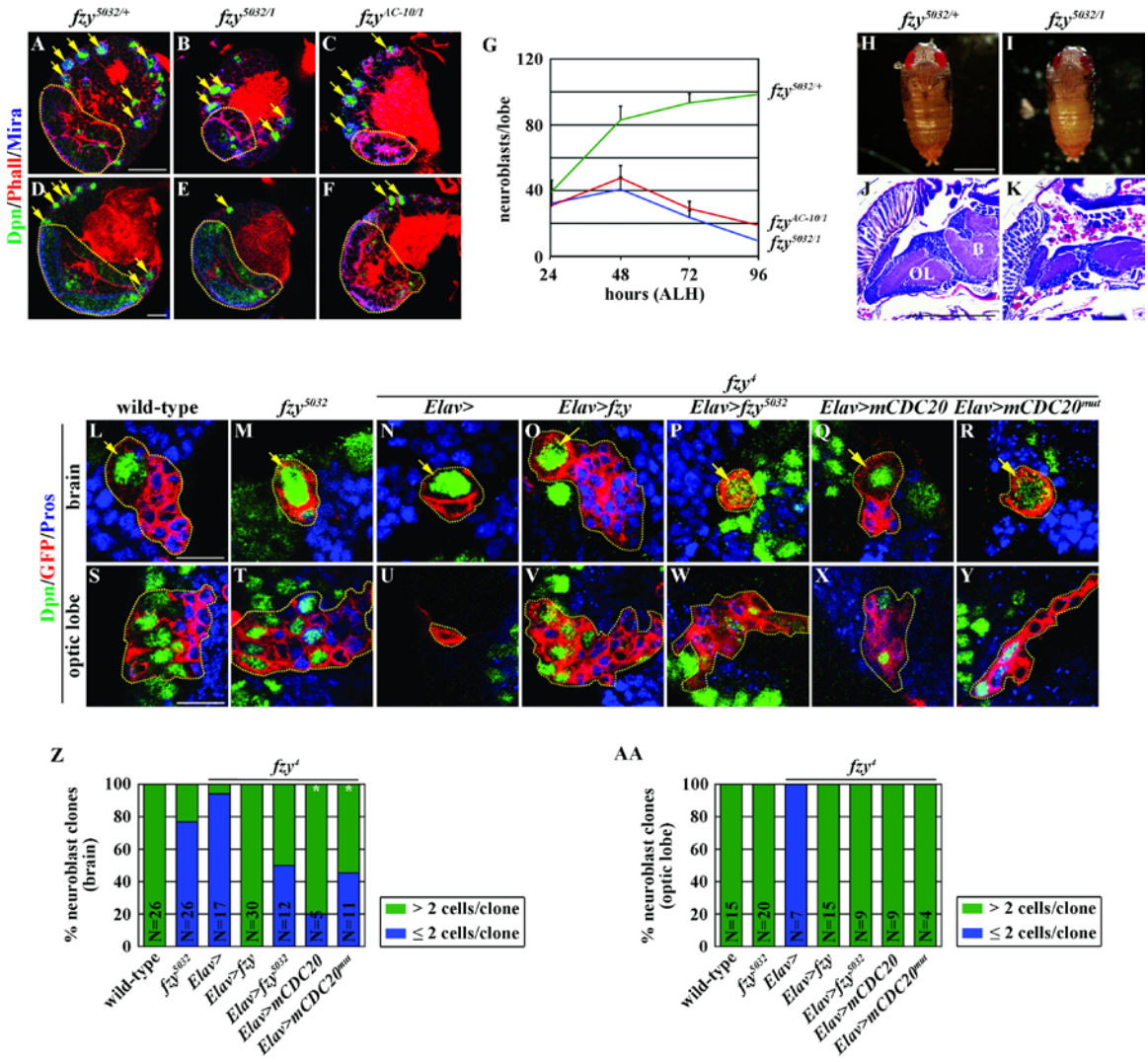

Figure 2.1. The *fzy*^{5032} mutation specifically attenuates the function of *fzy* in maintaining neuroblasts and does not perturb Fzy promoting cell proliferation.
(A-F) Larval brains of the genotypes indicated were stained for the neuroblast marker Deadpan (Dpn) and Miranda (Mira) and the cortex marker Phalloidin (Phall). Brains were dissected from larvae aged for 48 (A-C) or 72 (D-F) hours after larval hatching (ALH). Arrows mark neuroblasts and dotted lines mark optic lobes. Scale bars, 20μm.
(G) Average number of Dpn^{+} neuroblasts per larval brain lobe in the genotypes indicated during larval development (N = 10 brain lobes per genotype, error bars here and in all future graphs represent mean ± S.D).
(H, I) Dorsal view of pharate adult brains from control or *fzy* mutant pupae aged for 120 hours APF. Scale bar, 1mm.
(J, K) Horizontal sections of control and *fzy* mutant pupae brains at 96 hours after pupae formation (APF) visualized with hematoxylin and eosin. Scale bar, 200μm.
(L-R) GFP-marked central brain neuroblast mosaic clones of the genotypes indicated were stained for Dpn, GFP, and Prospero (Pros), a marker for immature neurons. Clones

were induced at 24 hours ALH and examined at 96 hours ALH. Arrows mark mother neuroblasts. Dotted lines mark clones. Scale bar, 10μm.
(S-Y) GFP-marked optic lobe neuroepthelial cell mosaic clones of the genotypes indicated were stained for Dpn, GFP and Pros. Clones were induced at 24 hours ALH and examined at 96 hours ALH. Dotted lines mark clones. Scale bar, 10μm.
(Z) Quantification of percentage of central brain neuroblast clones containing more than 2 cells versus percentage containing 2 or fewer cells for each genotype. Cell count includes mother neuroblast. Star indicates p-value < 0.001 as determined by chi-square test. All future stars will indicate $p<0.001$ for the marked genotype in comparison to the control or wild-type genotype in the same graph.
(AA) Quantification of percentage of optic lobe neuroepithelial clones containing more than 2 cells versus percentage containing 2 or fewer cells for each genotype.

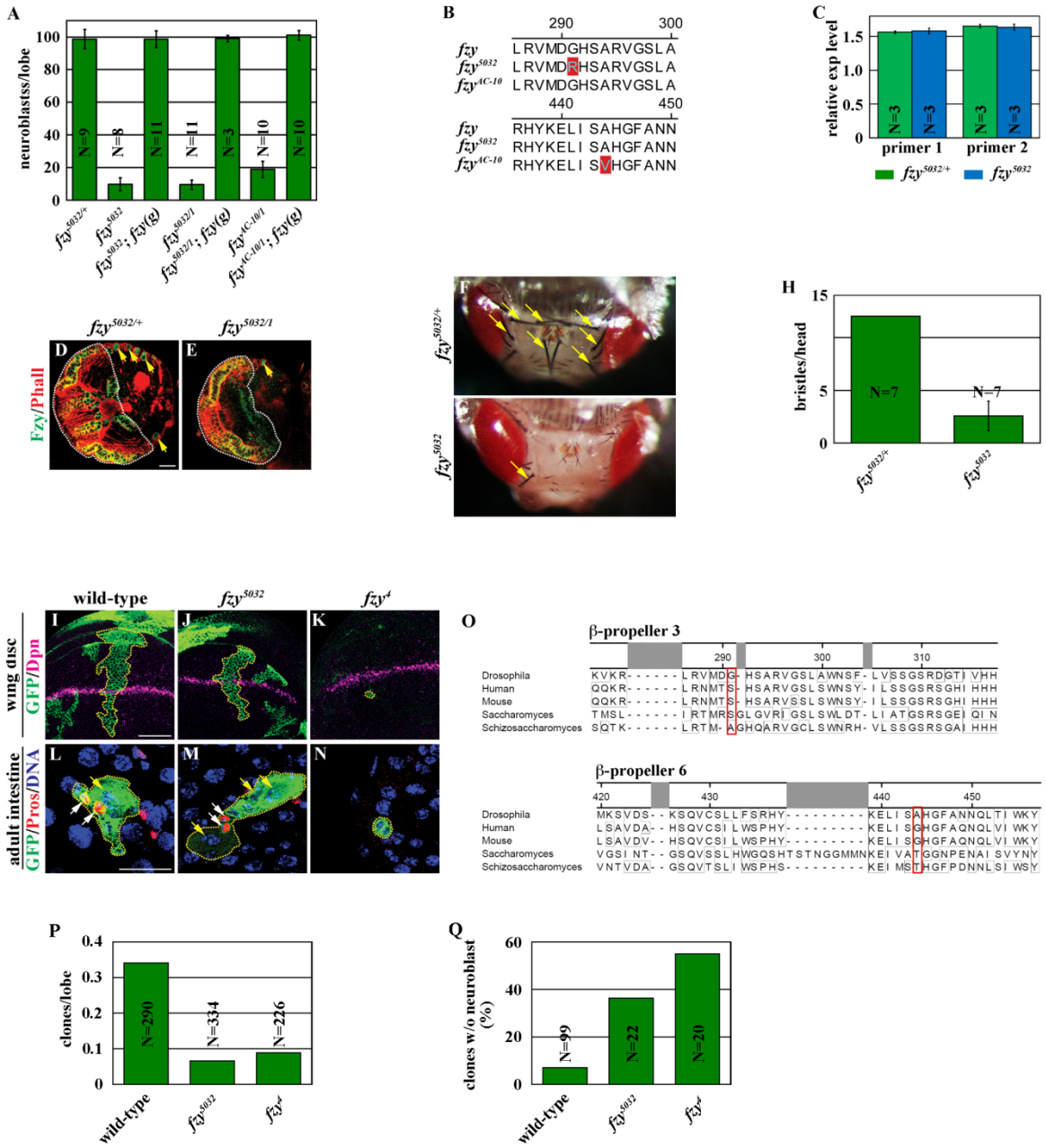

Figure 2.2. Fzy point mutations in the WD40 repeats specifically ablate neuroblast maintenance but allow cell cycle progression.

(A) Average number of Dpn^{+} neuroblasts per brain lobe in the genotypes indicated at 96 hours ALH. The *fzy* genomic transgene is abbreviated as *fzy(g)*. fzy^{5032} indicates fzy^{5032} homozygous (this and all similar graphs represent mean ± S.D.).

(B) The molecular lesions induced by the fzy^{5032} or fzy^{AC-10} mutation.

(C) The relative expression level of *fzy* mRNA to *rp49* mRNA in control or *fzy* mutant larvae (N = 3 reactions per genotype).

(D-E) Larval brains of the genotypes indicated at 96 hours ALH stained for Fzy and Phall. Arrows mark neuroblasts. Dotted lines mark optic lobe. Scale bar, 20μm.

