

Elucidating the non-apoptotic role of the caspases in the *Drosophila* intestinal system



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Abstract

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The deregulation of caspase activity and stem cell properties are often correlated with the appearance and propagation of cancer, including colorectal cancer. However, the caspase-dependent regulation of the intestinal system remains poorly understood. Taking advantage of the powerful genetic tractability of *Drosophila melanogaster*, I aimed to decipher the functional role of the caspases within the intestinal system, in addition to their role in tumour formation. In this thesis, I demonstrated that using an apical-caspase reporter, I was able to observe widespread and stereotyped patterns of caspase activation within the intestinal system. Furthermore, this activation was non-apoptotic and occurred in non-regenerative conditions. Upon conditional knockout of the mammalian *caspase-9/2* orthologue, *Dronc*, in intestinal progenitors, I demonstrated that *Dronc* was required to restrain proliferation and prevent the premature differentiation of Enteroblasts into Enterocytes. Additionally, this activity of *Dronc* relied on its catalytic activity but not the canonical apoptotic pathway. Using a *Dronc*-protein reporter I was able to correlate these functions with its specific accumulation within the Enteroblasts. Molecularly, the non-apoptotic activity of *Dronc* in this context relies on the activity of the Notch and Insulin-TOR pathways. Using the Drop-seq single-cell sequencing technique, I observed the misexpression of several regional and cell-specific genes following loss of *Dronc*. Furthermore, these *Dronc*-specific effects may result in the deregulation of the intestinal microbiota. Collectively, this data provides novel insights into the caspase-dependent but non-apoptotic regulation of the intestinal system in non-regenerative conditions. Furthermore, these findings could explain the correlation between caspase-9 misexpression and the bad prognosis observed in patients suffering from colon cancer.

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Publications resulting from this thesis

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Xu DC, **Arthurton L**, Baena-Lopez LA. Learning on the Fly: The Interplay between Caspases and Cancer. Biomed Res Int. 2018;2018:5473180. (Co-first)

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1. Introduction

1.1. The Caspases

The evolutionarily conserved family of enzymes termed the caspases, have been historically implicated in the regulation of apoptosis and inflammation. The discovery of the relationship between the caspase enzymes and apoptosis was elucidated in the nematode: *Caenorhabditis elegans* (1). The discovery of ced-3 as an executioner caspase in *C. elegans* lead to the discovery of the mammalian homologue, interleukin-1-beta-converting enzyme (ICE-1), subsequently re-termed caspase-1 (2). Presently, eighteen mammalian caspases are known to exist; however, their expression appears species-dependent. For example caspases-15, -17 & -18 are absent in placental animals; likewise, caspase-12 appears not to be present in humans, rabbits and cows (1, 3). Despite differences between species, the regulatory mechanisms of caspase activation and the apoptotic pathway appear highly conserved between mammals, *Drosophila melanogaster* and *Caenorhabditis elegans*.

Initially, caspases are synthesised as single-chain, inactive zymogens, termed procaspases (3). Structurally, most apical-caspases comprise of an N-terminal pro-domain, subsequently followed by a larger subunit and a smaller subunit. A subset of effector procaspases, encompass a small spacer sequence separating the subunits; later being excised from the zymogen during maturation of the enzyme (4). Activation of the caspases is achieved through either dimerization or multimerization, and later cleavage of a small catalytic domain (5, 6) Upon activation, they are potent cysteine-aspartic

proteases that mediate the enzymatic processing of multiple substrates (1, 7) This proteolytic activity is crucial for the destruction of all subcellular structures during apoptosis, thus facilitating the elimination of unnecessary or faulty cells.

1.2. The mammalian apoptosis pathway

The mammalian apoptosis pathway can be activated through the intrinsic, extrinsic or the Perforin/Granzyme pathway (8).

The intrinsic pathway

The intrinsic pathway encompasses all of the apoptotic processes which regulate the permeabilisation of the mitochondrial membrane and the release of cytochrome c, resulting in the activation of the caspases and subsequent cell death (9). The pathway itself can be stimulated through a diverse array of non-receptor mediated activators of the apoptosis programme, including viral and bacterial infections (8).

One such activation mechanism of the pathway is the tumour suppressor gene, p53, which is upregulated following catastrophic and unrepairable damage of cellular DNA (10). Increased levels of p53 result in the upregulation of the initiator of apoptosis factor, Bax, which in turn can override the suppression of the anti-apoptotic B-cell lymphoma 2 (BCL-2) factor (11). Furthermore, upregulation of p53 also results in the downregulation of the BCL-2 protein (12).

Within the mammalian apoptotic programme, the BCL-2 family of proteins regulate the apoptotic pathway (13). There are two subgroups of the BCL-2 family of proteins, which either initiate the apoptosis pathway (pro-apoptotic) or inhibit its activation (anti-apoptotic) [Figure 1-1] (14). The pro-apoptotic factors impart their effects either through regulating the permeability of the outer mitochondrial membrane or through inhibiting the activity of the anti-apoptotic proteins (14).

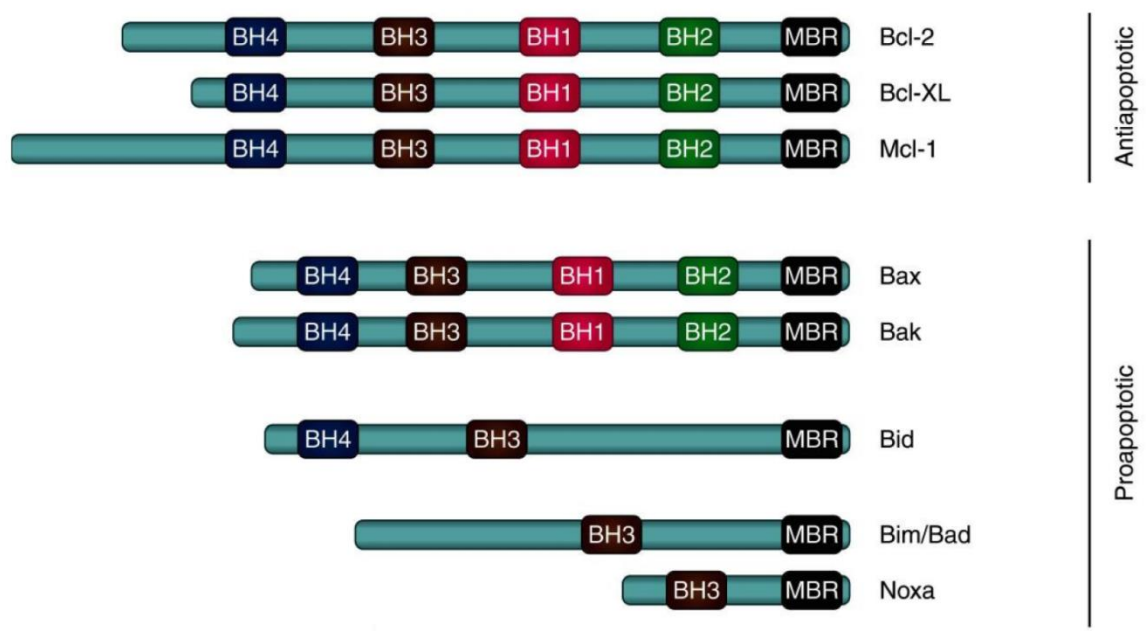


Figure 1-1: The domain structure of the BCL-2 family of proteins

The B-cell lymphoma 2 (BCL-2) family of proteins consists of two subgroups with either pro-apoptotic or anti-apoptotic activity. All proteins however, share homology in their structure with Bcl-2 homology (BH) regions, conserved across the different members. In addition to BH regions, membrane-binding regions (MBR) are present among all members of the family, facilitating their binding to the mitochondrial outer membrane or the endoplasmic reticulum (14). Figure adapted from (14).

Caspase-2 is also able to activate the apoptosis pathway upstream of the mitochondrial pathway; however, studies suggest that this activation occurs through alternative mechanisms to those utilised by other initiator caspases (15, 16). A study by Imre *et al.*, demonstrated that bacterial secretion of pore-forming toxins resulted in the efflux of potassium from the cell, activating caspase-2 (17). Additionally, other mechanisms such as heat-shock induced apoptosis have been shown to activate this caspase member (18).

The pro-apoptotic Bax forms pores in the mitochondrial membrane, resulting in the depolarisation and release of cytochrome c, endonuclease G (EndoG) and apoptosis-inducing factor (AIF), into the cytosol (19-21). The translocation of cytochrome c from the mitochondrial intermembranal space into the cytoplasm, allows its interaction with the apoptotic protease activating factor-1 (Apaf-1); these two proteins form the foundation of the apoptosome complex (22). EndoG and AIF translocate to the nucleus, where they mediate their apoptotic activity (21). AIF facilitates the condensation of chromatin and the fragmentation of DNA (23), similarly, EndoG cleaves chromatin DNA (24) [Figure 1-2].

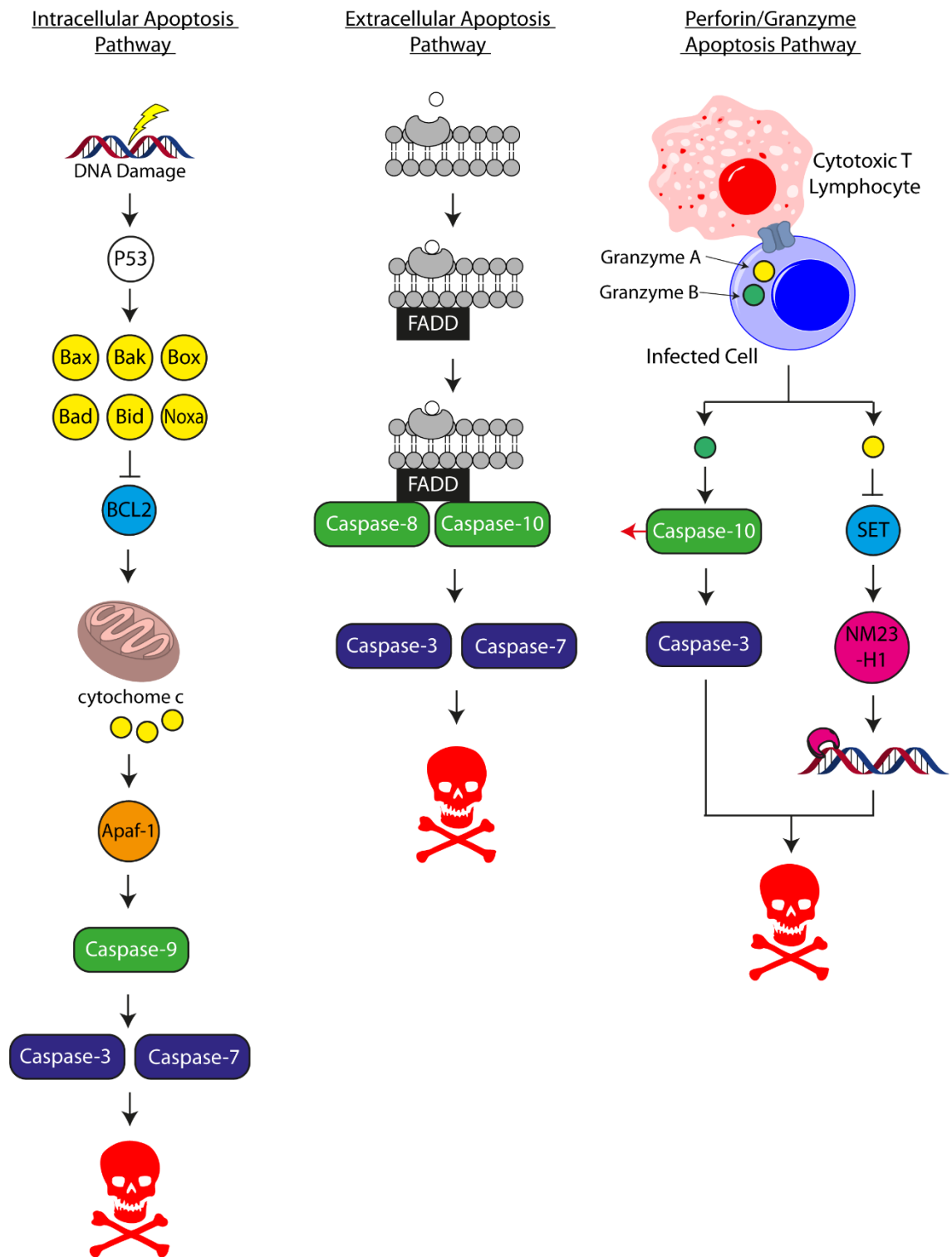


Figure 1-2: The simplified mammalian apoptosis pathways.