(F-G) Photograph of pharate adult head of the indicated genotypes. Arrows mark macrochaetes or large bristles that were counted. 12 stereotypical bristles were counted on each wild type head and the analogous bristles, if present were counted on mutant heads.
(H) Quantification of bristles per head on control versus *fzy*^{5032} mutant pharate adult heads.
(I-K) GFP-marked mosaic clones derived from wing imaginal disc cells of the genotypes indicated were stained for Dpn and GFP. Dotted lines mark clones. Scale bar, 40μm. All larval clones were induced at 24 hours ALH and examined at 96 hours ALH.
(L-N) GFP-marked mosaic clones of adult midgut stem cells derived of the genotypes indicated were stained for GFP, Pros and DNA. Clones were induced at 3 days post-eclosion and examined 10 days post-eclosion. Yellow arrows mark enterocytes, identifiable by their large 8n nuclei. White arrows mark enteroendocrine cells, identifiable by anti-Pros antibody. Unmarked cells in clones are likely stem cells or progenitors [84]. Dotted lines mark clones. Scale bar, 20μm.
(O) Protein sequence alignment of CDC20 between five species: *D. melanogaster*, *H. sapiens*, *M. musculus*, *S. cerevisiae*, and *S. pombe*. Only β propellers 3 and 6 of the WD40 repeats are shown. Numbering marks Drosophila Fzy residue position. Red boxes mark residues mutated in *fzy*^{5032} and *fzy*$^{AC-10}$ mutants.
(P) Quantification of total wild-type versus *fzy* mutant clones per lobe. Total clones include all clones containing at least 3 cells, with or without a mother neuroblast.
(Q) Percentages of clones which were missing the mother neuroblast among wild-type versus *fzy* mutant clones. Only clones containing at least three cells were counted. Small percentages of wild-type clones missing neuroblast were likely derived from intermediate neural progenitors.

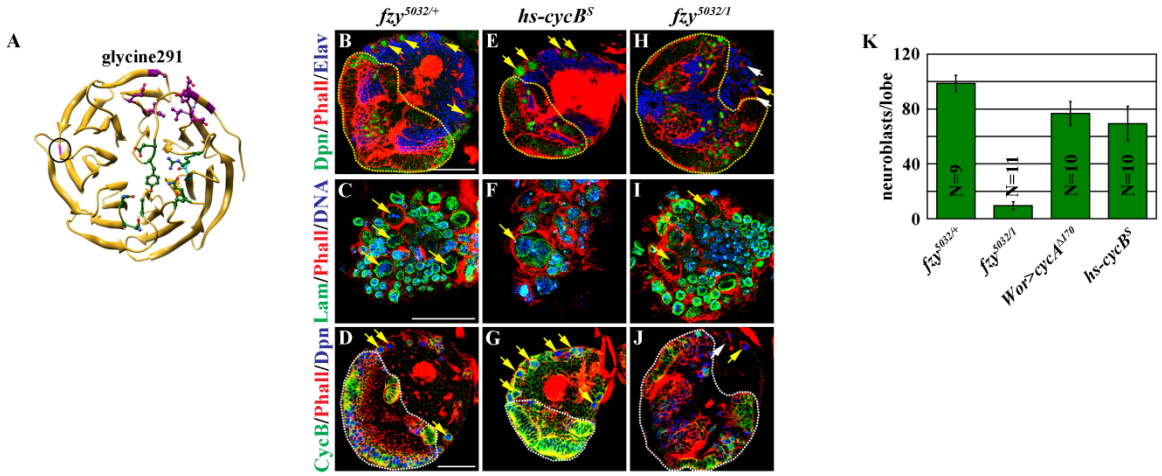

Figure 2.3. Premature loss of neuroblasts in *fzy* mutant brains unlikely occurs as a consequence of mitotic catastrophe.

(A) The structure of the WD40 repeats of *S. pombe* Slp1 protein. The analogous residues to those mutated in Fzy^{5032} (pink) and Fzy^{AC-10} (cyan) are marked, as determined by sequence alignment between Slp1 and Fzy. D box-binding (green) and KEN box binding (purple) residues are marked.

(B-D) Wild type brains at 96 hours ALH stained for indicated markers.

(E-G) Larval brains at 96 hours ALH from animals expressing non-degradable Cyclin B ($CycB^{s}$) under the control of a heat-shock promoter.

(H-J) *fzy* mutant brains at 96 hours ALH.

In B, E, and H, yellow arrows mark Dpn^{+} neuroblasts and white arrows mark Dpn^{-} dying neuroblasts. Elav marks differentiating neurons. Scale bar, 40μm.

In C, F, and I, arrows mark nuclei. Lamin (Lam) marks nuclear envelope. DNA visualized by Hoechst. Scale bar, 20μm.

In D, G, and J, arrows mark neuroblasts. Scale bar, 40μm.

All dotted lines mark optic lobes.

(K) Average number of Dpn^{+} neuroblasts per brain lobe in the genotypes indicated at 96 hours ALH. Expression of the non-degradable CyclinA ($CycA^{\Delta 170}$) transgenic protein was driven by a neuroblast-specific *Wor-Gal4* driver.

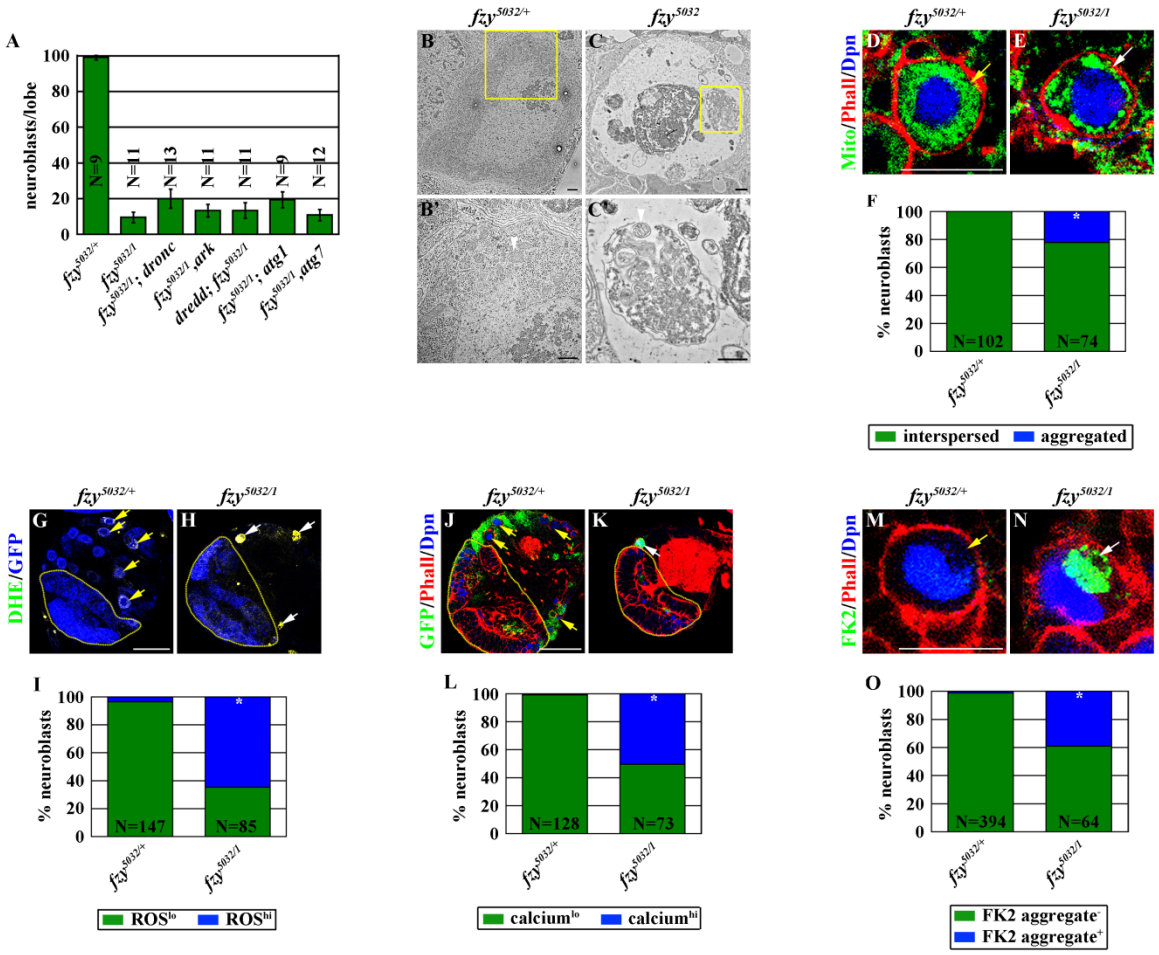

Figure 2.4. *fzy* mutant neuroblasts undergo necrotic cell death.
(A) Average number of Dpn^{+} neuroblasts per brain lobe in the genotypes indicated at 96 hours ALH. *ark dronc* and *dredd* encode proteins required for apoptosis. *atg1* and *atg7* encode proteins required for autophagy.
(B-C) Transmission electron micrographs of neuroblasts in the genotypes indicated at 96 hours ALH. B', C' are higher magnifications of areas marked by yellow boxes in B, C. Red stars mark mitochondria. Scale bars, 500nm.
(D-E) Neuroblasts in larval brains of the genotypes indicated at 72 hours ALH were stained for ATP5a (complex V of mitochondria ATP synthase), Phall and Dpn. Yellow arrow marks neuroblast with no mitochondrial aggregates. White arrow marks neuroblast with aggregates. Scale bar, 10μm.
(F) Quantification of Dpn^{+} neuroblasts with evenly interspersed mitochondria (green) versus compacted or aggregated mitochondria (blue) in larval brains of the genotypes indicated at 72 hours ALH.
(G-H) Unfixed larval brains of the genotypes indicated at 96 hours ALH stained with dihydroethidium (DHE), a small molecule whose signal indicates presence of superoxide free radicals[61]. Yellow arrows mark neuroblasts which were identifited by expression of PCNA::3XEmGFP and had low or no detectable ROS. White arrows mark neuroblasts with high ROS. Dotted lines mark optic lobes. Scale bar, 40μm.

(I) Quantification of PCNA::3XEmGFP^{+} neuroblasts with high levels of ROS (green) versus low levels of ROS (blue) in larval brains of the genotype indicated at 96 hours ALH.
(J-K) Larval brains of the genotypes indicated at 72 hours ALH were stained for GFP, Phall and Dpn. The levels of calcium ion were assessed by expression of the GCaMP3.0 transgenic protein driven by *Wor-Gal4*, indicated by the GFP activity. Yellow arrows mark Dpn^{+} neuroblasts with low or no calcium. White arrows mark neuroblasts with high calcium. Dotted lines mark optic lobes. Scale bar, 40μm.
(L) Quantification of neuroblasts with low calcium (green) or high calcium (blue) based on GCaMP3.0 signal in larval brains of the genotype indicated at 72 hours ALH.
(M-N) Neuroblasts in larval brains of the genotypes indicated at 72 hours ALH stained for ubiquitin-conjugated species (antibody FK2), Dpn, and Phall. Yellow arrow marks neuroblast with no aggregates. White arrow marks neuroblast with aggregates. Scale bar, 10μm.
(O) Quantification of neuroblasts with (blue) or without (green) ubiquitin-conjugated aggregates in larval brains of indicated genotypes at 72 hours ALH.

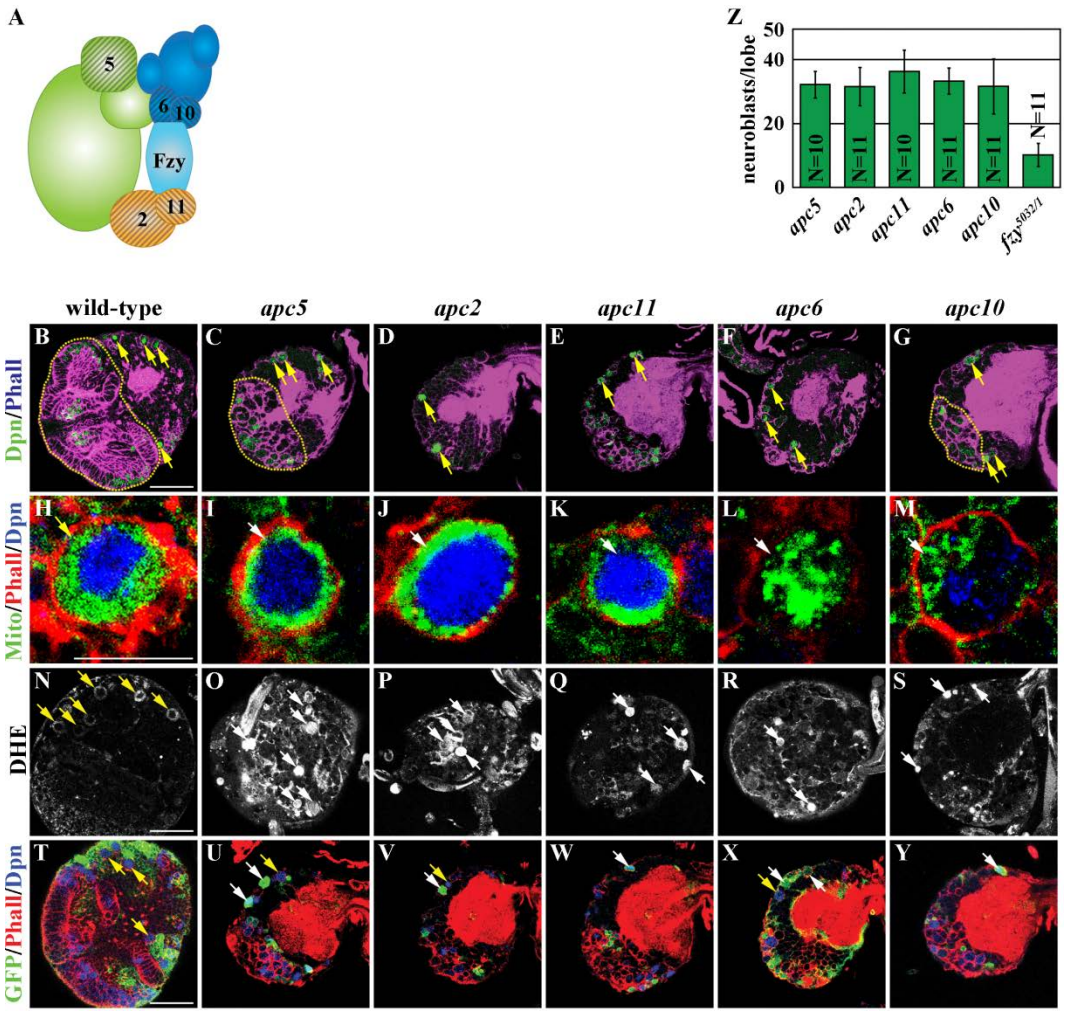

Figure 2.5. Loss of APC function leads to neuroblast necrosis.
(A) Summary diagram of the APC/C-Fzy complex). Scaffold subunits (green); catalytic subunits (orange); substrate recruitment subunits (blue). Crosshatches mark subunits whose mutants were analyzed.
(B-G) Larval brains of control and APC/C subunit mutants at 96h ALH stained for Dpn and Phall. Arrows mark Dpn^+ neuroblasts. Scale bar, 40μm.
(H-M) Neuroblasts in larval brains of control and APC/C mutants at 96h ALH stained for ATP5a, Dpn and Phall. Yellow arrow marks neuroblast with no mitochondrial aggregates. White arrows mark neuroblasts with aggregates. Scale bar, 10μm.
(N-S) Unfixed larval brains of APC/C mutants at 96h ALH stained for DHE. Yellow arrows mark neuroblasts with low or no detectable ROS. White arrows mark neuroblasts with high ROS. Scale bar, 40μm.
(T-Y) Larval brains of control and APC/C subunit mutants over-expressing *GCaMP3.0* at 96h ALH stained for GFP, Dpn and Phall. Yellow arrows mark Dpn^+ neuroblasts with low or no detectable calcium. White arrows mark Dpn^+ neuroblasts with high calcium. Scale bar, 40μm.
(Z) Average number of Dpn^+ neuroblasts per brain lobe in the *apc* mutants.

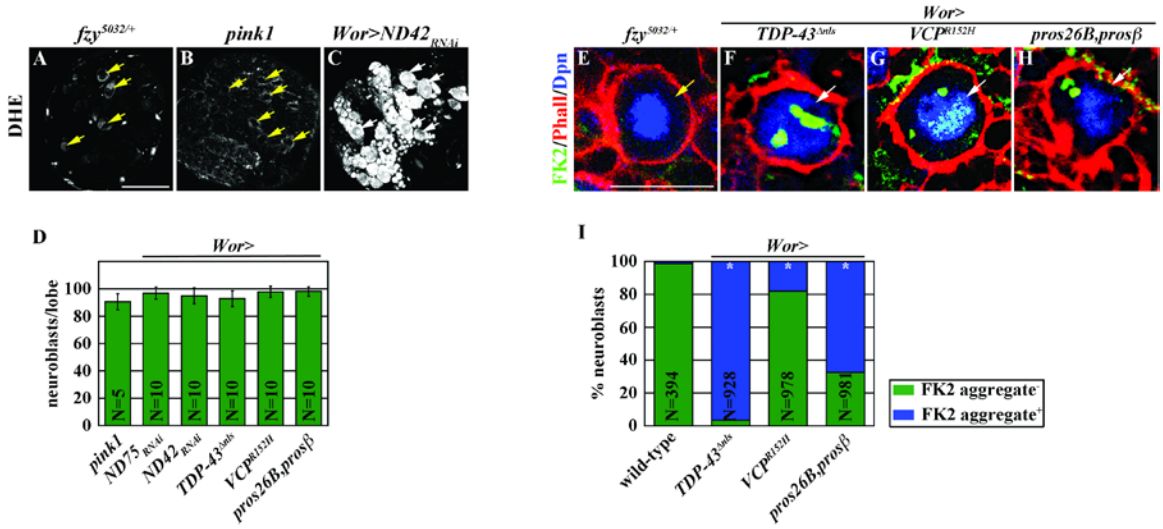

Figure 2.6. Necrosis markers are not sufficient to induce neuroblast necrosis.
(A-C) Unfixed larval brains of the genotypes indicated were stained with DHE. Wild type and *pink1* mutant animals were raised at 25°C and sacrificed at 96 hours ALH. *Wor>ND42$_{RNAi}$* animals were raised at 33°C and sacrificed at 72 hours ALH. All RNAi knockdown animals in this study co-expressed *dcr2*. Yellow arrows mark neuroblasts with low or no detectable ROS. White arrows mark neuroblasts with high ROS . Scale bar, 40μm.
(D) Quantification of neuroblasts in brains of indicated mutants. *pink1* mutant animals were raised at 25°C and sacrificed at 96 hours ALH. All others were raised at 33°C and sacrificed at 72 hours ALH.
(E-H) Neuroblasts in brains of larvae of indicated genotypes at 72h ALH stained for ubiquitin-conjugated aggregates, Dpn, and Phall. Yellow arrows mark neuroblasts with no aggregates. White arrows mark neuroblasts with aggregates. Scale bar, 10μm.
(I) Quantification of neuroblasts with (blue) or without (green) ubiquitin-conjugated aggregates in indicated genotypes.

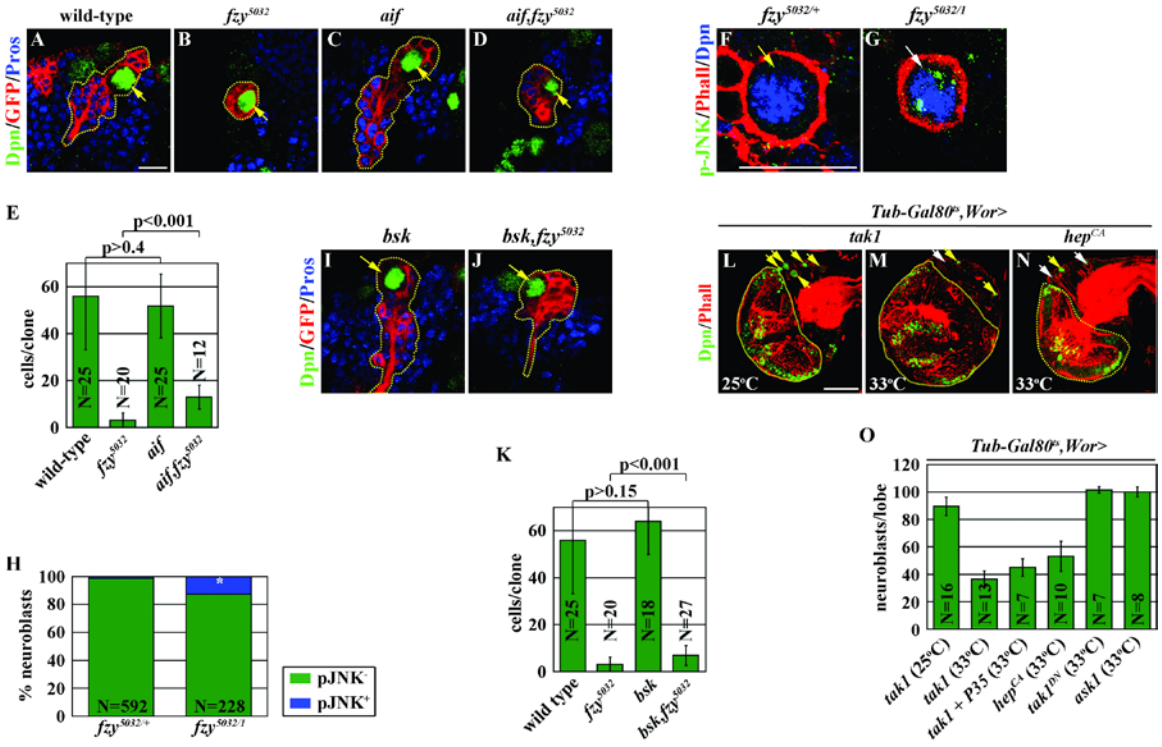

Figure 2.7. Fzy prevents neuroblast necrotic cell death through Aif and JNK dependent mechanisms.

(A-D) GFP-marked mosaic clones derived from neuroblasts of the genotypes indicated were stained for Dpn, GFP and Pros. Dotted lines mark clones. Scale bar, 10μm.

(E) Average number of cells per mosaic clone of the genotypes indicated. All clones with a single Dpn^+ neuroblast were counted.

(F-G) Neuroblasts in larval brains of indicated genotypes at 72h ALH stained for p-JNK, Dpn and Phall. Scale bar, 10μm.

(H) Quantification of $p\text{-}JNK^+$ (blue) versus $p\text{-}JNK^-$ (green) neuroblasts in brains of indicated genotypes.

(I-J) GFP-marked mosaic clones derived from neuroblasts of the genotypes indicated were stained for Dpn, GFP and Pros. Dotted lines mark clones. Scale bar, 10μm.

(K) Average number of cells per mosaic clone of the genotypes indicated. All clones with a single Dpn^+ neuroblast were counted.

(L-N) Larval brains over-expressing *tak1* or constitutively active *hep* weakly (25°C) or strongly (33°C) stained for Dpn and Phall. Animals were sacrificed at 72 hours ALH. White arrows mark Dpn^+ neuroblasts. Yellow arrows mark Dpn^- dying neuroblasts. Scale bar, 40μm.