The mammalian apoptosis pathway can be activated through intracellular, extracellular or cytotoxic T-lymphocyte stimulation. Despite the separation, upon activation the pathways can overlap in a context-dependent manner. The intracellular pathway is activated through DNA

damage, leading to the upregulation of P53. P53 activity subsequently activates the pro-apoptotic BCL-2 family of proteins: Bax, Bak, Box, Bad, Bid and Nova. The pro-apoptotic proteins initiate the release of cytochrome c from the mitochondria and in collaboration with Dark and Drac, form the apoptosome. The apoptosome activates the effector caspases, caspase-3 and caspase-7, leading to cell death. The extrinsic pathway of activation is instigated by the binding of an apoptosis activating ligand, to specific extracellular receptors. Binding of a ligand to the FAS receptor results in the recruitment and formation of the FADD complex, and the subsequent recruitment of the initiator-caspases. This complex activates the effector caspases and results in cell death. The Perforin/Granzyme pathway is activated through the release of Granzyme A and Granzyme B from cytotoxic T-lymphocytes into an infected or tumorigenic cell. Granzyme A activates machinery tasked with the destruction of DNA material. Granzyme B activates the initiator caspase-10, before activating effector caspase-3. Note caspase-10 activation can also lead to cytochrome c release from the mitochondria/

The formation of the apoptosome complex leads to the recruitment and subsequent activation of procaspase 9, forming the activated apoptosome. The activation of this complex results in the activation of the downstream effector caspases, caspase-3 and caspase-7 (25) [Figure 1-2]. The effector caspases activate numerous factors responsible for chromatin margination, DNA fragmentation and nuclear collapse; in addition to inactivating the X-linked inhibitor of apoptosis protein (X-IAP), culminating in apoptotic death (25). The subsequent characteristic blebbing of apoptotic cells is achieved through the caspase-3 dependent cleavage of Rock-I, resulting in the phosphorylation of the myosin regulatory light chain (MLC) (26).

The extrinsic pathway

The extrinsic pathway is initiated through the extracellular binding of pro-apoptotic ligands to specific cell membrane receptors, i.e. tumour necrosis factor (TNF) receptor, tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors and the FAS receptor (27-29). This interaction between the FAS receptor-ligand complex instigates the intracellular recruitment of platform proteins, containing the FAS-associated death domain (FADD). This complex attaches to the pro-domains of the initiator caspases, such as caspase-8, encouraging dimerisation and their subsequent activation. The complex formed by this association is termed the death induced signalling complex (DISC). Caspases activated through DISC, such as caspase-8 and -10, can proceed to activate effector caspases, resulting in the commencement of apoptosis (30) [Figure 1-2].

Perforin/Granzyme pathway

Similar to the extrinsic pathway, the perforin or granzyme pathway is mediated by external factors. In this instance however, the apoptotic pathway is initiated by cytotoxic T lymphocytes in response to tumorigenic or virally infected cells (8, 31). Apoptosis is orchestrated through the formation and secretion of the transmembrane pore-forming protein, perforin. The construction of the pore at the cellular membrane allows the release of cytoplasmic granules into the dying cell, including the serine proteases: granzyme A and granzyme B (32) [Figure 1-2].

Granzyme A initiates the apoptosis pathway indirectly of the caspases. Once inside the cell, granzyme A activates the DNase, NM23-H1, leading to nicking of the DNA (8, 33). Furthermore, it cleaves the inhibitor of the NM23-H1 enzyme, the nucleosome assembly protein, SET. This combination of events leads to the apoptotic destruction of cellular DNA. Additionally, the degradation of SET leads to the inhibition of chromatin structure maintenance and DNA repair. Thus, likely contributing to the apoptotic process (8, 34). Granzyme B can cleave proteins at aspartate residues, therefore possessing the ability to activate caspases. In particular, the cleavage of procaspase-10 and factors such as the inhibitor of caspase-activated DNase (ICAD) (8, 35). Additionally, this process can also activate the mitochondrial pathway of apoptosis, through cleavage of Bid and subsequent induction of cytochrome c release (36). However, subsequent studies have also demonstrated that the release of cytochrome c can occur independently of the cleavage of Bid, Bax and Bak (37). In addition to targeting the upstream targets of the apoptosis pathway, granzyme B can also directly activate the effector caspase-3 (38). All three mammalian pathways of apoptosis are summarised in [Figure 1-2]

1.3. The *Drosophila melanogaster* apoptosis pathway

The *Drosophila* apoptosis pathway is highly conserved when compared to its mammalian counterpart, with both pathways possessing the ability for both intrinsic and extrinsic activation (39).

The Intrinsic pathway

The intrinsic pathway can be activated through damage to the cellular DNA; however, the pathway of apoptosis appears contingent on the DNA damaging agent and the developmental stage of the *Drosophila*. Irradiation can lead to G2 arrest and subsequent apoptosis of the cells. G2 arrest is achieved through the activity of both Chl2 and grp, and in part through 14-3-3-ε (40). Subsequent apoptosis of the cell requires the combination of these proteins, and the upregulation of p53, in addition to the pro-apoptotic proteins, reaper, hid, grim and sickle (40). Ultraviolet induced DNA damage in early and middle stage embryos, activates the apoptosis pathway through a different mechanism. In early embryos, apoptosis is achieved through E2F and hac-1. In middle stage embryos, the same DNA damage leads to apoptosis through mei-41 and activation of the pro-apoptotic factor, reaper (40).

Whilst the initial DNA damage response and subsequent activation of the apoptosis process varies, the execution of the pathway is generally preceded by the activation of the closely linked pro-apoptotic factors: reaper, hid and grim (analogous to the BCL-2 proteins: Bid, Bax and Bad (39, 41)). The upregulation of these proteins compete with the *Drosophila* inhibitor of apoptosis homologue of XIAP (DIAP1), through multiple mechanisms. The first is through the hid-mediated poly-ubiquitination of Diap1, resulting in the degradation of the protein. Another mechanism is through the suppression of the translation of DIAP1, through the activity of Reaper and Grim (42). Despite the presence of four inhibitor of apoptosis proteins encoded by the *Drosophila* genome, DIAP1, DIAP2, dBruce and Derterin (43), DIAP1 appears to be the most

important for the inhibition of apoptosis (44). Interestingly, evidence also suggests that hid and reaper can directly bind to the mitochondria, leading to changes in the mitochondrial ultrastructure and the release of cytochrome c (45).

Loss of DIAP1 prevents the inhibitory ubiquitination of the *Drosophila* caspase-9/2 homologue, Dronc. In non-apoptotic conditions, DIAP1 binds to the Dronc via its BIR2 domain and utilising its RING finger domain, ubiquitinates Dronc [Figure 1-3] (46). Furthermore, binding of DIAP1 to this domain impedes the binding of hid, reaper and grim to Dronc. Interestingly, the ubiquitination-dependent degradation of Dronc does not appear to be the main mechanism of inhibition. Instead, ubiquitination blocks the processing of Dronc, impeding the activation of the caspase (47). In addition to Dronc, DIAP1 can directly inhibit the activity of downstream executioner caspases, through binding via its BIR1 domain [Figure 1-3] (44, 48).

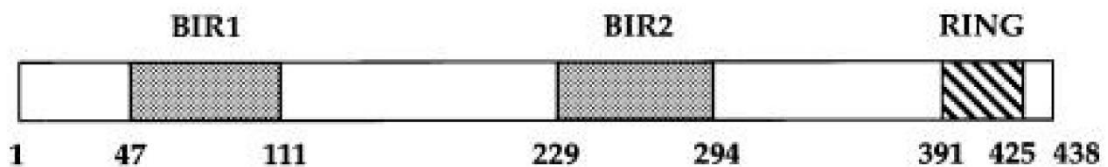


Figure 1-3 Domain structure of DIAP1

DIAP1 inhibits the activation of the apoptosis pathway through the binding of its BIR2 domain to Dronc. Subsequently, the RING domain ubiquitinates Dronc impeding its activation. The BIR1 domain of DIAP1 can bind directly to the effector caspase drICE, also contributing to pathway inhibition. Figure obtained and adapted from (49).

Removal of DIAP1 suppression, allows the recruitment of the *Drosophila* Apaf-1 homologue (Dark) with Dronc, forming the apoptosome. Initial structural studies suggested that the *Drosophila* apoptosome consisted of 8 molecules of the Dark protein bound to Dronc, forming a single ring structure. Within this structure, the caspase recruitment domains (CARDs), formed a crown on the central hub (50). However, subsequent cryo-Electron microscopy studies were able to obtain a resolution of the apoptosome at 4.1 Å or below, determining the presence of 16 molecules of Dark within the apoptosome. Structural analysis of the data suggested the existence of two stacked rings of Dronc-CARD, which are encompassed by two octameric rings of the Dark protein [Figure 1-4 A-B] (51). Furthermore, conversely to its mammalian counterpart, the *Drosophila* apoptosome does not require the presence of cytochrome c to form (52).

The formation of the apoptosome facilitates the activation of Dronc through the initial auto-cleavage of the protein at residue E352; separating the two subunits; thus stabilising the Dronc homodimer. This leads to the activation of the catalytic site (C318). [Figure 1-4 C-D] (53). During apoptosis, the CARD domain is cleaved at D135, suggesting that once activated, Dark is no longer necessary for the processing of substrates (53) [Figure 1-4 C-D]. Interestingly following loss of the CARD domain, DIAP1 becomes unable to repress the activity of Dronc, suggesting an interaction of DIAP1 with this domain (49).

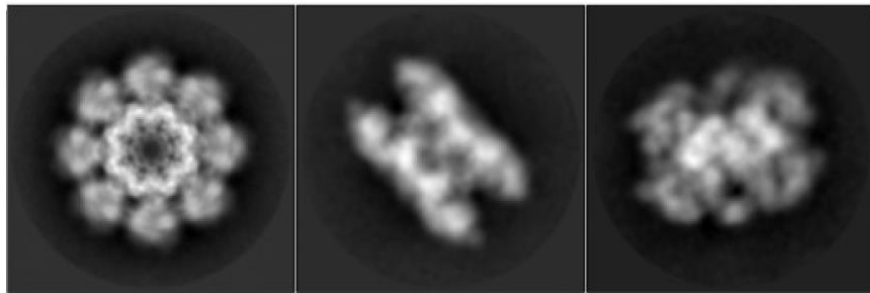
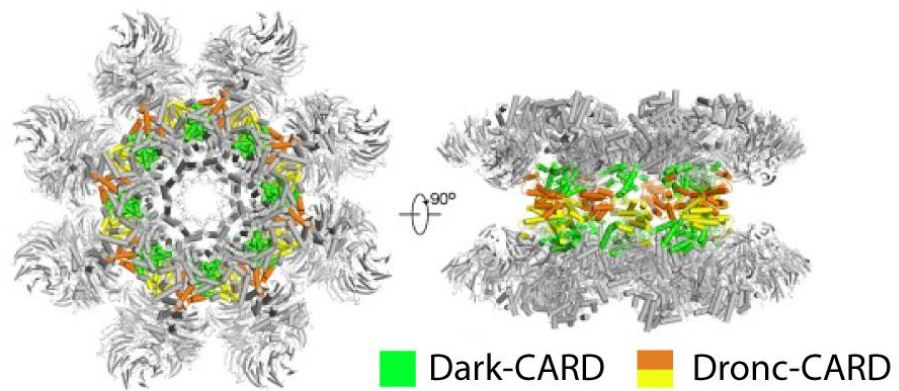
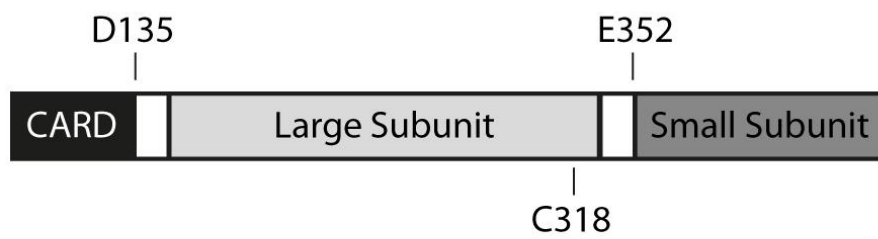
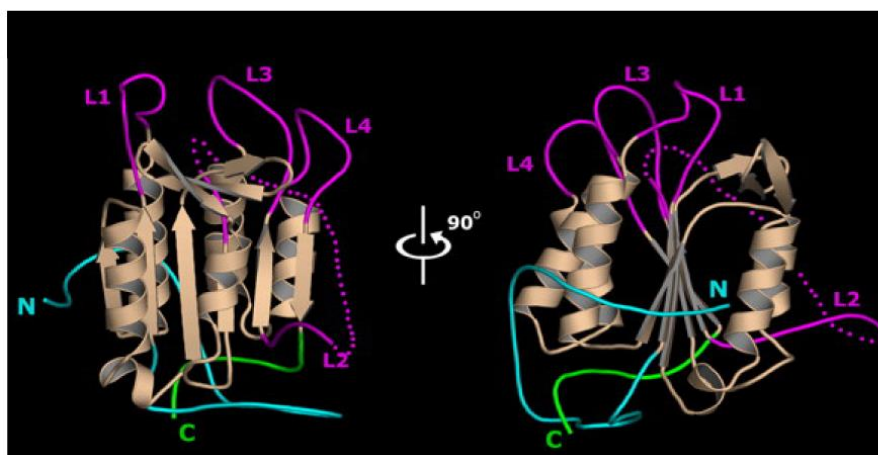
A**B****C****D**

Figure 1-4 Structure of the *Drosophila* apoptosome and the domain structure of Dronc

(A) A representative cryo-EM image of the *Drosophila* apoptosome observed from three contrasting views. Note the presence of two ring-like structures easily observable from the middle panel. (B) The overall structure of the apoptosome, with the Dark-CARD domain observable in green and the Dronc-CARD domains depicted in yellow and orange. The CARD domains interact within the central area of the structure, ensuring the two octameric rings of Dark do not interact. (C) Domain structure of Dronc. Dronc is fully activated through cleavage of the Glutamic acid residue at position 352 leading to the separation of the two subunits, forming a stable homodimer. This cleavage event results in the activation of the catalytic site present at residue C318. During cell death, the CARD domain cleavage occurs at residue D135. (D) The structure of the Dronc zymogen in two perpendicular views. The core of the protein can be observed in brown. The active site loop structures are shown in magenta. The C and N terminus can be observed in green. (A) and (B) obtained and adapted from (51), (C) is adapted from (54) and (D) from (55).