(O) Quantification of neuroblasts in larval brains over-expressing the indicated transgenes. Animals were sacrificed at 72h ALH.

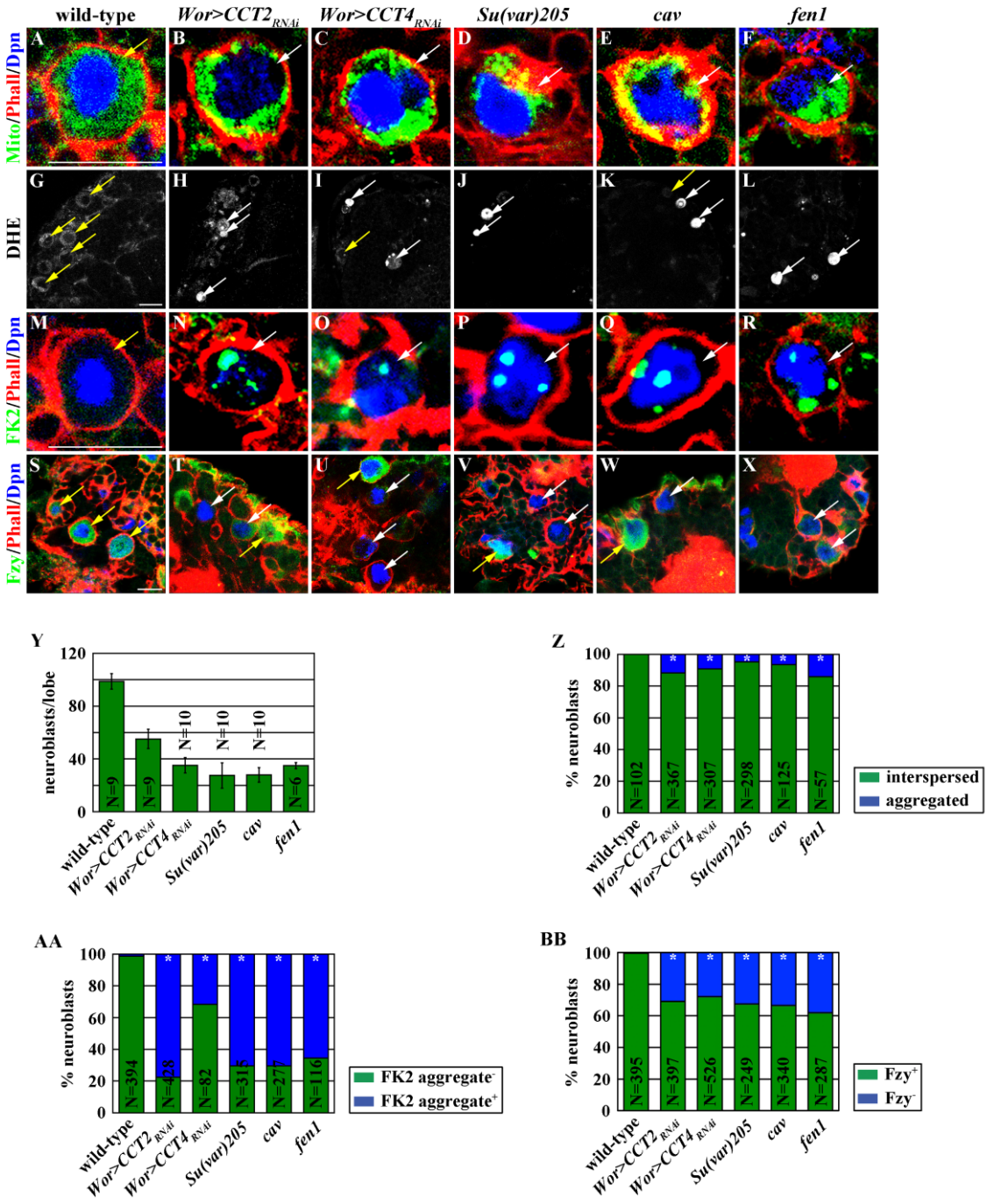

Figure 2.8. Catastrophic genotoxic stress causes loss of Fzy expression and neuroblast necrosis.

(A-F) Neuroblasts in larval brains of the indicated genotypes stained for ATP5a, Dpn and Phall. Yellow arrow marks neuroblast with no mitochondrial aggregates. White arrows mark neuroblasts with aggregates. Scale bar, 10μm.

(G-L) Unfixed larval brains of the indicated genotypes stained for DHE. Yellow arrows mark neuroblasts with low or no detectable ROS. White arrows mark neuroblasts with high ROS. Scale bar, 20μm.

(M-R) Neuroblasts in larval brains of the indicated genotypes stained for ubiquitin-conjugated aggregates, Dpn and Phall. Yellow arrows mark neuroblasts with no aggregates. White arrows mark neuroblasts with aggregates. Scale bar, 10μm.
(S-X) Neuroblasts in larval brains of the indicated genotypes stained for Fzy, Dpn and Phall. Yellow arrows mark Dpn^+ neuroblasts with Fzy. White arrows mark Dpn^+ neuroblasts with no detectable Fzy. Scale bar, 10μm.
(Y) Quantification of neuroblasts in larval brains of the indicated genotypes.
(Z) Quantification of neuroblasts with interspersed (green) or aggregated (blue) mitochondrial morphology in larval brains of the indicated genotypes.
(AA) Quantification of neuroblasts with (blue) or without (green) ubiquitin-conjugated aggregates in larval brains of indicated genotypes.
(BB) Quantification of neuroblasts with (green) or without (blue) Fzy expression in larval brains of indicated genotypes.

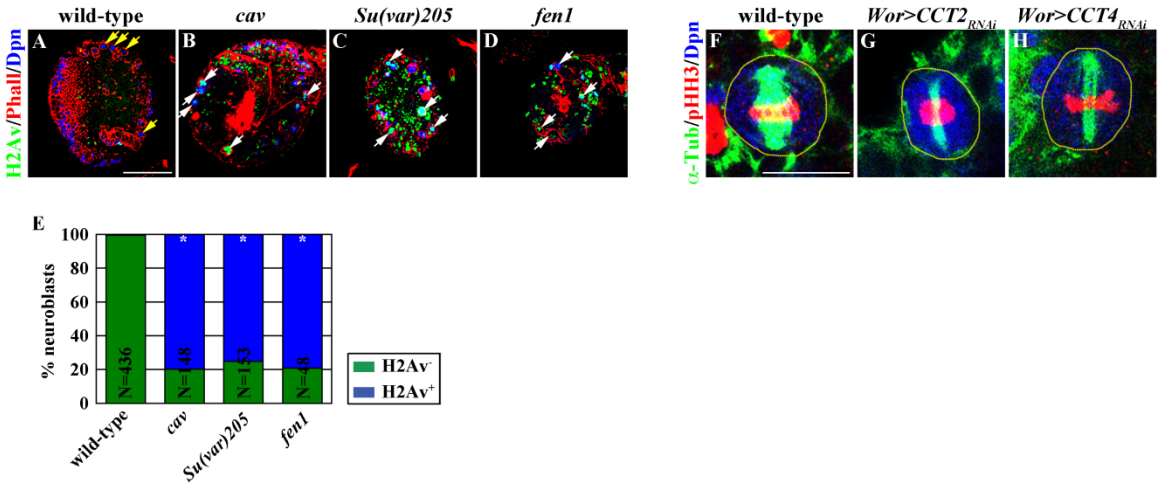

Figure 2.9. Loss of telomere capping or chaperonin function lead to DNA or cell cycle damage.

(A-D) Larval brains of indicated genotypes stained for DNA damage marker phosphorylated histone H2Av, Dpn and Phall. Yellow arrows mark Dpn^{+} neuroblasts with no detectable DNA damage. White arrows mark neuroblasts with DNA damage. Scale bar, 40μm.

(E) Quantification of neuroblasts with (blue) or without (green) phopho-H2Av marker in larval brains of the indicated genotypes.

(F-H) Representative images of metaphase neuroblasts in larval brains of the indicated genotypes at 72h ALH. Stained for alpha-Tubulin which marks the mitotic spindle, Dpn and Phall. Scale bar, 10μm.

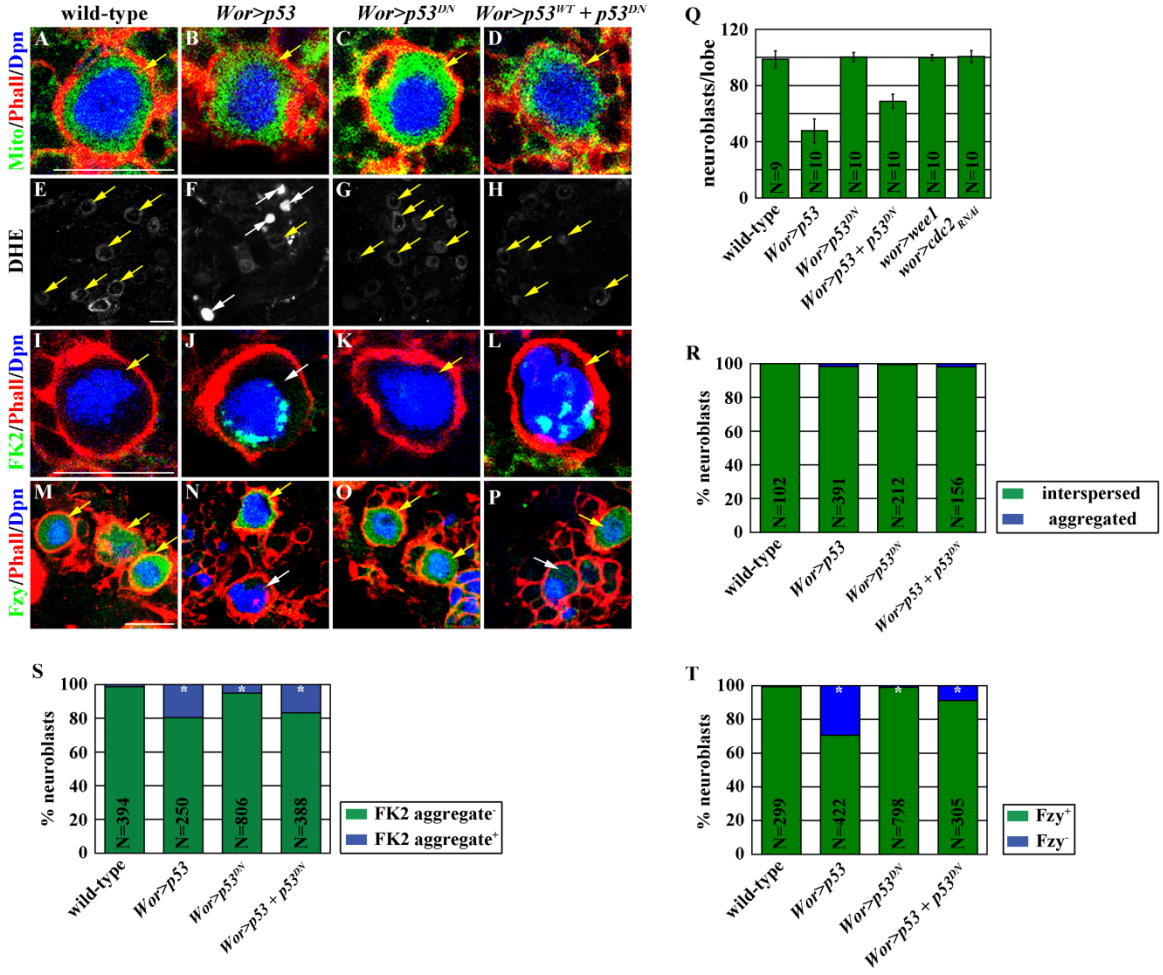

Figure 2.10. Up-regulation of p53 causes loss of Fzy expression and neuroblast necrosis.

(A-D) Neuroblasts in larval brains of the indicated genotypes stained for ATP5a, Dpn and Phall. Yellow arrows mark neuroblasts with no detectable mitochondrial aggregates. Scale bar, 10μm.

(E-H) Unfixed larval brains of the indicated genotypes stained for DHE. Yellow arrows mark neuroblasts with low or no detectable ROS. White arrows mark neuroblasts with high ROS. Scale bar, 20μm.

(I-L) Neuroblasts in larval brains of the indicated genotypes stained for ubiquitin-conjugated aggregates, Dpn and Phall. Yellow arrows mark neuroblasts with no aggregates. White arrows mark neuroblasts with aggregates. Scale bar, 10μm.

(M-P) Neuroblasts in larval brains of the indicated genotypes stained for Fzy, Dpn and Phall. White arrows mark neuroblasts with Fzy expression. Yellow arrows mark neuroblasts with low or no detectable Fzy. Scale bar, 10μm.

(Q) Quantification of neuroblasts in larval brains of the indicated genotypes.

(R) Quantification of neuroblasts with interspersed (green) or aggregated (blue) mitochondrial morphology in larval brains of the indicated genotypes.

(S) Quantification of neuroblasts with (blue) or without (green) ubiquitin-conjugated aggregates in larval brains of indicated genotypes.

(T) Quantification of neuroblasts with (green) or without (blue) Fzy expression in larval brains of indicated genotypes.

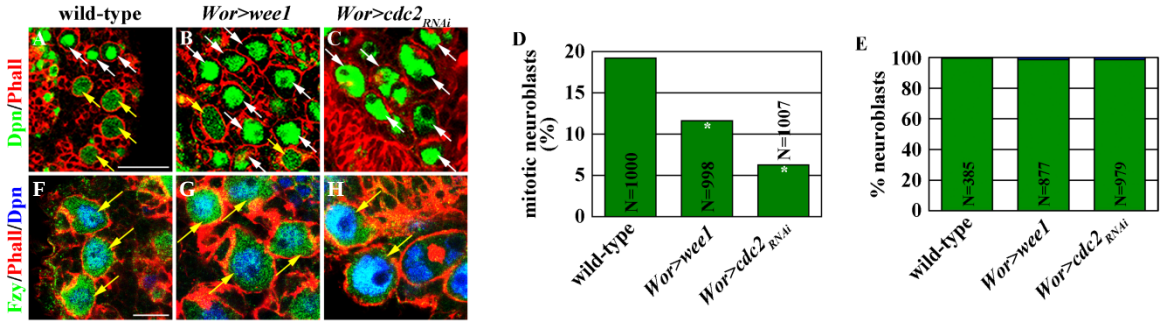

Figure 2.11. p53-mediated cell cycle arrest does not cause cell death.
(A-C) Larval brains of indicated genotypes stained for Dpn and Phall. Yellow arrows mark Dpn^{+} neuroblasts with cytoplasmic Dpn localization. White arrows mark neuroblasts that display nuclear Dpn localization. Scale bar, 20µm.
(D) Quantification of percentage of neuroblasts in indicated genotypes with cytoplasm localized Dpn.
(E) Quantification of percentage of neuroblasts in indicated genotypes with Fzy expression (green) versus without Fzy expression (blue).
(F-H) Nuroblasts in larval brains of indicated genotypes stained for Fzy, Dpn, and Phall. Yellow arrows mark neuroblasts with wild-type Fzy expression. Scale bar, 10µm.

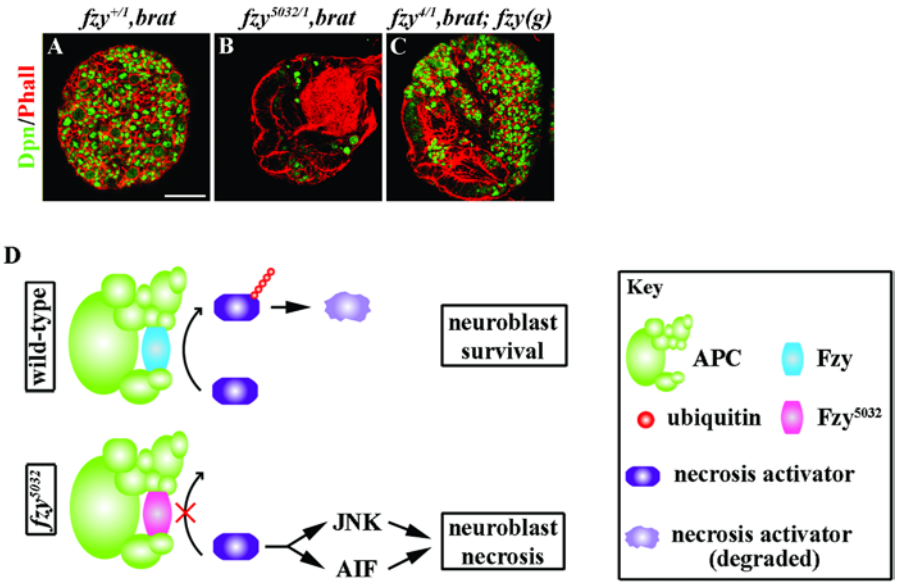

Figure 2.12. Fzy-mediated necrosis kills tumorigenic neuroblasts.
(A) Larval brain of *brat* mutant at 96h ALH demonstrating strong supernumerary neuroblast phenotype. Optic lobe is largely displaced by Dpn^{+} central brain neuroblasts. Scale bar, 40µm.
(B) Larval brain of fzy^{5032} mutant in brat mutant background at 96h ALH.
(C) Larval brain of *fzy* null mutant in brat mutant background, with one copy of wild type *fzy* genomic transgene at 96h ALH.
(D) Model of how Fzy maintains neuroblast viability. Wild-type Fzy (blue) is able to activate APC (green) and recruit endogenous substrates for ubiquitination (red), including the presumed immediate downstream factor(s) required for activating necrotic cell death (purple). Fzy^{5032} (pink) is not able to recruit the necrotic cell death activator(s), leading to perdurance of the factor and eventually necrotic cell death of the neuroblast.

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CHAPTER 3

FUTURE DIRECTIONS AND PERSPECTIVES

Downstream Mechanisms

The identification of *fzy* as a neuroblast maintenance gene has provided a starting point for uncovering the nature of cell death occurring in fzy^{5032} mutant neuroblasts. We know that the terminal morphology closely resembles necrotic cell death. We also identified several markers which are consistent with necrosis, namely increased intracellular calcium, increased reactive oxygen species (ROS), and altered mitochondrial morphology (Fig 3.1). These markers which serve as signs indicating that necrosis is occurring may also give clues as to what the terminal mechanisms of killing are.

The appearance of superoxide species as detected by DHE is has been a consistent indicator of neuroblast necrosis. In mammalian models of death receptor mediated necrosis, ROS are often produced and believed to be important for killing. In many cell death studies, ROS have been implicated as possible agents of cell death simply by oxidizing nonspecific cellular targets and inducing massive damage. ROS are also believed to trigger downstream signaling pathways or other mechanisms in certain contexts[1]. ROS have been implicated in nonapoptotic cell death after ischemia-reperfusion injury. Experiments in primary chick cardiomyocyte cultures suggested that ROS production after ischemia promoted the opening of the mitochondrial permeability transition pore (mPTP), leading to loss of mitochondrial integrity and necrotic cell death[2]. Thus, there are multiple options for what ROS may be doing, if anything, in neuroblast necrosis. However, current data suggests that ROS production alone is not sufficient to induce any sort of neuroblast loss. This would argue against the model that suggests ROS triggers a mitochondrial alteration or signaling pathway to kill neuroblasts. There is

still the possibility that ROS are contributing to the damage in the terminal stages of necrosis. It remains to be seen if reduction in ROS production by over-expressing transgenes encoding ROS scavengers will attenuate the loss of neuroblasts in fzy mutant brains.

The production of ROS is closely tied to mitochondrial health, since the primary source of ROS is leakage from the electron transport chain. Thus it is not surprising that dying neuroblasts showed increased ROS as well as altered mitochondrial morphology. In fact, in cardiac ischemia-reperfusion injury studies, the opening of the mPTP caused by overload of calcium and ROS is thought to trigger necrosis[3,4]. This would conveniently account for several of the markers seen in *fzy* mutant neuroblasts.

One possibility is that loss of mitochondrial function is leading to energy depletion and death. However, loss of Pten-induced kinase 1 (Pink1), which has been shown to be required for assembling the oxidative phosphorylation machinery in flies, did not result in any loss of neuroblasts[5,6]. This suggests that simple metabolic deficiency is not the cause of neuroblast necrosis. In various models of regulated necrosis, it is thought that release of mitochondrial factors contributes to cell death, much like release of cytochrome c in mammalian apoptosis[7,8]. The questions, then, are what factors may be facilitating this release and what are the released products that then execute cell death? It remains a possibility that calcium influx and ROS production function together induce mPTP opening in necrotic neuroblasts. The peptidylprolyl-isomerase cyclophilin D (CypD) has been proposed to be a critical component of the mPTP. Studies in mice and cell culture have shown that loss of CypD can attenuate apoptosis and necrosis, particularly in oxidative stress situations[9–11]. There a few orthologs of the CypD in the *Drosophila* genome, all of which are poorly characterized. It would be interesting to see if loss of CypD could modify the necrosis phenotype in *fzy* mutant animals.

Acitvation of poly(ADP-ribose) polymerase 1 (PARP1) has been shown in mammals to lead to various forms of nonapoptotic cell death[12–14]. PARP1 is normally a nuclear DNA repair protein which has the capability of responding to genotoxic stress. Various models of PARP1 activation suggest that PARP1 translocates to the mitochondria to trigger release of downstream factors that lead to necrosis. One of the key mitochondrial proteins released by PARP1 activation is apoptosis-inducing factor

(AIF), which has been shown to trigger the terminal events of cell death[12,15–17]. Loss of AIF partially rescued loss of neuroblasts in *fzy* mutant clones, suggesting that AIF may be functioning downstream of *fzy* to mediate necrosis. It remains to be seen if PARP is acting as an intermediary in this mechanism. AIF itself has been shown to coordinate large scale DNA fragmentation both in apoptosis and necrosis[18–20]. Future experiments should help delineate the players involved in DNA degradation during neuroblast necrosis.

A fascinating study recently demonstrated that p53 interacts with CypD to facilitate mPTP formation and cerebral necrosis in a mouse stroke model. While loss of p53 did not appear to rescue the loss of neuroblast phenotype in *fzy* mutant brains, it is possible that the strong mutant background is not sensitive enough to detect modification by loss of p53. It may be worthwhile to test if loss of p53 can rescue loss of neuroblasts in *fzy* mutant clones. Another intriguing possibility is that mitochondrial fragmentation or fission is required for necrosis to occur. This has been shown in recent work investigating the mitochondrial phosphatase PGAM5 in cell lines[21,22]. In mammalian cells, PGAM5 organizes and facilitates the RIP1/RIP3 complex to phosphorylate and activate the mitochondrial fission protein Drp1. Loss of Drp1 was able to attenuate programmed necrosis. Demonstrating a role for PGAM5, Drp1, or mitochondrial fission/fusion in the neuroblast necrosis model would be significant.