The activation of Dronc and the apoptosome leads to the subsequent activation of the effector caspases, Death caspase-1 (Dcp-1), Death associated molecule related to Mch2 caspase (Damm), Death related ICE-like caspase (drICE) and Death executioner caspase related to Apopain/Yama (Decay) [Figure 1-5]. The effector caspases, in addition to their position downstream of the apoptosis pathway, are classified as such, owing to the absence of a long pro-domain within their structure [Figure 1-5] (56). While there are four effector caspases, the caspase-3 homologue, drICE, appears the most important with redundancy reported for DCP-1 (Caspase-7), Damm and Decay in several processes (44). DrICE is activated initially through cleavage of pro-drICE by Dronc, at the junction

between the large and small subunit [Figure 1-5]. This cleavage event results in two subunits which remain bound, and through a conformational change, establish the enzymatic activity of the caspase (57). Activation of the effector caspases leads to the death of the cell.

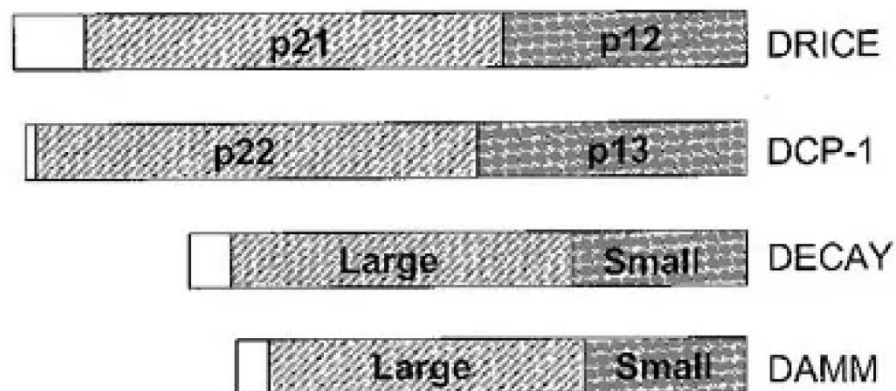


Figure 1-5 Domain structure of the effector caspases.

Diagram depicting the domain structure of the four known effector caspases in *Drosophila melanogaster*. drICE contains a 21 kDa large subunit, termed p21, in addition to a smaller 12 kDa Subunit. The cleavage between the large and small subunit of drICE by Dronc leads to its activation. DCP-1 varies slightly in structure, with the subunits larger in size when compared to drICE. Both Decay and Damm are smaller, however still retain the large and small subunit structure. Figure obtained and adapted from (58).

The Extracellular pathway

Extracellular activation of the apoptosis pathway in *Drosophila* is similar to its mammalian counterpart, with its activation orchestrated through a multitude of different receptors such as the Tumour necrosis factor- α (TNF- α) and the PDGF- and VEGF-receptor related (Pvr) (59, 60). Additionally, the pathway can be activated as a

consequence of the immune response, particularly in the resistance of infection by gram-negative bacteria through the immune deficiency pathway (IMD) (61).

Recognition of bacteria is achieved through the binding of *meso*-diaminopimelic acid-type peptidoglycans, to the peptidoglycan recognition receptor (PGRP) (62). Following binding, the receptors dimerise or multimerise, and the signal is transmitted intracellularly to the adaptor protein, IMD (63). Subsequently, IMD recruits dFADD and the caspase-8 homologue, Dredd (61, 64, 65). Dredd contains two death effector domains, essential for the interaction with the dFADD adapter (66, 67). The presence of this complex activates subsequent downstream immune responses (62). Additionally, upregulation of this pathway can lead to apoptosis, likely through subsequent activation of, at least in part, the effector caspases; owing to the rescue of apoptotic phenotypes following expression of the P35 inhibitor of effector caspase activity (57, 68).

Collectively, many of the caspase members and pathways in *Drosophila* share homologs with their mammalian counterparts, in addition to the nematode worm *Caenorhabditis elegans* [Figure 1-6]

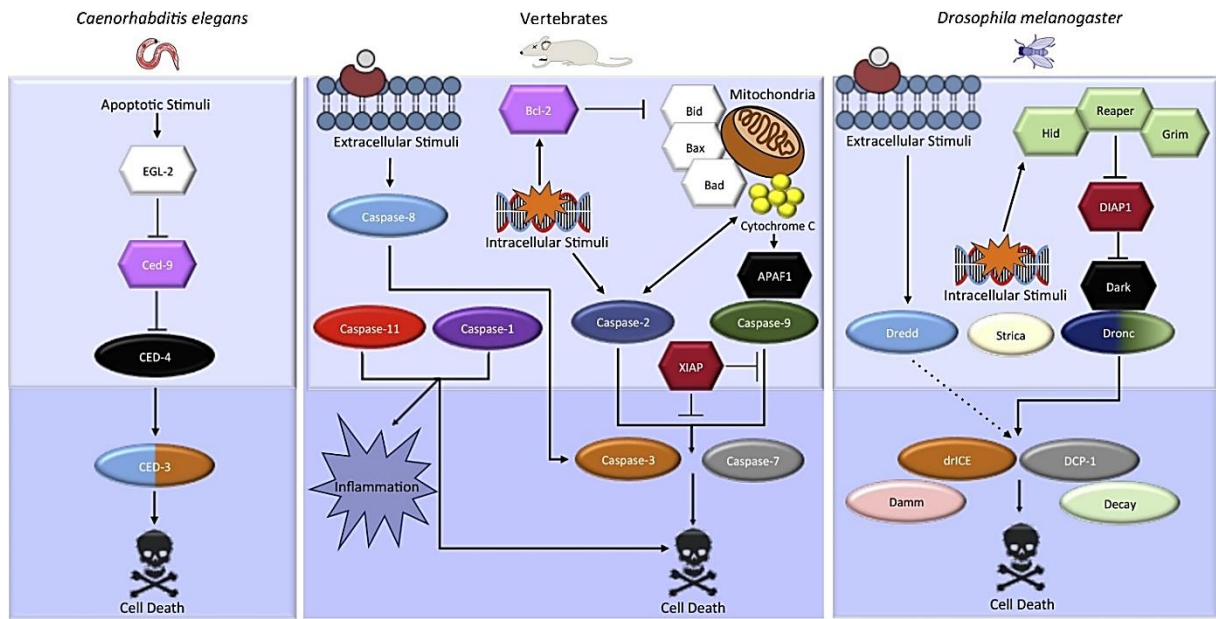


Figure 1-6: The evolutionary conservation of the main regulators of the apoptosis pathway.

The left panel denotes the *Caenorhabditis elegans* apoptotic pathway, the middle denotes the vertebrate and the right denotes the *Drosophila melanogaster* apoptotic pathway. The ellipsoid objects denote members of the caspase family, whereas the hexagons are accessory proteins necessary for the apoptotic process. Proteins sharing homology are represented in the same colours across the three pathways; the light blue background depicts apical caspases and the darker region the effector caspases. Figure adapted from (39).

1.4. Non-apoptotic roles of the Caspases

In addition to their apoptotic roles, the caspases have been implicated in a plethora of functions not related to apoptosis. These functions appear essential for the maintenance of organism homeostasis and physiology; including the modulation of the immune response, proliferation, differentiation, cell fate decisions and cytoskeletal reorganisation (69). Despite the extensive and dedicated study into the non-apoptotic roles of these enzymes for over 17 years, many open questions remain. First and foremost, what is the mechanism by which the activation and regulation of these enzymes occur, without the occurrence of cell death? Secondly, how are the specific substrates recognised by activated caspases which orchestrate their non-apoptotic roles within the cell? Thirdly, how does this caspase signalling activity affect organism-wide phenotypes and finally, are these novel non-apoptotic functions physiological, or are they simply a consequence of the rescue of important cells from the apoptosis process, through the so-called anastasis phenomena (69, 70)? Equally unknown, is the consequence of non-apoptotic caspase-deregulation in the manifestation of pathologies relating to stem cells, and the formation of cancer (71).

1.5. Non-apoptotic caspase regulation of embryonic stem cells

Stem cells are precursor cells possessing the ability for unlimited or prolonged replication, as well as the potential to generate at least one type of more specialised cell. Collectively, these cells contribute to the development of the organism or the

maintenance of homeostasis (39, 72, 73). There are two classes of stem cells known to exist, termed embryonic and adult stem cells. Embryonic stem cells are characterised as the group of cells formed following cleavage of the zygote, but before the formation of the blastocyst. These cells are considered totipotent and thus possess the ability to develop into any specialised cell within the host organism (73). Adult stem cells are cells which originate from postnatal tissues, such as those present in the bone marrow. While still retaining the ability to self-renew, these stem cells are only capable of generating certain types of cells (74). Despite the distinction between adult and embryonic stem cells, the activity of the caspases appears to be able to regulate the properties of both subtypes.

A seminal study in 2002 suggested a regulatory role for caspase-8 in the regulation of mice embryonic stem cells. Through the phenotypic analysis of *caspase-8* mutant mice, they were able to observe lethality of these mice at early stages of development. Furthermore, these mice had neural tube defects, which could not be attributed to apoptotic flaws, but instead resulted from the downregulation of neurogenic markers and the aberrant differentiation of the neural precursors (75). Despite this initial observation, functional and mechanistic evidence was not obtained until 2008 in the work of Fujita *et al.* In this study, mice embryonic stem cells mutant for both *caspase-9* and *caspase-3* were unable to differentiate into more specialised cells. This experimental evidence was further reinforced by the use of specific caspase inhibitors, which recapitulated the aforementioned observations. Additionally, utilising a mutated version of the stem cell factor, Nanog, the authors were able to determine that the

caspase-mediated cleavage of Nanog was required to permit the differentiation of these embryonic cells into more specialised daughter cells (76). Interestingly, the caspase cleavage sites present in Nanog are conserved in the human orthologue of this factor (76).

The ability of the caspases to modulate mice embryonic stem cells appears to be conserved in humans. Human embryonic stem cells and induced pluripotent stem cells continuously undergo cell differentiation and anoikis (a specialised form of programmed cell death), in the absence of treatment with basic fibroblast growth factor. Caspase-3 inhibition prevents apoptosis, but also the excessive differentiation of these cells (77).

More recent studies have speculated that non-apoptotic activation of the caspases in murine embryonic stem cells promotes the proliferation and differentiation of c-Kit⁺/α-actinin^{low} cardiac progenitor cells. Conversely, the inhibition of the caspases lead to a reduction in the differentiation of cardiomyocytes (78). Additionally, the downregulation of caspase-3 and caspase-7 has also been implicated in the reduction of differentiated myocytes in the developing heart in murine models (79).

Interestingly, evidence also suggests that caspase-3 and caspase-8, rather than solely mediating the differentiation of stem cells into more specialised cells, are also implicated in the reversion of specialised cells into induced pluripotent stem cells. This occurs through the caspase-dependent cleavage and degradation of the retinoblastoma

protein. Furthermore, the peak of the expression occurring during the reprogramming process appears to be mediated by the stem cell factor Oct-4 (80).

1.6. Non-apoptotic caspase regulation of adult stem cells

In addition to the regulation of embryonic stem cells, the caspases also regulate the activity of the adult stem cells and indeed, most of our understanding relating caspase-mediated stem cell properties originate from these cell types (39). For example, an abundance of literature has suggested a crucial role for the caspases in the regulation of the hematopoietic precursors, in addition to the lymphoid and myeloid derivatives (39). Studies have shown that haematopoietic precursors from *caspase-3* mutant mice demonstrate phenotypes of excessive proliferation and an untimely exit from a quiescent state. Furthermore, loss of *caspase-3* also appeared to correlate with aberrant differentiation and an insufficient number of mature cells within the peripheral tissues. Importantly, these phenotypes originated independently of apoptotic defects (81). Caspase-2 also appears to participate in the regulation of the haemopoietic cells through its non-apoptotic activity. Loss of *caspase-2* in murine models results in a significant increase in the total number of hematopoietic precursors. In aged mice, this leads to the overrepresentation of myeloid progenitors compared to controls, resulting in the increased number of granulocyte macrophages and erythroid progenitors. Additionally, the phenotypes were also suggested to contribute to tumour and aneuploidy (82).

Defects in stem cell homeostasis following the loss of caspase activity are not restricted to the haemopoietic stem cells, but can frequently be observed in the more mature precursors present in the peripheral tissues. Mice splenic B-cells demonstrate phenotypes of excessive proliferation following the loss of *caspase-3*, through the formation of stable p21-PCNA complexes (83). Likewise, the activity of caspase-8 is required for T-cell proliferation following stimulation through cytokines (84). Additionally, murine *caspase-8* mutant hematopoietic myeloid progenitors, demonstrate a diminished ability to proliferate and form colonies, following specific signalling stimulation (85). Further studies have shown a requirement for caspase-3 and caspase-8 activity in the differentiation of myocyte cells into dendritic cells and macrophages, respectively (85). Strikingly, a reduction in caspase-3 activity in erythroblasts increases their susceptibility to death, possibly through the autophagic pathway (86). Collectively, this data demonstrates an essential requirement for caspase activity in the regulation of haemopoietic stem cell function.

In addition to the haemopoietic system, a substantial body of evidence has suggested an important requirement for caspase activity in the neuronal system. In *Drosophila*, the caspase-2/9 homologue, Dronc, has been demonstrated to cleave the *Drosophila* GSK-2- β homologue, Shaggy. This cleavage is required for preventing the specification of excessive sensory organ precursor cells (87). Later studies were able to determine that Dronc was regulated through the activity of the *Drosophila* inhibitor of apoptosis, DIAP1 (88). Additionally, the activity of Dronc in regulating the nervous system of *Drosophila* is not restricted to the peripheral neurons, but also exerts a regulatory role

on its central nervous system. The type II neuroblasts utilise protein-protein interactions as opposed to the proteolytic activity of Dronc, in order to downregulate the activity of the Notch pathway regulator, Numb. The regulation of this protein is essential to limit the over-proliferation of these neuroblasts through Notch deregulation (89). Importantly, the caspase-mediated regulation of neuronal stem cells is conserved in mammalian organisms. Caspase-3 has been shown to modulate the differentiation of neuronal precursors in mice models, through PAK1 and the regulation of the cytoskeleton of the cell (90). Similarly, in the Bergmann glial cells and the neurons of the cerebellar cortex, caspase-3 has been implicated in orchestrating the correct timing of the differentiation of these neurons (91, 92). Depletion of *caspase-1* in PC12 cells, derived from the rat pheochromocytoma, precludes the expression of the Fodrin and Clapain markers of differentiation, crucial for the timely differentiation of PC12 cells (93). Along the same vein, human NT-2 cells are well utilised in the study of neuronal differentiation. The depletion of *caspase-9* within these cells prevents their differentiation, and their ability to express markers of differentiation. The authors hypothesise that this is likely due to the ineffective processing of the class III histone deacetylase, Sirt1 (94). In the same cellular scenario, caspase-2 performs a counteracting role, obstructing the commitment of these cells to differentiate through mechanisms which remain elusive (94).

Caspases have also been demonstrated to partake in the regulation of stem cells resident in the bone, muscle and skin. For example, in the osteoblast cell line, MC3T3-E1, BMPs induce the cell cycle arrest of these cells in the G0/G1 transitional stage, in

addition to the activation of differentiation markers. These events correlate with an upregulation in the activity of caspase-2, caspase-3 and caspase-8, without evidence of apoptotic phenotypes (95, 96). *Caspase-3* null mice exhibit a reduction in osteoclast proliferation and differentiation; this reduction correlates with the expression of senescence markers such as p53 and p21 (96). Furthermore, primary osteoclast numbers are reduced and differentiation cannot be triggered through binding of the ligand to the NF- κ B receptor (97).

Skeletal muscle stem cells also appear regulated by the caspases. Loss of *caspase-3* activity leads to deficient myotube and myofibril formation, and a reduction in differentiation into skeletal muscle progenitors, termed myoblasts (98). Further studies established that the proteolytic activity of caspase-3 was indispensable for the differentiation of skeletal muscle precursors, occurring through the proteolytic processing of the Mammalian Sterile Twenty-like kinase (Mst-1). More recent studies by the same group have demonstrated that cleavage of the Inhibitor of Caspase Activated DNase, ICAD, also occurs during this process of differentiation (98). Remarkably, the activation of this enzyme leads to DNA double-strand breaks, upregulating the transcription of p21 and through inhibition of CDK-Cyclin complexes, facilitates the differentiation of these cells. Later in the differentiation program, caspase-3 also cleaves the master regulator of proliferation in muscle cells, Pax7, committing the cell to differentiate (99). In C2C12 cells, despite being normally downstream of the apoptotic process, caspase-9 appears activated by the mitochondrial pathway, resulting in the activation of caspase-3 and subsequent entry into the

myoblastic differentiation program. Interestingly, the caspase-mediated differentiation of muscle cells is not restricted to skeletal muscle cells but also extends to cardiac muscle. Cardiomyocyte differentiation can be triggered by Wnt-11 leading to the activation of caspase-3 and caspase-8, and the subsequent degradation of β -catenin. This ultimately inhibits Wnt signalling responses and stimulates the cardiomyocytes to differentiate (100).

In the skin, caspase-3 is a major regulator of the interfollicular embryonic keratinocytes, controlling the cell fate decision of these cells from proliferation to differentiation (101). Furthermore, in hair follicles, the mast cells exhibit excessive proliferation following the loss of *caspase-7* (102). Additionally, recent literature has suggested that caspase-3 can contribute to the progression of squamous carcinoma, with caspase-3 upregulation resulting in hyperproliferation and thus formation of tumours (103).

Finally, the caspases also partake in critical regulatory processes within the regulation of the intestinal system and particularly, the regulation of the intestinal stem cells. In *Drosophila*, caspase-mediated regulation of the Intestinal Stem Cells has been observed as a result of the apoptotic process, interacting with the chromatin remodelling factor, Brahma (104). In the *Drosophila* intestinal system, the Hippo pathway is an important regulator of proliferation and the survival of cells within the epithelium (105). In conditions absent of cell death, Brahma, a regulator of the Hippo pathway, promotes the proliferation of Intestinal Stem Cells. Conversely, in its absence cell proliferation is inhibited. The caspases can cleave the caspase-cleavage site present in the Brahma

protein, inactivating its proliferative activity in apoptotic conditions (104). Importantly, the caspase-mediated regulation of intestinal cells is not a curiosity of *Drosophila* but conserved in vertebrate models. Interestingly, in some circumstances, caspase activation can lead to cell survival as opposed to the initiation of the cell death process. *Caspase-11* deficient mouse intestinal cells demonstrate enhanced susceptibility to treatment with dextran sodium sulphate, leading to increased levels of cell death and reduced proliferation (106). Similarly, mouse intestinal cells mutant for *caspase-3* demonstrated symptoms of increased cell death, organ damage and severe colitis in response to chemical and environmental stress (107). In addition to studies demonstrating pro-survival roles for the caspases in the intestinal system, the deregulation has also been attributed to pathologies, including the initiation and onset of cancer. In *caspase-1* null mice, an excessive proliferative response is observed following sustained damage to the intestinal epithelium, resulting in an increased propensity for the formation of tumours (108). Furthermore, *caspase-9* deregulation has been observed in colon adenomas and carcinomas. Intriguingly, compared to normal mucosa, higher expression of *caspase-9* was observed in adenomas, whilst appearing lower in cancer, both at the mRNA and protein levels. Additionally, the downregulation of caspase-9 was more frequently observed in poorly differentiated cancers. Using cell lines, the same study determined that overexpression of caspase-9 reduced colony formation and the rate of proliferation (109). Unfortunately, the molecular mechanisms orchestrating these caspase-9 dependent defects remain elusive.

As previously demonstrated, a frequently used model organism to elucidate the non-apoptotic regulation of stem cell function is *Drosophila melanogaster* and it is the primary model organism utilised in this thesis.

1.7. The model organism: *Drosophila melanogaster*

Drosophila melanogaster is routinely used to explore pathways associated with human diseases (110, 111). *Drosophila* genes and their pathways share strong homology with their mammalian counterpart, with 75% of human disease genes considered to have a functional homologue in the fly (112). The apoptotic pathway also demonstrates strong homology with its mammalian counterparts (58, 113).

The first reported scientific use of *Drosophila* was in the laboratory of William Castle in Harvard in 1901. Despite this, the use of *Drosophila* as a model organism did not become widespread until it was adapted by the “father” of *Drosophila* research, Hunt Morgan. Hunt Morgan advanced the theory of inheritance, proposed initially by Mendel; these discoveries consequently lead him to receive the Nobel Prize in Physiology or Medicine in 1933, for his work on chromosomal inheritance using *Drosophila melanogaster*. Subsequently, the use of *Drosophila* has become prevalent within the scientific community (114, 115).

The power of the model organism, *Drosophila melanogaster*, stems from the complexity and flexibility of the genetic tools which can be adapted for use in this organism relatively inexpensively, when compared to vertebrate models. Early methods of genetic study involved the use of random mutagenesis, achieved through various chemical or physical methods, such as ethyl methanesulfonate (EMS) or X-rays respectively (116). Subsequent advancements lead to slightly more targeted gene disruption, through the use of P-elements within the *Drosophila* genome. Transgenic *Drosophila* lines were generated through subcloning small interfering RNA or cDNA sequences, with identifying marker genes (such as white), between two inverted repeating elements, into a plasmid. These plasmids were then injected into *Drosophila* embryos, enabling the subsequent generation of transgenic lines (116).

Subsequently, more advanced methods of genetic manipulation were developed for use in *Drosophila*, such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). This enabled more accurate and targeted manipulation of genes within the genome (117). However, the discovery of CRISPR/Cas9 genome editing techniques revolutionised genetic analysis studies in *Drosophila* (118).

The clustered regularly interspaced short palindromic repeat (CRISPR/Cas9) system is a bacterial defence mechanism, utilised to protect against infection from viruses and plasmids (119-121). The principle of this system has been successfully imported for use in *Drosophila*, and is now routinely used. Despite the presence of multiple variations of this gene-editing technique, the basic principles remain the same. Firstly, single guide

RNAs (sgRNAs) are designed, which specifically target the gene of interest. These sgRNAs are subsequently injected or expressed in the *Drosophila* embryo. Along the same vein, the Cas9 protein is also either expressed or injected into the embryo. The result of this combination is the targeted cleavage of the DNA at the site dictated by the sgRNA, inducing either single or double-strand breaks (122). Importantly however, the Cas9 protein will only cleave at protospacer adjacent motif (PAM) sequences, and thus sgRNA sequences should reflect this requirement (123). Through injection or expression of a third complementary template sequence, it is possible to insert specific DNA sequences into the locus through homology-directed repair during the DNA repair process (122).

The process of selecting and inserting specific reporters or cDNAs into a gene locus was simplified through the development of plasmids produced by *Baena-Lopez et al.*, and the inclusion of the attP/attB PhiC31-mediated insertion tool, in combination with the CRISPR/CAS9 genome editing protocol (124). In this protocol, the complementary sequence for inclusion during the homology directed repair processes, contain an attP landing site and a removable Pax-Cherry selection marker. The attP once included within the locus of the gene of interest, acts as a landing site for the subsequent insertion of constructs containing an attB site, mediated through the PhiC31 integrase enzyme (124). The ease at which these techniques have been adapted to *Drosophila* has ensured its continued and invaluable use as a model organism, aiding with our understanding of signalling pathways and cellular physiology.

The power of genome editing in *Drosophila* through the ages, has led to the incorporation of techniques which allow the targeted and controlled expression of genes through bipartite systems. The most commonly utilised in *Drosophila* being the yeast-derived, Gal4/UAS system (125). The *Gal4* sequence, once inserted into the regulatory region of the gene of interest (e.g. through P-element insertion or CRISPR/CAS9 etc.), is expressed under the regulation of its promoter. The UAS promoter, which remains mostly inactive until the presence of its activator, Gal4, can drive the expression of specific transgenes (126). The characterisation of many genes as specific markers of cell identity has led to the ability for tissue or cell-specific, genetic interrogation of homeostatic and pathological signalling processes (126-130). In addition to spatial specificity, temporal regulation has been achieved through the use of a thermosensitive Gal4 repressor, ubiquitously expressed under the tubulin promoter, TubGal80^{ts} (131). Further similar bipartite systems have subsequently been developed such as the QF/QUAS and LexA/LeXOP systems (126, 132-134).

Arguably, one of the strongest advantages of using *Drosophila* over other model organisms is the presence of so-called “balancer chromosomes”. Deleterious or recessive lethal mutations as part of natural selection, are selected out of an inbred population over time (135). Balancer chromosomes contain multiple inversions or rearrangements within the chromosome in order to inhibit recombination events during meiosis (136). The presence of these balancers allows the cultivation of an infinite number of stocks with relative ease.

In addition to signalling pathways, there appears to be a high degree of conservation between organs, for example the *Drosophila* intestinal system with the mammalian intestinal system (137). Collectively, the aforementioned examples and advantages of *Drosophila melanogaster*, ensure its suitability and usefulness in the elucidation of the non-apoptotic roles of the caspases within the *Drosophila* Intestinal system.

1.8. The development of the adult *Drosophila* Intestinal System

The gastrointestinal system is an essential organ for the processes of digestion, excretion and the protection of the organism (138). The *Drosophila* intestinal system, despite subtle differences, shares strong conservation with its mammalian equivalent, ensuring its frequent use in improving our understanding of the mammalian intestinal system (139).

The adult midgut progenitors commence development around embryonic developmental stage 11. The resultant epithelium consists of differentiating midgut cells (i.e larval enterocytes) and the undifferentiated adult midgut precursors (140, 141). These adult midgut precursors initially manifest as spindle-shaped cells located at the exterior of the midgut epithelium, before their subsequent relocation to the basal surface. Here, they persist throughout larval development, regulated by Epidermal growth factor (EGF), decapentaplegic (Dpp) and Notch signalling (140, 142). During pupation and the first few hours of metamorphosis, the adult midgut precursors combine into a continuous epithelial tube, overgrowing the larval tissue and

differentiating into the adult midgut. Simultaneously, the larval gut is broken down and degenerates (143).

1.9. The structure of the adult *Drosophila* intestinal system

The adult *Drosophila* midgut, similar to most other insects, consists of a simple epithelium encapsulated with muscles, nerves and tracheae (144, 145). Among other functions, the muscles participate in the movement of ingested material through the gastro-intestinal passage through peristalsis (146). The neuronal system regulates this process in addition to fluid balance and appetite (147).