A topic that has not been touched upon in our studies thus far is the role of lysosomes and lysosomal proteins in cell death. Lysosomal proteases and rupture of lysosomes have been shown to play critical roles in different forms of nonapoptotic cell death[23]. Lockshin and Williams' studies on silk moth muscle degradation found that an increase in cathepsin, a lysosomal protease, coincided with cell death[24]. Proper acidification of lysosomes has been shown to be important for cell death in yeast and *C. elegans* models[25–27]. Fly germ cell models have consistently reported that lysosome membrane permeabilization and release of lysosomal DNAse and proteases play integral roles in nonapoptotic cell death execution[28,29]. Rupture of the lysosome can typically be detected with a probe that responds to cytosolic pH change. The possible roles of lysosomal proton pumps, DNAses, and proteases should be tested in the appropriate *fzy* mutant background.

Removal of Necrotic Fragments

The final fate of all cells programmed to die is typically engulfment by either professional phagocytes or neighboring non-professional phagocytes. The mechanisms by which apoptotic bodies are taken up after death are relatively well characterized while phagocytosis of necrotic cell fragments is more poorly understood[30–32]. Many of the advances in phagocytosis of necrotic cells have been made studying macrophage clearance of necrotic immune cells. Some mechanisms for engulfment of cell corpses in *Drosophila* have come into focus recently as well. The cell surface receptor Draper has been found to promote engulfment in multiple cell death contexts, including apoptosis, autophagy, and death following neuronal injury[33–37]. In the adult *Drosophila* brain, a special subset of glia engulfs cell fragments after axonal injury[38]. We hypothesize that cortical glia, which are in contact with and encase single neuroblasts and their lineage during larval development, may be responsible for engulfment of necrotic cell fragments in *fzy* mutant brains.

There is currently little evidence for engulfment of necrotic neuroblasts. A simple experiment could be done to determine if the neighboring glia truly are involved in phagocytosis. Neuroblasts and glia may be marked in a cell specific manner with intracellular β-galactosidase and a membrane bound CD8-GFP, respectively. These manipulations could be performed in a *fzy* mutant background and examination for β-galactosidase localization within GFP^+ cells would allow us to determine if necrotic cell fragments are being engulfed by glia. Involvement of Draper or other candidate engulfment receptors could be analyzed genetically.

While beyond the scope of this discussion, glial engulfment could potentially trigger extracellular signals to other tissues in the organism. In mammals, this process can result in repair of damaged tissues nearby, but can also cause local or systemic inflammation. Thus, the possible downstream functions of glia in managing neuroblast necrosis would be of great interest in future studies.

Fzy Substrate Specificity

Using a homology model of Fzy based on the fission yeast Cdc20 crystal structure, we found that both *fzy*^{5032} and *fzy*$^{AC-10}$ cause amino acid substitutions of residues far from the surfaces required for canonical Fzy function[39]. This distance suggested that these mutations should not perturb the ability of Fzy to bind to its canonical mitotic substrates and perform its well-known function in promoting cell cycle progression. Indeed, further studies showed that the Fzy^{5032} mutant protein is capable of promoting cell cycle progression but is not able to maintain neuroblasts. Altogether, these led us to hypothesize that the *fzy*^{5032} and *fzy*$^{AC-10}$ point mutations are located on uncharacterized surfaces of the WD40 protein binding domain of Fzy. These surfaces are important for maintaining neuroblast viability. Disruption of protein-protein interactions on these surfaces may lead to an inability of Fzy to recruit a substrate(s) for ubiquitin-mediated degradation. In turn, the increased level of this substrate leads to premature necrosis of neuroblasts.

Targeted mutagenesis will be used to test this hypothesis (Appendix 1). Transgenic flies encoding *fzy* with point mutations located in the WD40 repeats will be generated. These mutations will fall into one of two classes: mutations that are expected to disrupt canonical *fzy* function as a cell cycle regulator and mutations that are expected to disrupt *fzy* function in preventing necrosis. Of the first class, two mutations will disrupt the KEN-box and one will disrupt the D-box binding surfaces. These surfaces are reported to be required for *fzy* to bind to its canonical mitosis substrates[39]. Five point mutations will disrupt the surface of the WD40 repeats near the mutated residue in *fzy*^{5032}, while three point mutations will disrupt the WD40 repeats near the mutated residue in *fzy*^{AC10} (Fig 3.2). The ability of these transgenes to rescue the *fzy* mutant phenotype will be examined.

The likely outcome for the KEN-box and D-box mutant transgenes would be their inability to rescue cycle arrest in *fzy* null mutant cells, such as in wing imaginal disc clones or intestinal stem cell clones. They may, however, still be able to rescue the neuroblast necrosis phenotype, either in a *fzy*^{5032} mutant background or in *fzy*^{5032} mutant neuroblast clones. This would suggest that the D-box and KEN-box binding surfaces are

functionally distinct from the neuroblast maintenance surfaces. Another likely possibility is that the KEN-box and D-box mutant transgenes will only partially rescue the neuroblast necrosis phenotype, which would suggest some shared interaction between the canonical surfaces and the new surfaces.

The ability of the new transgenes, which have mutated residues near the Fzy^{5032} and Fzy^{AC-10} lesions, to modify the cell cycle or necrosis phenotypes will be much less predicatable. We initially thought that fzy^{5032} was nearly wild type in promoting cell cycle progression. However, recent experiments in wing imaginal disc clones suggested that fzy^{5032} may be slightly deficient in cell cycle promotion. Over-expression of fzy^{5032} was able to rescue the cell cycle arrest phenotype of *fzy* null wing imaginal disc clones, but the clones were still smaller than those rescued by wild type *fzy* over-expression (data not presented). Thus, it is likely that both fzy^{5032} and fzy^{AC-10} cause minor defects in canonical *fzy* function. However, their ability to maintain neuroblasts is very severely compromised, while their ability to promote the cell cycle remains largely intact. The new transgenes might therefore not be able to rescue the necrosis phenotype in fzy^{5032} neuroblasts, and they might also have cell cycle defects in *fzy* null neuroblasts. The extent to which the transgenes rescue each phenotype would help us begin to map out the interaction surface of Fzy WD40 repeats in greater detail. Finally, if the direct targets of Fzy are discovered in the future, the nature of their interactions with the WD40 repeats may be tested using targeted mutagenesis.

The Search for the Necrosis Activator

The current model of how loss of Fzy activates necrosis suggests that there must be a downstream target of Fzy, or perhaps multiple targets, which is normally phosphorylated by APC/C-Fzy and degraded. Thus, this necrosis activator does not function to kill in the presence of intact Fzy but can build up to lethal levels in *fzy* mutant backgrounds. No likely candidates for such a factor have been found in the literature yet, prompting us to employ unbiased approaches.

The most direct approach would be to attempt to immunoprecipitate Fzy protein with these potential factors bound, then dissociate and perform mass spectrometry to identify them. The only tissue that would suffice for this experiment is larval brain. The *fzy* mutant necrosis phenotype appears to be incredibly context dependent, since only the neuroblasts have shown to exhibit the phenotype thus far. This likely depends on multiple factors, such as appropriate expression APC/C-Fzy, the potential targets, and proper modification and regulation of both. However, using the larval brain as the source of material for a co-immunoprecipitation presents its own challenges. There are only 100 central brain neuroblasts in each brain lobe, which makes it tedious to collect a large enough sample for analysis, and potentially introduces non-specific binding due to the volume of brain taken up by other cell types. Therefore, we propose to use a mutant background that will amplify the number of neuroblasts at the cost of other cell types.

Mutation of the gene *brain tumor* (*brat*) was demonstrated to cause a dedifferentiation phenotype in a subset of neuroblasts[40,41]. Loss of brat in an otherwise wild type background or with heterozygous loss of *fzy* led to a severe supernumerary neuroblast phenotype. Importantly, *fzy* is required to maintain even the supernumerary neuroblasts, since the fzy^{5032} allele in a *brat* mutant background caused massive loss of neuroblasts. In a *fzy* null, *brat* null double mutant brain, the presence of a single copy of a wild type *fzy* genomic fragment was able to completely restore the *brat* mediated supernumerary neuroblast phenotype. These results suggest that it may be possible to express a tagged Fzy protein to rescue the fzy null phenotype in a brat mutant background. This combination would presumably provide an enrichment of neuroblasts and give a tagged Fzy species with a stoichiometric advantage for co-

immunoprecipitation experiments. Future work will focus on obtaining a tagged Fzy transgene that is capable of rescuing the *fzy* mutant phenotype.

A genetic method for finding downstream factors is by using a dominant modifier screen. The key to this method is to establish a sensitized genetic background that will allow for modification by haploinsufficient chromosomal deletions. A library of fly chromosomal deletions, or deficiencies, can then be crossed into the background and the phenotype can be assessed. Our lab has used this method in a *brat* mutant background to identify genes that interact with brat to regulate neuroblast self-renewal[42].

We obtained a *fzy* mutant allele caused by an insertion of a transposable P-element, called fzy^{EP1028} or simply fzy^{EP} [43]. This allele was homozygous viable, unlike most other loss of function *fzy* alleles, suggesting that if it had a phenotype, it would be weak. Indeed, $fzy^{5032/EP}$ transheterozygotes showed an intermediate loss of neuroblast phenotype compared to $fzy^{5032/-}$ animals (Fig 3.3A,D). This phenotype was highly consistent across independent replicates. Screening a library of *Drosophila* genome deficiencies covering a large portion of the 3rd chromosome has yielded several candidate loci which either suppress or enhance the sensitized phenotype (Fig 3.3A-C,E, Appendix 2). Importantly, most of these modifier loci overlapped with another modifier locus, suggesting that a gene in the overlap region was interacting with the *fzy* mutant phenotype and making it unlikely to due to a background mutation in the deficiency chromosome. Some modifier loci or overlapping regions were small enough that single genes may be tested for their interaction with *fzy* (Fig 3.3F).

While the results of the screen thus far are encouraging, more work need to be done to analyze the genes within the identified modifier loci. Further analysis will include experiments to examine the epistasis of the candidate genes with *fzy* and potentially each other. This method does not guarantee that the genes found will function upstream or downstream, or that they will directly interact with *fzy*, only that they interact genentically. In contrast, a co-immunoprecipitation is more likely to reveal the necrosis activator that may be functioning directly downstream of APC/C-Fzy.

Balancing Neuroblast Death versus Survival

The neuroblast has proven to be a unique system for investigation of cell death in response to injury. Two interesting features are the ability of the neuroblasts to withstand cellular damage without arresting cell cycle or dying and the suppression of apoptosis. Both of these features may be related to the unusual circumstances of the neuroblast's function. Most of the larval neuroblasts are required to generate a vast number of cells without the benefit of transit amplification. Each asymmetric division by a Type I neuroblast produces a differentiating cell that can only produce two final post-mitotic progeny. Therefore, neuroblasts may prefer to divide as quickly as possible while ignoring any potential disruptions as long as possible.