At an anatomical level, the *Drosophila* intestinal system can be separated into seven morphological features, the crop, proventriculus, anterior midgut, copper cell region, posterior midgut, Malpighian Tubules and the hindgut [Figure 1-7] (148). The crop consists of a single thin layer of epithelial cells with a cuticle covered apex (149). Structurally, the epithelium is organised into continuous folds, allowing its expansion or reduction to aid its primary function as a food storage organ (149, 150). While the cells of the crop contain a small quantity of rough endoplasmic reticulum, golgi complexes and secretory granules; the digestive activities undertaken in this area are likely due to enzymes collected through secretion from the salivary glands and midgut cells (149). Evidence from other Dipterans suggests it may also partake in antimicrobial activity, through the mixing of immunological peptides from the labella glands with the eaten

food, in addition to nuptial gift-giving, trap-lining, bubbling and droplet disposition (151).

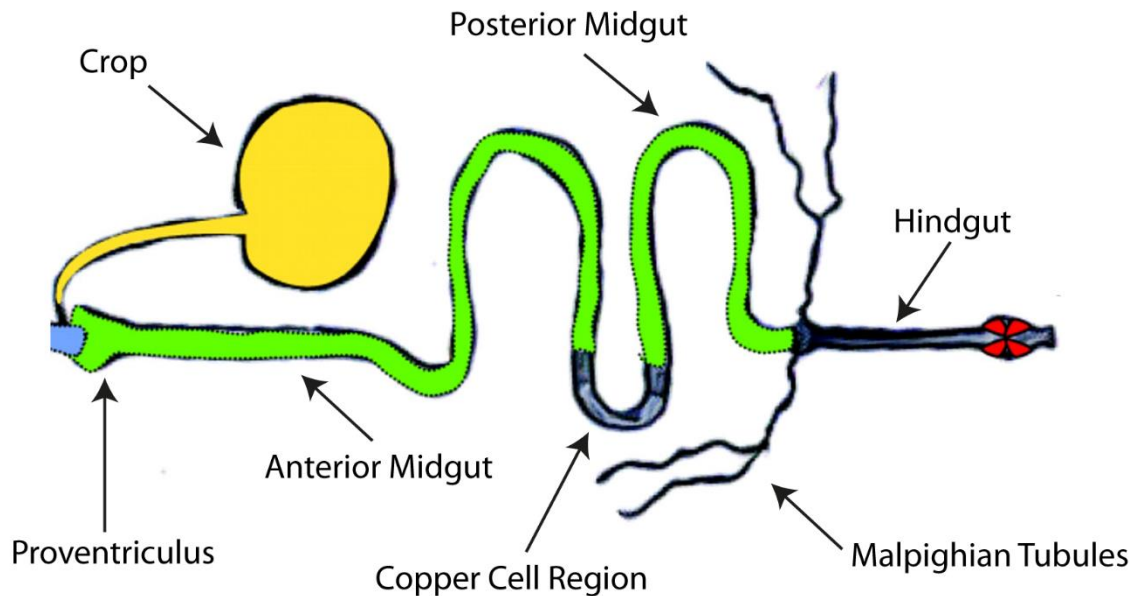


Figure 1-7: The structure of the *Drosophila* intestinal system

The *Drosophila* intestinal system contains 7 anatomically distinct regions, the proventriculus, crop, anterior midgut, copper cell region, posterior midgut, Malpighian Tubules and the hindgut.

Figure obtained and adapted from (137).

The proventriculus is a structure which resides at the boundary of the foregut and midgut, where the oesophagus, midgut and crop are situated [Figure 1-7]. The primary role of this structure is to act as a valve, regulating the passage of food into the anterior midgut and the crop, and in this respect, shares homology with the mammalian stomach (152, 153). The genetic control of the stem cells within this structure appears similar to mouse gastric cells, with JAK-STAT regulating proliferation, wingless regulating self-renewal, and the hedgehog pathway promoting differentiation (153).

The Malpighian Tubules form one part of the *Drosophila* excretion system, and its function analogous to the tubular section of the nephron within the human kidney. Their principal role is the generation of urine through the active transport of ions, organic solutes and water from the haemolymph (154-156). Anatomically there are four Malpighian tubules, of which a pair of tubes form the longer anterior, and the other pair, the shorter posterior. Each pair joins to form a common ureter connecting at the junction between the midgut and the hindgut [Figure 1-7]. The anterior Malpighian tubule is further subdivided into four sections, with the first segment storing calcium, magnesium and other solutes. The second section is the main secretor of ions generating the primary urine. The third acidifies the urine and reabsorbs K^+ , Cl^- and water, producing more concentrated urine, before its passage into the final ureter section for excretion (156-159). The posterior Malpighian tubules are smaller and have significantly fewer cells than the anterior tubule. These tubules mainly function through secretion at the main segment and reabsorption in the lower segment (156).

The hindgut is situated at the most posterior region of the *Drosophila* intestinal system, beginning at the midgut/Malpighian tubule boundary [Figure 1-7]. This structure can be subdivided into three discernible regions, the pylorus, ileum and the rectum. The pylorus, recognisable for its condensed ring of cells, primarily functions as a valve regulating the passage of digested material, through the constriction and relaxation of the sphincter muscles located in this region (160). The ileum has been shown to mediate the excretion of water and ions from the tract (160). Furthermore, studies have reported that a number of stem cells reside in the anterior region of the pylorus,

maintaining its structure throughout adulthood. The proliferation of Intestinal Stem Cells within this area, termed the hindgut proliferation zone, is regulated by locally produced wingless and hedgehog signalling. Collectively, these signalling pathways maintain a niche, promoting and maintaining the cells in a slow proliferative state. Additionally, dividing cells move away posteriorly from this zone and the wingless source, in a fashion similar to those described in the crypt of the mammalian intestine (161).

The copper cell region of the *Drosophila* intestinal system is a highly acidic ($\text{pH} < 3$) compartment, containing a group of acid-producing cells analogous to the mammalian gastric parietal cells of the stomach [Figure 1-7] (162). This area is discernible from its encompassing midgut structures due to the presence of two substantial reductions in luminal size, occurring at the extremities of this region (150). The acid-secreting cells within this area are some of the most well studied differentiated cells within the *Drosophila* intestinal system. These cells generate regional acidity through vacuolar H^+ ATPase pumps, using a carbonic anhydrase-catalysed pool of H^+ (162). This region also contains a distinct subtype of Intestinal Stem Cell, termed gastric stem cells (162). The stem cells in this region can produce the copper acid-secreting cells, interstitial cells and enteroendocrine cells. However, they remain largely quiescent and appear to be regulated by wingless signalling (163).

The remaining portions of the gut constitute the anterior and posterior midgut [Figure 1-7], analogous to the mammalian small intestine. The cells in this region are arranged

as a pseudostratified epithelium, in contrast to the larval gut where the cells are arranged cuboidally (164). These regions are perhaps the most well studied of all intestinal compartments, possibly due to the first characterisation of the Intestinal Stem Cells within this region (165, 166). As their name suggests, the Intestinal Stem Cells are the principle proliferative cells within the intestine, subsequently differentiating to renew cells damaged through environmental or pathogenic insults (167, 168); or to enhance reproductive success, as a consequence of intestinal remodelling following mating (169, 170).

1.10. The structure of the adult mammalian intestinal system

Despite the *Drosophila* anterior and posterior midguts sharing homology with the mammalian small intestinal system, the structural arrangement of cells within this epithelium are very different. Until mid-gestation, the intestinal epithelium forms as a stratified cuboidal epithelium (171, 172). During mid-late gestation, a series of epithelial-mesenchymal interactions, induced by signals emanating from the mesoderm-derived mesenchyme, lead to the invagination of the endoderm-derived epithelium to form villus and intervillus regions (172, 173). The intervillus regions subsequently invaginate, mediated through Myosin-11-driven apical constriction, to form a crypt [Figure 1-8]. Later, hinges form through cell constriction, which compartmentalises the crypts from the Villi. This process appears mediated through the process of Rac1 signalling (173). The small intestine is thought to contain around one million crypts, each producing around 300 cells per day (174).

Within the Crypt structure of the small intestine, around four to six Lgr5 positive intestinal stem cells reside at the very base of the Crypt (174, 175). Here, they proliferate forcing neighbouring cells to migrate upwards towards the intestinal lumen. Interestingly, mammalian Intestinal Stem Cells do not differentiate directly following division, instead differentiation into more specialised cells occurs during the process of continuous upward migration (174) [Figure 1-8].

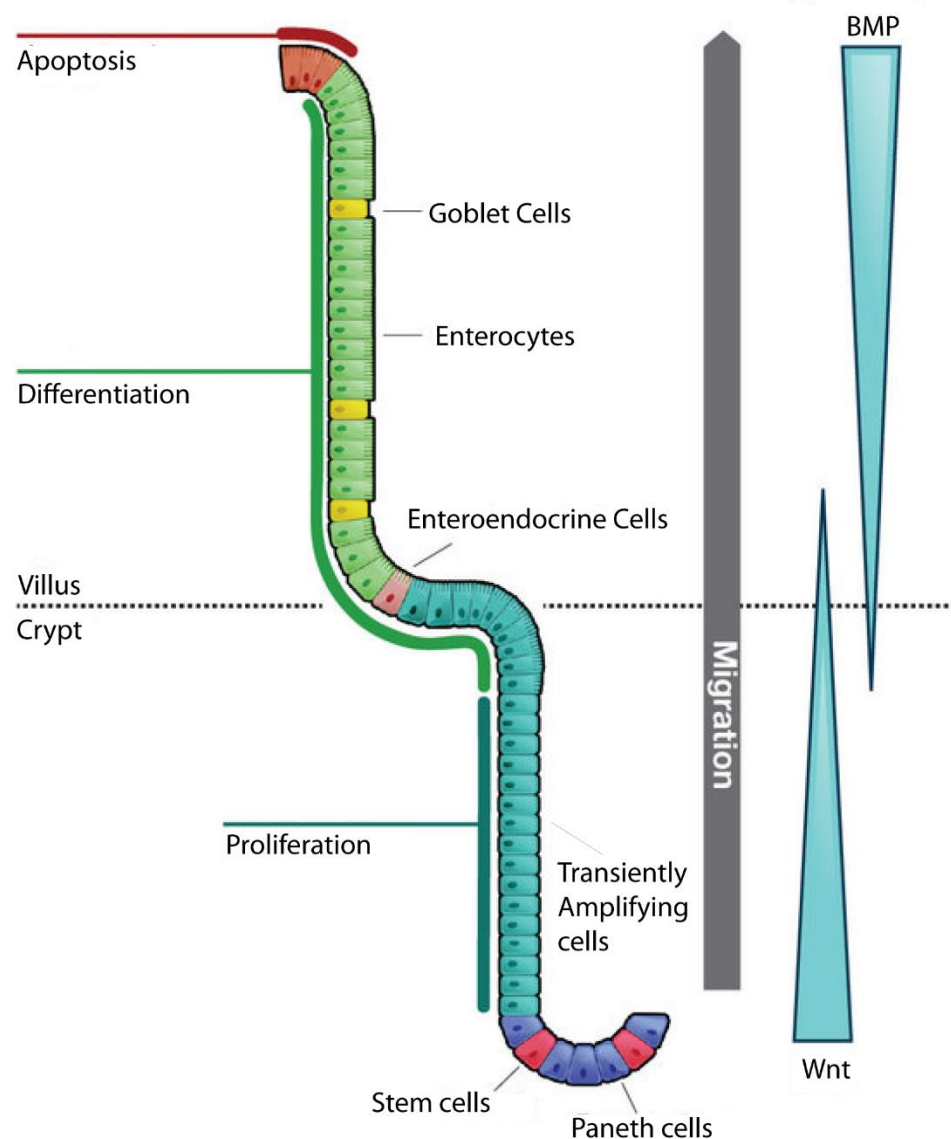


Figure 1-8: The Crypt and Villus structure of the mammalian small intestine

The mammalian small intestine consists of two segments, the lower Crypt structures and the upper Villus. The Crypt containing Intestinal Stem Cells and the Transiently Amplifying Cells constitute the primary proliferative zone of this structure. As cells migrate towards the Villus they differentiate into terminal differentiated Enterocytes, Goblet Cells or Enteroendocrine Cells. A signalling gradient between the Wnt and BMP pathways helps to regulate the transition from stemness into a differentiated cell. Figure obtained adapted from (176).

1.11. The cells of the *Drosophila* intestinal system

Compared to the mammalian intestinal system, the cellular subtypes present in the *Drosophila* intestine system although similar, are markedly simplified, with Paneth and Goblets cells absent from this system. Generally, the Intestinal Stem Cells can be characterised by the co-expression of the Snail family transcription factor, *escargot* (*esg*) and the expression of the Notch receptor ligand, Delta (177-179). The suppression of Notch targets through the Hairless-Suppressor of Hairless complex appear essential for the maintenance of stem cell identity. Further evidence from the same laboratory also determined that the transcription factor, *daughterless*, is also required for the maintenance of stemness, with the enhancer of split C factors promoting differentiation (180). Despite Intestinal Stem Cells appearing uniformly spread across anatomical regions, there is a substantial degree of variation in the transcription of genes within these cells, possibly contributing to their compartmental variations (181, 182).

The Transiently Amplifying-like cells, the Enteroblasts, also express the stem cell marker, *esg*. These cells however, are deficient in the expression of Delta, and instead are characterised by the expression of Suppressor of Hairless, Su(H) (128). Enteroblasts are formed following the asymmetric cell division of Intestinal Stem Cells, resulting in the formation of one Intestinal Stem Cell and an Enteroblast daughter cell (179). Unlike their mammalian counterpart, these cells appear quiescent and rarely, if ever divide (183).

The terminally differentiated Enterocytes, like their mammalian counterpart, are primarily involved in the absorption of nutrients from the intestinal lumen into the haemolymph (184). These cells are characterised by their larger cell and nuclei size, due to a series of endoreplication steps that occur during the differentiation process (146, 185). Likewise, the loss of *esg* expression precedes the expression of the POU-family transcription factor, *Pdm1*, as a marker of cellular identity (186). Alternatively, the overexpression of *Pdm1* can downregulate the expression of *esg* (186).

Similarly to mammalian enterocytes, the *Drosophila* enterocytes absorb carbohydrates, proteins, lipids, metals, ions and water. Initially, carbohydrates are broken down into fructose, galactose and glucose before absorption by enterocytes (187). Absorption of glucose is mediated through the glucose transporter (GLUT) protein transporter family, sharing homology with their respective mammalian equivalents (188). Amino acid transport relies upon the pathetic, minidisc, NAT1 and yin transporters (144).

The Enteroendocrine cells constitute the other terminally differentiated cell within the *Drosophila* intestinal system. The main task of this subtype of cell is the hormonal regulation of the intestinal system, independently of direct neuronal control. Numerous peptides such as allostatins , A, B and C, diuretic hormone 31, neuropeptide F and tachykinins have been shown to be produced by the enteroendocrine cells. Furthermore, despite being regularly dispersed along the intestinal system, there appears to be substantial variation between their activity, contingent on their location (189). Despite these observations, very little is known regarding the function and mechanism of action of the enteroendocrine cells and their peptides (190).

1.12. The signalling pathways mediating proliferation and differentiation of the *Drosophila* intestinal system

Until recently, the central dogma of *Drosophila* Intestinal Stem Cell differentiation into more specialised lineages, revolved around the direct transition of an Intestinal Stem Cell into a committed progenitor cell, termed an Enteroblast. Subsequently, Enteroblasts faced a cell fate decision to either differentiate into the major absorptive cell, the Enterocytes, or the main secretory cell, the Enteroendocrine cells (144, 170). However, new evidence has suggested that this differentiation pathway may be more nuanced than first thought. Recent evidence has revealed that Intestinal Stem Cells either directly, or through asymmetric or symmetric divisions, differentiate into Enteroendocrine cells, independently of Enteroblasts (191). Further evidence suggests that the Enteroblasts function as intermediate-like cells, prior to full terminal

differentiation into a mature Enterocytes (190). This process and the signalling pathways orchestrating these events are described in [Figure 1-9].

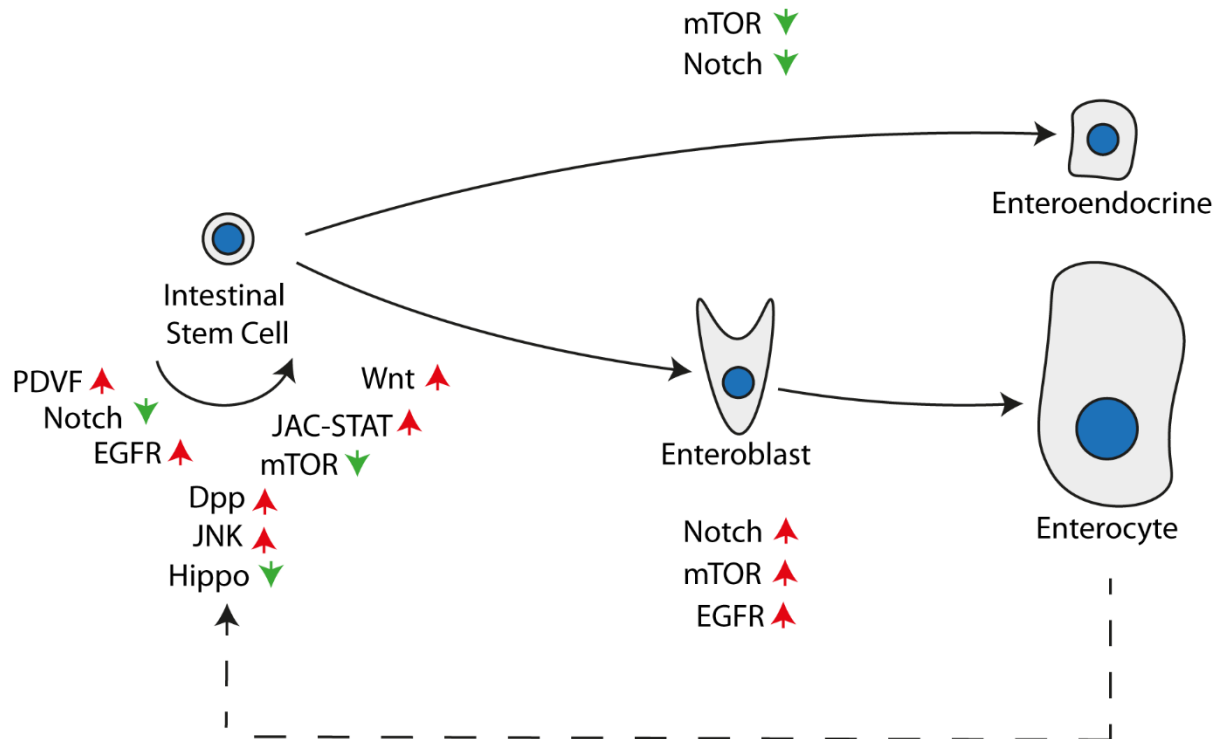


Figure 1-9: Differentiation pathway of cells in the *Drosophila* intestinal system

Intestinal Stem cells can differentiate directly into Enteroendocrine cells or Enteroblasts. Enteroblasts subsequently differentiate into Enterocytes. The signalling pathways orchestrating these events can be observed in the Figure. Note the red upward pointing arrow denotes increased activation of the pathway, whereas green downfacing arrows denote decreased activation.

In the *Drosophila* intestinal system, the main modulator of proliferation and differentiation of progenitor cells is the Notch pathway. Loss of *Notch* signalling within the Intestinal Stem Cells leads to the proliferation of these cells and the formation of Enteroendocrine cells (192), with differentiation into Enterocytes inhibited. Conversely, upregulation of Notch signalling within the progenitor cells precedes their exit from

stem cell fate and their differentiation into terminally differentiated enterocytes (193). A similar, all be it opposing role for the Notch signalling pathway exists in the mammalian intestinal system. Following loss of stemness, the Notch pathway subsequently determines the differentiation identity of the cell, with high Notch activity leading to specification as a secreting cell, and low Notch activity promoting differentiation into Enterocytes (194).

In addition to the Notch pathway, the Janus Kinase / Signal transducer and activator (JAK/STAT) pathway has also been shown to regulate the Intestinal Stem Cells following loss of Enterocytes through apoptosis. Upregulation of the pathway, though overexpression of the upd pathway ligand in *esg* expressing cells, results in the proliferation of the Intestinal Stem Cells. Furthermore, loss of JAK/STAT signalling impedes the differentiation of the Intestinal Stem cells into terminally differentiated cells. Interestingly, these effects were found to share epistasis with the Notch signalling pathway (195).

In addition to the JAK-STAT pathway, the mammalian target of rapamycin (mTOR) pathway also appears to modulate the activity of Notch, in order to regulate the intestinal system. Studies have demonstrated that the *Tuberous Sclerosis Complex 2* (*TSC2*) appears highly expressed in Intestinal Stem Cells, but downregulated in Enteroblasts. The resultant downregulation of TSC2 promotes the activation of the TOR pathway and differentiation of Enteroblasts into Enterocytes. On the contrary, maintenance of TSC2 activity promotes the differentiation of progenitor cells into

Enteroendocrine fate. The Notch pathway regulates the activity of the TOR pathway through the suppression of TSC2, via the activity of the Notch pathway regulator Su(H); thus acting as a cell fate determinant in this process (196, 197). Loss of the *TSC* in Intestinal Stem Cells results in larger sized cells, which are unable to proliferate and differentiate. The implications of this defect can be observed throughout the whole of the midgut, with the appearance of a thinner epithelial structure and increased susceptibility to damage (198).

The homologue of the mammalian Bmp pathway in *Drosophila*, the Dpp pathway, has been demonstrated to contribute to intestinal regulation. Additionally, the Dpp pathway also regulates the activity of the Notch pathway (199), with the secretion of Dpp and glass bottom boat (Gbb) from Enterocytes, antagonising Notch signalling in the Intestinal Stem Cells promoting proliferation (200). Conversely, activation of the Dpp pathway within the Enterocytes themselves inhibits the secretion of the ligands, impeding the proliferation of the Intestinal Stem Cells (201). In addition to the Enterocytes, haemocytes recruited to the intestinal epithelium following damage, have also been shown to secrete Dpp, inducing the proliferation of the Intestinal Stem Cells (202). Furthermore, studies have shown that Dpp is secreted from the encompassing visceral muscle (202). In contrast, activation of the Bmp pathway within mammalian Intestinal Stem Cells inhibits their proliferation, with its signalling activity most prominent at the tip of the villus and least active within the base of the crypt (203). The regulation of this gradient appears contingent on the activity of the BMP pathway inhibitor, Noggin. Studies have demonstrated that overexpression of this factor results

in the hyperproliferation of Intestinal Stem Cells and the formation of new crypts (204). The inhibitory effect of the BMP pathway on stemness is, in part, mediated through the inactivation of stem cell markers such as Lgr5⁺ (205)

The Epidermal Growth Factor Receptor (EGFR) pathway is also an important mechanism of Intestinal regulation. Numerous studies have demonstrated that high levels of di-phospho-ERK, an activated Mitogen-activated protein kinase (MAPK), are detected in Intestinal Stem Cells and Enteroblasts (183, 206, 207). EGFR signalling through the ligand, Vein, is released from the encompassing intestinal musculature and is required to maintain the optimal proliferation of the Intestinal Stem Cells (206, 207). Furthermore, in tissue-damaging conditions through the activation of several *rhomboids* and the expression of multiple EGFR ligands, the activation of the pathway results in the proliferation of Intestinal Stem Cells and their subsequent differentiation into Enterocytes (207). Additional studies have shown that the transcription factor, FOS, is required for EGFR-induced proliferation. However, it also acts as a mediator of the Jun N-terminal Kinase (JNK or basket (bsk) in *Drosophila*) pathway, increasing proliferation of the Intestinal Stem Cells in response to stress (206).

As alluded to previously, the JNK pathway is often activated in response to intestinal stress, and often in collaboration with other signalling pathways (208-210). Activation of the pathway through downregulation of *puckered* (puc), a JNK pathway suppressor, in the Enterocytes results in an increase in proliferation of the Intestinal Stem Cells. Importantly, the authors were able to demonstrate that the increase in proliferation

observed was not a consequence of apoptosis signalling, through the overexpression of the apoptosis inhibitor, P35 (57, 208). Another study observed JNK activation in response to oxidative stress, induced by infection with the pathogenic bacteria, *Erwinia carotovora carotovora* 15 (Ecc15). Infection with this gram-negative bacterium led to the activation of the JNK and JAK-STAT pathways. This activation induced the proliferation of Intestinal Stem Cells following epithelial damage. Conversely, the same study observed that indigenous bacteria also activate the pathway, however, its activation only led to a basal level of proliferation (209). In addition to JNK activation in response to epithelial damage, studies have also shown that its signalling is correlated with the age-related deterioration of the intestinal epithelium. As the *Drosophila* intestine ages, an increase in Intestinal Stem Cell proliferation and aberrant differentiation is observed. This phenotype was found to be a consequence of an interaction between the JNK and Notch signalling pathways, with the deregulation of the fine-tuning of JNK pathway and Notch pathways, leading to tissue degeneration and tumour formation (210).

The Hippo signalling pathway also regulates the activity and differentiation of Intestinal Stem Cells. In the Intestinal Stem Cells, activation of the Hippo pathway has been shown to restrict the proliferation of these cells within the adult midgut, with loss of this activity promoting proliferation. The activation of proliferation in response to damage through the tissue damaging reagent, DSS, was shown to result from the translocation of the Hippo pathway mediator Yorkie (Yki) to the nucleus (105, 211), with loss of this signalling member perturbing the damage response (105, 212). This proliferation was

demonstrated to occur in part through the upstream factors, Fat and Ds (212). Additionally, upregulation of *Yki* in damaged Enterocyte cells, leads to the expression of the *upd* genes. These genes mediate the non-cell autonomous action of the JAK-STAT pathway in neighbouring cells, through the secretion of the ligands (105, 213). Some studies have described Hippo pathway signalling as a component of the response to infection or bleomycin-induced damage, however this remains unclear (183).

Despite an important role for Wnt signalling in maintaining the stem cell niche in the crypt of the mammalian intestinal system (214); within the *Drosophila* intestinal system, Wnt signalling defects result in mild phenotypes (183). Studies conducted by *Lin et al.*, demonstrated through clonal analysis, downstream components of the Wnt pathway such as Frizzled, Dishevelled and Armadillo, were autonomously required for Intestinal Stem Cell proliferation. Additionally, these effectors were found to be downstream of the Notch pathway. The source of this Wnt signalling was found to originate from the surrounding circular muscle forming an Intestinal Stem Cell niche (215). Further studies by *Lee et al.*, observed that loss of *Adenomatous polyposis coli (Apc)*, a downstream effector of the Wnt pathway, results in loss of midgut homeostasis and the formation of hyperplasia within the tissue. Further investigations determined that Apc signalling is required to regulate Intestinal Stem Cell proliferation but not differentiation (216).