A rough estimate based on the size of a typical mosaic clone of a larval neuroblast would put its cell cycle length at approximately 1.5 hours. Wing imaginal disc cells, by comparison, have been reported to divide once every 8 hours[44]. Similarly, the size of typical adult midgut stem cell clones would put their cell cycle time in the range of 12-16 hours. Neuroblasts are forced to divide so quickly because they divide asymmetrically, mostly without producing transit amplifying cells, and so can only generate one progeny per division. Additionally, the short time window of larval development is when the neuroblasts must generalte almost all of the adult neural cells. Therefore, a proper cell cycle checkpoint may not be as useful in a neuroblast as it would be in rare adult tissue specific stem cells. Any neuroblast with its cell cycle blocked would be considered a useless neuroblast and fit for elimination, which is what happens in a developmentally timely fashion anyways.

The need to ignore most types of cell damage might explain the ability of neuroblasts to tolerate high production of ROS, mitochondrial fusion defects, and excess ubiqitin-conjugated aggregates without dying. It could be that none of these defects carry enough long term ramifications to the future progeny to warrant death of the neuroblasts. On the contrary, catastrophic loss of genomic integrity or defects in cell cycle progression directly threatens the production or survival of future progeny. This may be why defects

in telomere capping and spindle assembly cause neuroblast death. The preference to maintain neuroblasts' unusually high proliferative rate may also explain the suppression of apoptosis by Polycomb group proteins[45]. Apoptosis seems to be a common target for diverse pathways to integrate on in order to activate programmed cell death[46]. This may actually work to the detriment of cells that are particularly valuable. In the limited developmental time span during which neuroblasts need to continuously divide and avoid death, suppression of apoptosis in general forces to cell to stay alive and only die when a truly catastrophic event occurs.

A Stem Cell Health Checkpoint

Neuroblast necrosis seems to be a response to either catastrophic DNA damage or cell cycle disruption. This correlates with known functions of p53 as a master regulator of genomic integrity. Without apoptosis available to kill these cells, necrosis is activated instead. While p53 is commonly associated with either cell cycle arrest and DNA repair or apoptosis, it has also been found to activate cell death in a non-caspase dependent manner[47]. The connection between p53 and Fzy is unprecendented, since Fzy has never been implicated in maintenance of cell viability. However, Fzy may be well positioned to regulate necrosis in response to p53-mediated responses. Fzy is already a key component of the cell cycle machinery and thus its expression or function may serve as a good monitor for how healthy the neuroblast cell cycle is. Thus, p53 may simply be one of several pathways that converge on Fzy to trigger necrosis of the neuroblast.

The telomere capping genes appeared to promote neuroblast maintenance and were highly expressed in neuroblasts compared to neural progeny[48,49]. Loss of function mutations in both of these genes led to a loss of neuroblasts and similar marker expression as *fzy* mutant neuroblasts. Aside from telomere fusions and a DNA damage response, uncapped telomeres in *Drosophila* could also trigger the spindle assembly checkpoint (SAC)[50,51]. The SAC is normally activated during the metaphase to anaphase transition to prevent entry into anaphase until all kinetochores are anchored to microtubules. One of the mechanisms by which this is accomplished is formation of the Mad protein complex at unanchored kinetochores and sequestration of Fzy/Cdc20 from binding to APC/C. Without APC/C-Fzy activity, securin cannot be degraded and the kinetochores of the sister chromatids cannot separate[52]. Loss of *cav* was found to trigger inappropriate SAC with Mad complex formation at the kinetochores as well as accumulation of the Mad protein BubR1 at the exposed telomeres[50]. Interestingly, BubR1 is one of the Mad complex subunits which binds to Fzy in the SAC. Thus, loss of telomere capping may be causing BubR1 and Fzy to accumulate at the telomere, effectively eliminating Fzy activity. In this case, a partial loss of function of *cav* or *Su(var)205* might presumably be rescued by overexpressing Fzy and bypassing the inappropriate SAC. Furthermore, an independent mutation that would also be expected

to cause SAC activation should show a similar phenotype as neuroblast necrosis. However, over-expression of stabilized mitotic cyclins caused a metaphase arrest but was not able to mimic the neuroblast necrosis phenotype. Therefore, it seems unlikely that SAC activation alone would trigger neuroblast necrosis. Thus, the more likely cause for neuroblast death in telomere capping mutants is the DNA damage response.

It is still unclear why necrosis in the *fzy* mutants occurs specifically in the neuroblast. Curiously, despite most of the adult structures looking grossly normal in fzy^{5032} mutants, the bristle patterning appeared different. fzy^{5032} mutant adults had far fewer bristles than control animals. A *Drosophila* bristle originates from an epithelial cell which acquires a neural fate, and then undergoes a series of programmed asymmetric divisions to generate the four cells that make up each bristle organ[53]. There are several possibilities for why these bristles may not have formed, including improper cell type specification or failure to execute the asymmetric divisions properly. One way to interpret this is that somehow, only asymmetrically dividing progenitors of neural identity are affected by the fzy^{5032} mutation. However, the actual thread linking these two cell types and segregating them from the other dividing cells might be the speed of cell division.

We hypothesize that neuroblasts and perhaps other fast dividing cells may employ an altered cell cycle checkpoint which functions more like a cell health checkpoint. Cells that have encountered too much damage, such as in telomere capping mutants or CCT mutants, would trigger this checkpoint mechanism. An inability to progress through cell cycle in a timely manner will result in premature cell death, thereby eliminating the need to expend resources on that cell any further. Thus, this checkpoint is designed to kill rather than repair. Loss of *fzy* function would be acting as a downstream event in this checkpoint. Thus, the *fzy* mutant neuroblasts are undergoing necrosis without any upstream trigger. The telomere capping mutants and CCT mutants are likely activating intermediate mechanisms which eventually feed into loss of Fzy activity, activation of the checkpoint and death.

Final Remarks

Cells that die due to defects in mitosis are typically believed to undergo mitotic catastrophe[54]. This process has largely been studied and applied in cancer biology. It is generally thought of as cell death occurring during or after aberrant mitosis, which can trigger cell death. The Chk1 and Chk2 checkpoint regulators play particularly important roles in mitotic catastrophe. Some cancers can evade these mechanisms, either by evading the cell cycle arrest and continuing to divide with DNA damage, or by blocking cell death and gaining the opportunity to divide again. Initial morphological characterizations suggested that cells which suffered mitotic catastrophes may be triggered to die by apoptosis. However, blockade of apoptotic machinery has been found to lead to increased loss of cells in some situations, suggesting that this mitotic catastrophe might lead to a specialized cell death pathway[55,56]. The checkpoint that neuroblasts encounter may be a similar mechanism as mammalian mitotic catastrophe, in which irreparable damage to the cells leads to death. One difference is that many example of mitotic catastrophe describe cells that have accumulated genomic instability after undergoing or attempting flawed mitoses, leading to aneuploidy and multinucleated cells. This morphology was never observed in *fzy* mutant neuroblasts, suggesting that while the output may be the same, the mechanisms could be quite distinct. Further studies should determine if Chk1 and Chk2 checkpoint proteins as well as DNA damage response proteins such as ATM and ATR are activated prior to neuroblast death.

Neuroblasts' ability to withstand stress is another issue that requires elucidation. Sufficient genotoxic stress seems to trigger neuroblast necrosis. What other forms of stress can lead to this phenotype? In the literature, many different types of cellular injury have been associated with regulated cell death. Some examples include ischemia-reperfusion, protein stress or unfolded protein response, infection, and endoplasmic reticulum stress. Many of these can be induced by genetic or pharmacological means. It would be particularly interesting if protein aggregation in fly models of neurodegenerative disorders, such as Huntington's, Amyotrophic lateral sclerosis, and Parkinson's, led to the same necrotic cell death as in *fzy* mutant neuroblasts.

There are several features of neuroblasts that are somewhat reminiscent of cancer cells. Neuroblasts divide relatively quickly, can avoid apoptosis even under extreme stress, and appear to have altered checkpoint mechanisms. Neuroblasts seem to be resistant to quite a few stresses, such as increased ROS and mitochondrial dysfunction. Some of this is developmentally programmed, such as the inhibition of apoptosis by Polycomb group genes[45]. However, it could be argued that cancer cells acquire their unique properties by micro-evolution into a specialized developmental program. These characteristics allow neuroblasts to maximize their potential in their role, which is to generate a very large and complex organ in a short amount of time. Yet, neuroblasts are still susceptible to cell death of some sort provided enough damage has occurred. Even in the *brat* tumorigenic background, Fzy function is still required to maintain neuroblast viability. Thus, neuroblasts might provide a model system for examining pathways and strategies for killing cancers which have developed resistance to apoptosis or have bypassed cell cycle checkpoints.

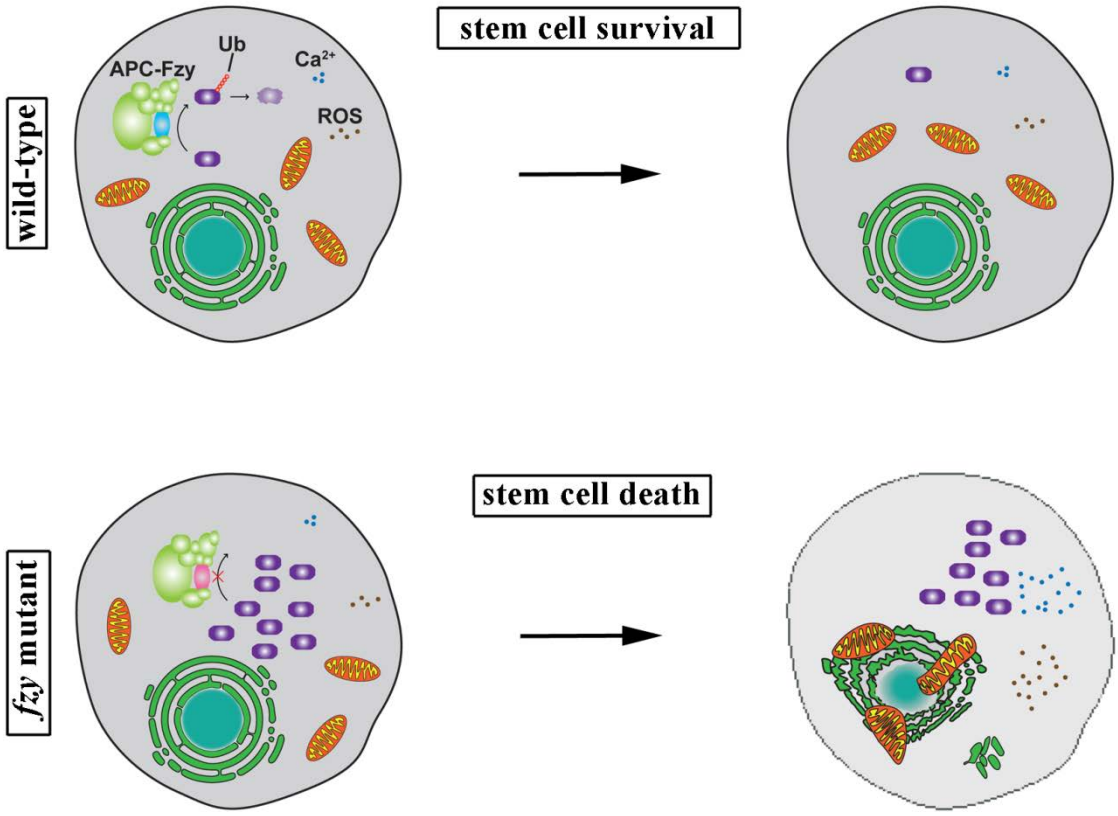

Figure 3.1. Graphical representation of neuroblast necrosis.