A number of other signalling pathways have also been linked to the regulation of stem cell properties within the *Drosophila* intestinal system. The Insulin pathway has been shown to be required for Intestinal Stem Cell proliferation, following DSS treatment or

expression of pro-apoptotic proteins (168). Additionally, inhibition of the Insulin pathway and subsequent downstream PI3Kinase effectors, result in the delay of ageing phenotypes (217).

Furthermore, premature ageing phenotypes observed following the induction of oxidative stress, through either treatment with paraquat or loss of catalase function, is correlated with an increase in PDGF (VEGF in mammals) signalling within the midgut. Moreover, increased levels of the PVF2 ligand, resulted in increased proliferation of the Intestinal Stem Cells (218).

1.13. Thesis outline

Despite various studies demonstrating a role for the caspases in the regulation of the *Drosophila* intestinal system, most studies observe their activation in response to damaging stimuli (104). Furthermore, these roles appear to rely on the effector caspases (104), with the initiator caspases often overlooked.

The elucidation of a role for the apical-caspases is particularly pertinent, as studies have demonstrated that caspase-9 deregulation can contribute to the onset and manifestation of colon cancers (109). Furthermore, this caspase-deregulation has been shown to contribute to the differentiation state of the tumour (109), appearing in line with other reported non-apoptotic roles of the caspases (39). Understanding caspase-

deregulation within colon tumours is important for patients, as its deregulation in cancer is often correlated with a poor prognosis (109).

Using new powerful protein sensors and conditional knockout tools developed by the Baena-Lopez laboratory, in conjunction with the highly conserved *Drosophila* intestinal system, I explored the function of the caspase-9/2 homologue, Dronc. In this thesis, I observed non-apoptotic Dronc activity, in non-regenerative conditions (Chapter 3). This activation appeared important for regulating the proliferation and differentiation of intestinal precursors, and the prevention of hyperplasia. Furthermore, although the catalytic activity was required, the regulation of Dronc was independent of the canonical apoptotic pathway (Chapter 4). The activity of Dronc appeared specific for the Enteroblast cells, and this was correlated with its accumulation within the cell; with the activity of Dronc requiring the activity of both of the Notch and Insulin-TOR pathways (Chapter 5). Single-cell Drop-seq analysis determined the misexpression of a number of genes reported to be region and cell-specific. Furthermore, loss of *Dronc* within the intestinal system may result in microbiome compositional changes (Chapter 6). Collectively, these findings could aid in our understanding of the caspase regulation of the intestinal system, and the onset of cancer often attributed to its deregulation.

2. Material and Methods

2.1 Fly Stains and Experimental Genotypes

2.1.1 Fly Handling

Drosophila were maintained on media consisting of agar, water, malt, molasses, maize, yeast, soya, nipagin mix and acid mix (Oxford Food) at 25°C unless specified otherwise. Additionally, live yeast was added liberally to each tube before addition of *Drosophila* unless specified otherwise.

2.1.2 *Drosophila melanogaster* stocks

All Balancer chromosomes and markers have been previously described on flybase.org.

The complete list of *Drosophila* lines used in this thesis are listed in table 1.

Table 1: *Drosophila* lines used in this thesis

Figure	Genotype	Description
3-3, 3-4, 3-8	<i>w¹¹¹⁸ DBS-S-QF UAS-CD8-GFP QUAS-dTomato-HA;</i>	Apical-caspase activity reporter.
3-3	<i>w¹¹¹⁸ DBS-S-QF UAS-CD8-GFP QUAS-dTomato-HA / w¹¹¹⁸ Su(H)-LacZ;</i>	Apical-caspase activity reporter and the Enteroblast marker, Su(H)-LacZ
3-6	<i>w¹¹¹⁸ DBS-S-QF UAS-CD8-GFP QUAS-dTomato-HA; QUAS-Flp / Cyo; Actin > Stop > LacZ / TM6B</i>	Apical-caspase activity reporter with permanent labelling.

3-8, 4-2	<i>w¹¹¹⁸; esg-Gal4 UAS-mCD8-GFP / Cyo; UAS-HistoneRFP TubG80ts / TM6B</i>	ReDDM reporter
3-9	<i>w¹¹¹⁸; esg-Gal4 UAS-mCD8-GFP / UAS-P35; UAS-HistoneRFP TubG80^{ts} / UAS-P35</i>	ReDDM reporter overexpressing two copies of P35, under the regulation of the ReDDM reporter
4-2, 4-6, 4-10, 4-11, 4-12, 5-10, 6-2, 6-3, 6-4, 6-6, 6-7, 6-8, 6-10, 6-11	<i>w¹¹¹⁸; esg-Gal4 UAS-mCD8-GFP ; UAS-HistoneRFP TubG80ts Dronc^{KO} / SM6A-TM6B</i>	ReDDM reporter in a <i>Dronc</i> heterozygous background.
4-3	<i>w¹¹¹⁸; esg-Gal4 UAS-mCD8-GFP / QUAS-LacZ; UAS-HistoneRFP TubG80ts Dronc^{KO} / UAS-FLP FRT Dronc-GFP-APEX FRT QF</i>	ReDDM, <i>Dronc^{KO}</i> & <i>Dronc</i> -QF rescue cassette
4-6, 5-10 6-2, 6-3, 6-4, 6-6, 6-7, 6-8, 6-10, 6-11	<i>w¹¹¹⁸; esg-Gal4 UAS-mCD8-GFP / +; UAS-HistoneRFP TubG80ts Dronc^{KO} / UAS-Flp FRT Dronc-GFP-APEX FRT Suntag-HA-Cherry</i>	ReDDM, <i>Dronc^{KO}</i> & <i>Dronc</i> -GFP-APEX to <i>Suntag-HA-Cherry</i> Cassette Knockout
4-8	<i>w¹¹¹⁸; esg-Gal4 UAS-mCD8-GFP / +; UAS-HistoneRFP TubG80ts / UAS-Flp FRT Dronc-GFP-APEX FRT Dronc-Suntag-HA-Cherry</i>	ReDDM, <i>Dronc</i> -GFP-APEX to <i>Dronc-Suntag-HA-Cherry</i> Cassette
4-10	<i>w¹¹¹⁸; esg-Gal4 UAS-mCD8-GFP / +; UAS-HistoneRFP TubG80ts Dronc^{KO} / UAS-Flp FRT Dronc-GFP-APEX FRT Dronc-FLCAEA-Suntag-HA-Cherry</i>	ReDDM, <i>Dronc^{KO}</i> & <i>Dronc</i> -GFP-APEX to <i>FLCAEA-Suntag-HA-Cherry</i> cassette (a catalytically and enzymatically inactive version of <i>Dronc</i>)
4-10	<i>w¹¹¹⁸; esg-Gal4 UAS-mCD8-GFP / +; UAS-HistoneRFP TubG80ts Dronc^{KO} / UAS-Flp FRT Dronc-GFP-APEX FRT Dronc-ΔCAEA-Suntag-HA-Cherry</i>	ReDDM, <i>Dronc^{KO}</i> & <i>Dronc</i> -GFP-APEX to <i>FLCAEA-Suntag-HA-Cherry</i> cassette (a catalytically and enzymatically inactive version of <i>Dronc</i> , also lacking the CARD domain)
4-11	<i>w¹¹¹⁸; esg-Gal4 UAS-mCD8-GFP / UAS-P35; UAS-HistoneRFP TubG80^{ts} Dronc^{KO} / UAS-P35</i>	ReDDM reporter in <i>Dronc</i> heterozygous conditions overexpressing two copies of P35, under the regulation of the <i>esg</i> promoter.

4-11	<i>w¹¹¹⁸; esg-Gal4 UAS-mCD8-GFP / UAS-Drice RNAi UAS-Decay RNAi; UAS-HistoneRFP TubG80^{ts} Dronc^{KO} / UAS-Damm RNAi UAS-DCP1 RNAi</i>	ReDDM reporter in <i>Dronc</i> heterozygous conditions expressing RNAi against all effector caspases, under the regulation of the <i>esg</i> promoter.
4-12	<i>w¹¹¹⁸; esg-Gal4 UAS-mCD8-GFP / UAS-RHG miRNA; UAS-HistoneRFP TubG80^{ts} Dronc^{KO} / +</i>	ReDDM reporter in <i>Dronc</i> heterozygous conditions expressing a micro RNA against the pro-apoptotic factors Reaper, Hid and Grim, under the regulation of the <i>esg</i> promoter
5-2	<i>w¹¹¹⁸; + / QUAS-LacZ; TubG80^{ts} Dronc^{KO} Delta-Gal4 / UAS-FLP FRT Dronc-GFP-APEX FRT QF</i>	<i>Dronc^{KO}</i> & <i>Dronc</i> to QF rescue cassette, under the regulation of the <i>Delta</i> promoter
5-3	<i>w¹¹¹⁸; + / +; TubG80^{ts} Dronc^{KO} Delta-Gal4 / TM6B</i>	<i>Dronc^{KO}</i> heterozygous <i>Drosophila</i> with Intestinal Stem Cells labelled with GFP.
5-3	<i>w¹¹¹⁸; + / +; TubG80^{ts} Dronc^{KO} Delta-Gal4 / UAS-FLP FRT Dronc-GFP-APEX FRT Suntag-HA-Cherry</i>	<i>Dronc^{KO}</i> & <i>Dronc-GFP-APEX</i> to <i>Suntag-HA-Cherry</i> rescue cassette under the regulation of the <i>Delta</i> promoter
5-4	<i>DBS-S-QF UAS-CD8-GFP QUAS-dTomato-HA / +; + / +; Dronc^{Gal4} / TM6B</i>	DBS-S-QF reporter & <i>Dronc</i> transcriptional reporter
5-6	<i>w¹¹¹⁸; + / +; Actin-Dronc-GFP-MYC / TM6B</i>	Dronc-protein localisation reporter. The Dronc protein is tagged with a modified GFP and Myc tag.
5-6	<i>w¹¹¹⁸ / Su(H)-LacZ; + / +; Actin-Dronc-GFP-MYC / TM6B</i>	Dronc-protein localisation reporter in combination with the enteroblast marker, Su(H).
5-8	<i>w¹¹¹⁸ UAS-Notch RNAi; esg-Gal4 UAS-CD8-GFP / +; TubG80^{ts} UAS-Histone-RFP DroncKO / +</i>	ReDDM reporter in a <i>Dronc</i> heterozygous background overexpressing RNAi against Notch, under the regulation of the <i>esg</i> promoter.
5-8	<i>w¹¹¹⁸ UAS-Notch RNAi; esg-Gal4 UAS-CD8-GFP / +; TubG80^{ts} UAS-Histone-RFP DroncKO / UAS-FLP FRT Dronc-GFP-APEX FRT Suntag-HA-Cherry</i>	ReDDM, <i>Dronc^{KO}</i> & <i>Dronc-GFP-APEX</i> to <i>Suntag-HA-Cherry</i> knockout, under the regulation of the <i>esg</i> promoter. Under the same promoter, RNAi against Notch is expressed.
5-9	<i>w¹¹¹⁸; esg-Gal4 UAS-CD8-GFP / UAS-Notch Intra; TubG80^{ts} UAS-Histone-RFP DroncKO / +</i>	ReDDM reporter in a <i>Dronc</i> heterozygous background overexpressing the Notch

		intracellular domain, under the regulation of the <i>esg</i> promoter.
5-9	<i>w¹¹¹⁸; esg-Gal4 UAS-CD8-GFP / UAS-Notch Intra; TubG80ts UAS-Histone-RFP DroncKO / UAS-Flp FRT Dronc-GFP-APEX FRT QF</i>	ReDDM, <i>Dronc^{KO}</i> & <i>Dronc-GFP-APEX</i> to QF knockout under the regulation of the <i>esg</i> promoter. Under the same promoter, the Notch intracellular domain is expressed.
5-12	<i>w¹¹¹⁸; esg-Gal4 UAS-CD8-GFP / UAS-Insulin Receptor DN; TubG80ts UAS-Histone-RFP DroncKO / +</i>	ReDDM reporter in a <i>Dronc</i> heterozygous background overexpressing the dominant-negative form of the Insulin Receptor, under the regulation of the <i>esg</i> promoter.
5-12	<i>w¹¹¹⁸; esg-Gal4 UAS-CD8-GFP / UAS- Insulin Receptor DN; TubG80ts UAS-Histone-RFP DroncKO / UAS-Flp FRT Dronc-GFP-APEX FRT QF</i>	ReDDM, <i>Dronc^{KO}</i> & <i>Dronc-GFP-APEX</i> to QF under the regulation of the <i>esg</i> promoter. Under the same promoter, the dominant-negative form of the Insulin Receptor is expressed.

2.1.3 Generation of Fly lines used in this thesis

Details of the cloning techniques used to generate the DBS-S-QF reporter can be found (219), and for all other lines (220). Note, I did not generate any of the fly lines described in this thesis and this section is for reference only.