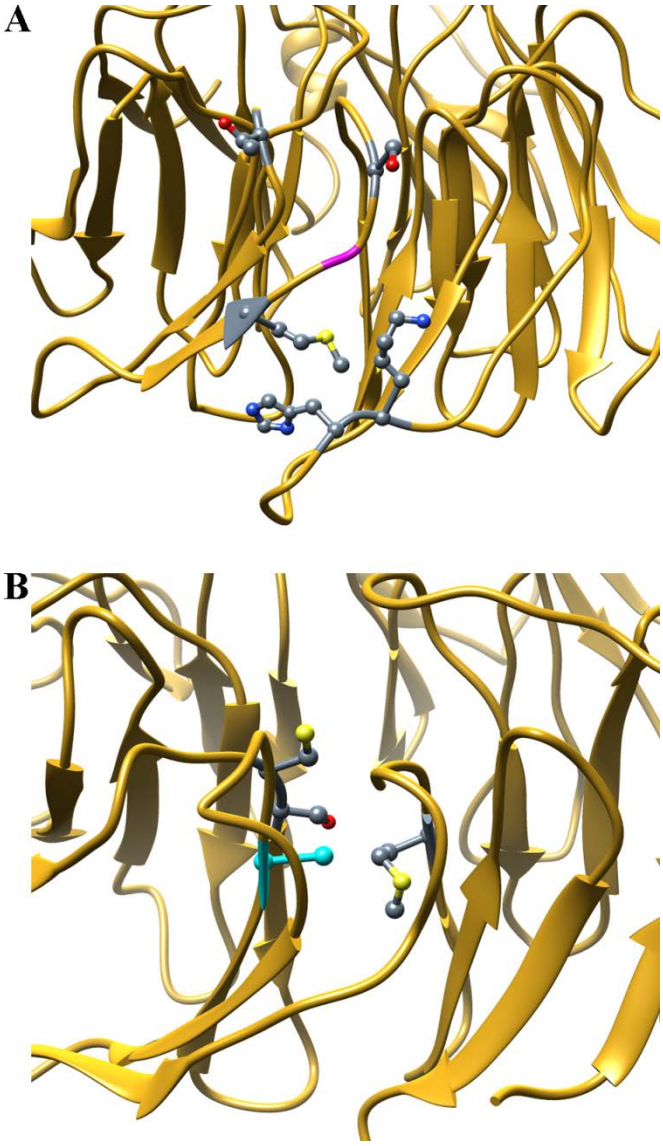

Figure 3.2. Proposed mutations to disrupt Fzy maintenance of neuroblasts.
Model of Fzy WD40 repeats is based on sequence similarity to fission yeast Slp1/Cdc20[39]. Regions containing **(A)** *fzy*^{5032} (pink) substitution and **(B)** *fzy*$^{AC\text{-}10}$ (cyan) substitution are shown. Nearby residues with potential to form protein-protein interactions shown in grey. Proposed substation mutations will change polar or hydrophilic side chains to nonpolar or hydrophobic side chains in an attempt to disrupt potential interactions.

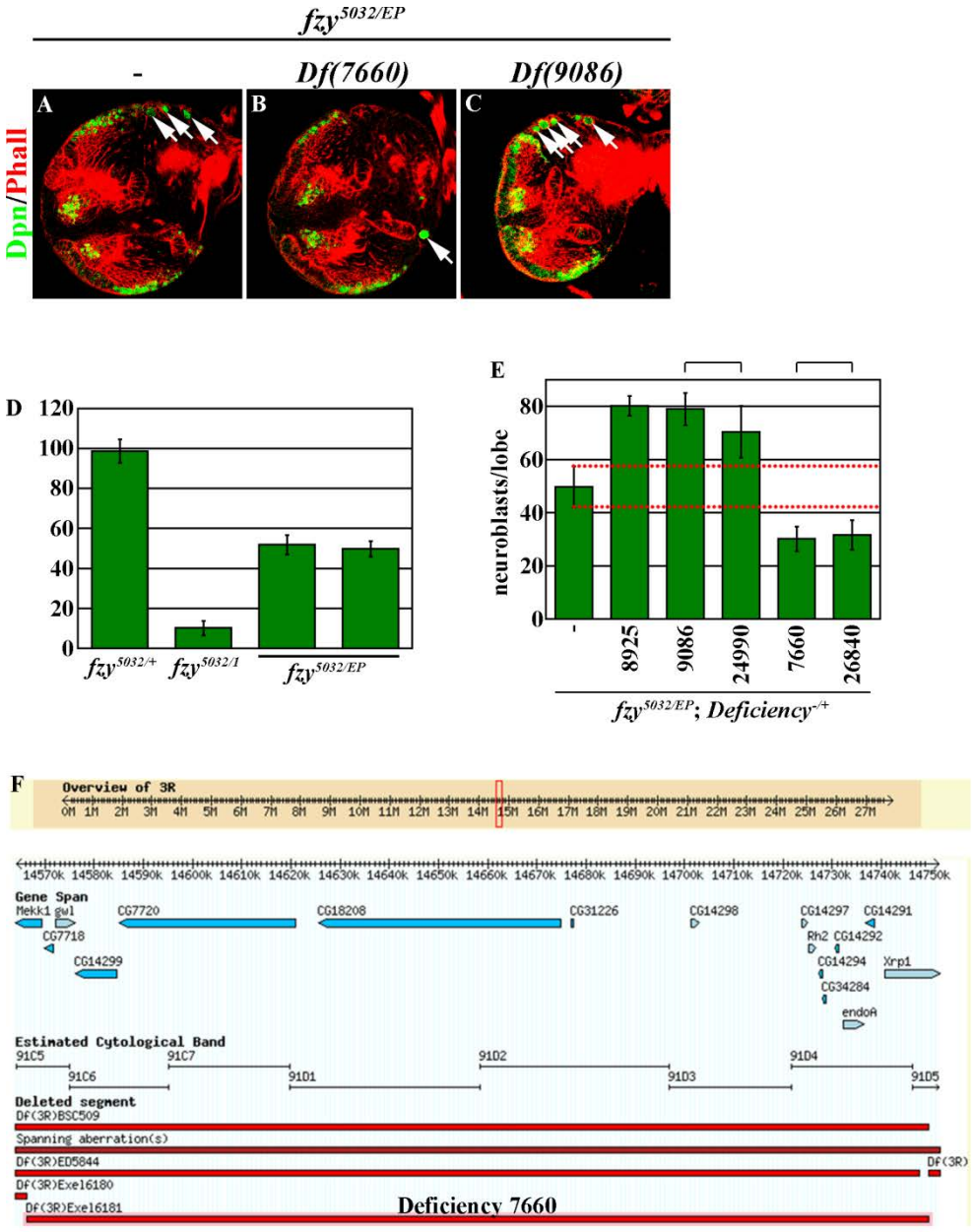

Figure 3.3. *fzy* genetic dominant modifier screen.

(A-C) Larval brains of *fzy* sensitized genetic background **(A)** without a modifier, and with heterozygous chromosomal deficiencies that dominantly **(B)** enhance or **(C)** suppress the sensitized background. Arrows mark Dpn^{+} neuroblasts.

(D) Quantification and comparison of *fzy* mutant backgrounds. Two independent replicates of *fzy* sensitized ($fzy^{EP/5032}$) background are shown (mean ± S.D.).

(E) Quantification of fzy sensitized background control and several chromosomal deficiencies that consistently suppressed or enhanced the background. Brackets indicated deficiencies which overlap. Red dotted lines encompass 95% confidence interval of control quantification (mean ± S.D.)

(F) Map of genomic locus deleted in modifier deficiency 7660, an enhancer [57].

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APPENDIX 1
FZY POINT MUTATIONS CLONING PRIMERS

D-box mutations:

Val226Met
For: GACAATATAGTGGCTATGGCCTTGGGCAGTT
Rev: AACTGCCCAAGGCCATAGCCACTATATTGTC

Ala384Phe
For: ACGACCATCAAGCTTTTGTGCGTGCCTTGGC
Rev: GCCAAGGCACGCACAAAAGCTTGATGGTCGT

KEN-box mutation:

Asn354Ala / Asn356Ala / Gln428Ala / Arg472Leu
(used 3 pairs of stitching primers)
For1: GCTAGCGGAGGCGCCGACGCTCTGGTGAATGTT
Rev1: AACATTCACCAGAGCGTCGGCGCCTCCGCTAGC
For2: TGGACTCCAAGTCGGCGGTCTGTTCTCTGC
Rev2: GCAGAGAACAGACCGCCGACTTGGAGTCCA
For3: CTGGACACACGTCACTAGTTCTCCAGATGGC
Rev3: GCCATCTGGAGAACTAGTGACGTGTGTCCAG

Fzy^{5032} adjacent mutations:

Thr272Val
For1: CCATCGGCAACAGCGTCGGTGCCGTGGAGC
Rev1: GCTCCACGGCACCGACGCTGTTGCCGATGG

Met289 (to Leu, codon CTG)
For2: AGCGTCTGCGAGTGCTGGATGGACACAGTG
Rev2: CACTGTGTCCATCCAGCACTCGCAGACGCT

Ser293Ala
For3: TGATGGATGGACACGCTGCCCGAGTGGGAT
Rev3: ATCCCACTCGGGCAGCGTGTCCATCCATCA

His325 Val
For4: TGCGTGCACGTGAGGTCAAGCTTTCCACAT
Rev4: ATGTGGAAAGCTTGACCTCACGTGCACGCA

Lys326 Leu
For5: GTGCACGTGAGCACCTGCTTTCCACATTGT
Rev5: ACAATGTGGAAAGCAGGTGCTCACGTGCAC

Fzy^{AC-10} adjacent mutations:

Cys430Ala
For1: CCAAGTCGCAGGTCGCTTCTCTGCTCTTTT
Rev1: AAAAGAGCAGAGAAGCGACCTGCGACTTGG

Ser431Ala
For2: AGTCGCAGGTCTGTGCTCTGCTCTTTTCTC
Rev2: GAGAAAAGAGCAGAGCACAGACCTGCGACT

Met476 Leu
For3: TCACGAGTTCTCCAGCTGGCCATGTCTCCGG
Rev3: CCGGAGACATGGCCAGCTGGAGAACTCGTGA

Each primer pair is complementary and used in combination with *fzy* 5’ and 3’ primers to generate *fzy* transgene fragments containing new point mutations.

Highlighted bases represent mutations sites.

APPENDIX 2
FZY DOMINANT MODIFIER SCREEN RESULTS

Genetic background: fzy^{5032}/fzy^{EP1028}

Initial screening candidates: Bloomington Stock Center third chromosome deficiencies

Suppressor locus 1:

Overlap of Df(9086) and Df(24990)
Includes genes: *CG18616, CG18530, CG11598, CG11600, CG11608, CG6753, CG6234, CG6225, CG14395, CG6188, CG43630, mbo, Cyp313a4, kar, Su(fu), Arp1, CG31347, CG14391, NijC, Past1, CG12279 , mus308, Men*

Suppressor locus 2:

Overlap of Df(669) and Df(8925)
Includes genes: *CG2014, CR43613, Dr, CG7567, CG11470, CG31041, alph, CG7568, Diedel2, CG1907*

Enhancer locus:

Overlap of Df(7660) and Df(26840)
Includes genes: *Mekk1, CG7718, gwl, CG14299, CG7720, CG18208, CG31226, CG14298, CG14297, Rh2, CG14294, CG34284, CG14292, endoA, CG14291, Xrp1*