2.1.4 Experimental Fly Handling

Drosophila were crossed at 25°C. Subsequent progeny were collected for two days, before flipping into fresh media and maintained at 25°C. *Drosophila* were then flipped into fresh media every two days. Despite only female *Drosophila* being used for experimental study, both male and female flies were maintained until dissection in order to replicate homeostatic conditions.

For experiments where a TubG80^{ts} transgene was incorporated to repress the expression of constructs under the regulation of the UAS promoter, *Drosophila* were crossed at 18°C (131). Subsequent progeny were flipped into fresh media and maintained at 29°C to remove TubG80^{ts} repression. Experimental *Drosophila* were then flipped into fresh media every two days and dissected at 3- or 7-days post-temperature shift (131) [Figure 2-1] .

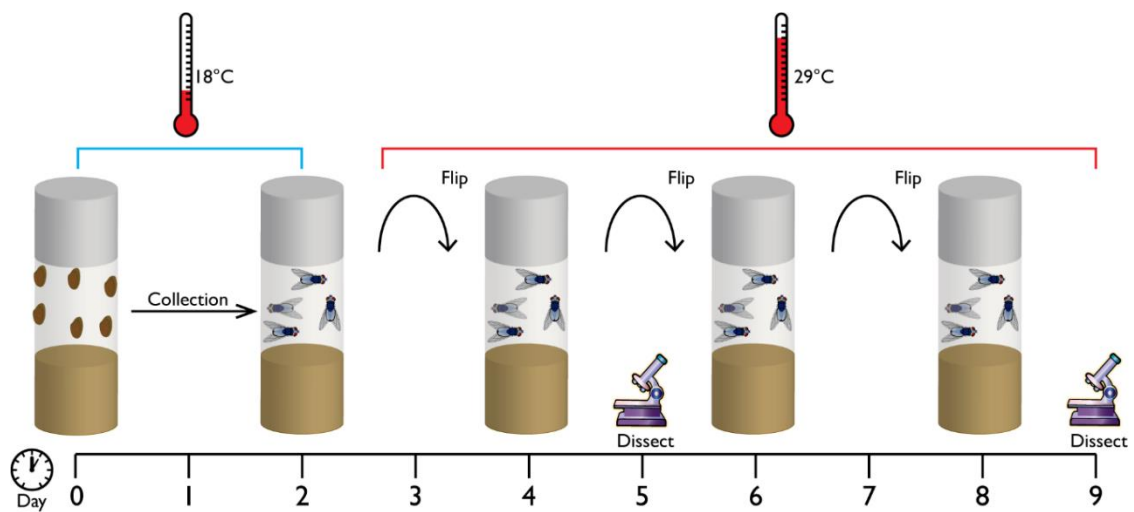


Figure 2-1: TubG80^{ts} Experimental Fly-Flipping Procedures

Drosophila were crossed at 18°C and the progeny collected for 2-days, before transfer to fresh media at 29°C. *Drosophila* were then flipped every two days to new media and dissected at 3- or 7-days post temperature shift (131).

2.1.5 Paraquat Fly Treatment

Drosophila were crossed at 25°C, or 18°C for experiments including a thermosensitive G80 construct (TubG80^{ts}), as discussed in section 2.1.3. Six days following collection or temperature shift, the *Drosophila* were dry starved for 4 hours. Subsequently, they were

left for 16 hours on a “Flug” (Dutscher Scientific Ltd) soaked in a solution of 5% sucrose (Sigma) and 6.5mM Paraquat (Sigma).

2.1.6 *Drosophila* Quick Mix (Blue) Preparation

Media was obtained from Blades Biological. 1g of dry media was added to an empty *Drosophila* vial and the food mixed with 3 ml of ddH₂O. The media was then pressed using a 15 ml falcon tube before being left for 60 seconds, as recommended by the manufacturer. *Drosophila* were subsequently added to the media as per the requirements of the experiment.

2.2 Dissection of *Drosophila* Intestines

Drosophila were anaesthetised using CO₂, with adult mated female flies of the required genotypes collected. Selected *Drosophila* were rapidly washed once in PBS (Sigma) and subsequently in PBT (PBS & 0.03% Triton). Fly intestines were then dissected in ice-cold PBS following the protocol outlined in (221) and the Malpighian tubules subsequently removed (156). The dissected intestines were transferred to a meshed basket submerged in fresh ice-cold PBS, in a well of a 24-well plate.

2.3 Fixation of *Drosophila* Intestines

The meshed basket containing the dissected intestines was removed from the ice-cold PBS. The basket was then submerged in wash solution (0.7% NaCl, 0.05% Triton X-100, ~90°C) for 6 seconds before being immersed in a well containing ice-cold wash solution [Figure 2-2]. The Intestines were then sequentially washed in a well containing 1x PBS and then 3 wells containing PBT (1x PBS, 0.03% Triton X-100).

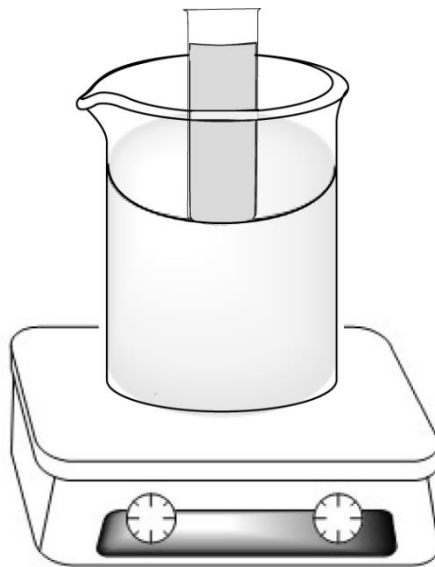


Figure 2-2: “Double-Boiler” intestinal fixation set-up

A large beaker is placed on top of a hotplate and filled with wash solution. A smaller beaker is submerged within the larger beaker and filled with wash solution. This smaller beaker is suspended within the wash solution using a supporting device. When the wash solution in the larger beaker begins to boil, the temperature within the smaller beaker is approximately 90°C. Baskets containing intestines for fixation can then be immersed in the hot wash solution using forceps.

2.4 Immunofluorescence

2.4.1 Primary Antibodies

Fixed intestines were blocked in 1% BSA PBT (1x PBS, 0.03% Triton X-100) for at least an hour. Primary antibodies were diluted in the blocking solution and incubated overnight at 4°C, on a 4-way rotator. The following primary antibodies used were:

Table 2: Primary Antibodies used in this thesis.

Protein	Concentration	Species	Catalogue Number
Armadillo	1:50	ineints	Developmental Studies Hybridoma Bank, N2 7A1, armadillo-c
Prospero	1:20	Mouse	Developmental Studies Hybridoma Bank, MR1A
HA	1:1000	Rabbit	Cell Signalling, C29F4
LacZ	1:500	Chicken	Abcam Ab9361
GFP	1:200	Goat	Abcam ab6673
P35	1:100	Rabbit	Novus Biologicals NB100-56153SS
Pdm1	1:2000	Rabbit	Gift from Yu Cai

2.4.2 Secondary Antibodies

Following primary antibody incubation, the intestines were washed 3x for at least 10 minutes each time in PBT (1x PBS, 0.03% Triton X-100). Secondary antibodies were diluted in the blocking solution (see 2.4.1) and incubated with the intestines for 2 hours at room temperature, on a 4-way rotator. The following secondary antibodies were used:

Table 2: Secondary Antibodies used in this thesis.

Protein	Concentration	Species	Catalogue Number
DAPI	1:10,000	N/A	Thermo Scientific 62248
Alexa-488 anti-Mouse	1:200	Donkey	Life Technologies A21202
Alexa-555 anti-Rabbit	1:200	Donkey	Life Technologies A31572
Alexa-647 anti-Chicken	1:200	Goat	Life Technologies A21449
Alexa-488 anti-Goat	1:200	Donkey	Life Technologies A1105
Alexa-647 anti-Rabbit	1:200	Donkey	Life Technologies A31573
Alexa-647 anti-Chicken	1:200	Donkey	Jackson 703-605-155

2.4.3 Mounting of Drosophila intestines

Following secondary antibody incubation, the intestines were washed 3x for at least 10 minutes each time in PBT (1x PBS, 0.03% Triton X-100). Using a P1000 pipette with a widened tip, the intestines were removed from the Eppendorf tubes and deposited onto a Poly-L-lysine slide (Poly-Prep Slides, Sigma P0425). The intestines were then separated using dissecting forceps, and most of the PBT solution removed using a pipette. The intestines were subsequently arranged on the slide. Once arranged, the slide was inverted 90° and the remaining PBT solution wiped away with a cloth. Antifade mounting media with DAPI (Vectashield) was then liberally applied to the slide using a pipette, before a coverslip (Marienfield High Precision No. 1.5) was placed on top. Nail varnish was used on two sides to secure the coverslip to the slide, and was left to dry. All slides were stored at 4°C.

2.4.4 Confocal Microscopy

All confocal microscopy was performed using an Olympus FV1200 scanning confocal microscope. Imaging was performed using either the 10x or 40x objective lenses, and all intestines were acquired in the same orientation, using the dense band of cells in the R5/hindgut region as a reference. Z-stacks were taken with a step size of $0.28\ \mu\text{m}$ from the basal membrane into the intraluminal space to achieve a representative image of the intestinal structure.

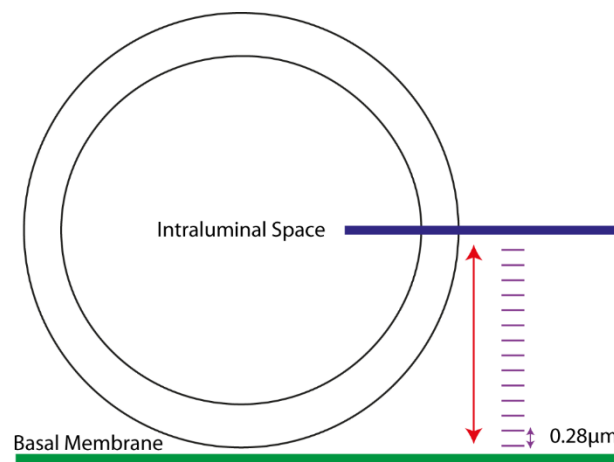


Figure 2-3: Representation of intestinal confocal Z-stack imaging.

Black circles define the borders of the intestinal cells surrounding the Intraluminal Space. Confocal Z-stack images were taken from the basal membrane (green line) into the intraluminal Space (blue line) shown by the red arrow. The Z-stack images were taken at $0.28\ \mu\text{m}$ steps, illustrated by the purple lines.

2.5 Image Processing

Images were processed in the ImageJ2 / Fiji image analysis software (222, 223).

2.5.1 Presentation of Images

Representative focal planes of Images were projected using the *Stk > Z Project...* function. Generally, colour was ascribed to the channel depending on the Alexa flour utilised (i.e 405 = blue, 488 = green, 555 = red, 647 = grey). The colour was then enhanced using the *Image > Adjust > Colour Balance...* function, ensuring the image remains representative. In composite images, the scale bar was included using the *Analyze > Tools > Scale Bar...* function.

2.5.2 DBS-S-QF Heat Maps

Representative focal planes (as described in Figure 2-3) of the DBS-S-QF images were projected using the average intensity function (*Stk > Z Project...*) and the channels split (*Image > Color > Split Channels*). The DBS-S-QF channel was selected and saved, all other information was discarded. Once all of the images had been processed, the DBS-S-QF channels were reloaded and combined into a new Z-stack of projections (*Image > Stacks > Images to Stack*). An average intensity projection was applied (*Stk > Z Project...*) and the “16 colors” LUT was applied (*LUT > 16_colors*).

2.6 Quantification of Images

Images were either quantified using ImageJ macros (automated quantification) or manually quantified. Automated quantification was the preferred method; however, when this was not possible, the image was quantified manually or discarded.

2.6.1 Automated Quantification

Automated quantification was used to quantify either cell size, nuclear size, cell number or nuclear number. The image was loaded, and the channels split. The channel of interest was then projected using the “Max Intensity” setting. The image was then thresholded using the automatic setting, and the “Despeckle” function was used to reduce noise. Subsequently, the “Watershed” function was used to separate objects inadvertently joined by the image processing. The “Analyse Particles...” function was then used to count both the number and the size of all the objects in the image that passed through the size filter. This was repeated for all images, and the subsequent data was saved.

2.6.2 Manual Quantification

Manual quantification was used to quantify the number of cells or cell nuclei when automated quantification was not possible. The image was loaded, and the focal planes of the Images were projected using the *Stk > Z Project...* function. The counting was performed using the *Plugins > Analyse > Cell Counter* plugin.

2.7 Cell Dissociation

Drosophila were anaesthetised using CO₂ and female flies of the required genotypes were collected. The selected *Drosophila* were rapidly washed once in PBS (Sigma) and subsequently in PBT (PBS & 0.03% Triton). Fly intestines were then dissected in ice-cold PBS following the protocol outlined in (221) and the Malpighian tubules subsequently removed (156).

The intestines were subsequently dissociated using the following protocol adapted from (224). The intestines were transferred to a 1.5 ml Eppendorf tube containing ice-cold PBS-DEPC (150µl). The DEPC-PBS was removed and replaced with 100 µl of elastase solution (1 mg/ml) (Sigma E0258-5MG), diluted in cell dissociation buffer (Gibco 13151-014). The intestines and solution were incubated for 1 hour at 27°C on an Eppendorf Thermoblock, shaking at 1500rpm. The intestines were agitated every 15 minutes using a P200 pipette with a widened tip, pipetting up and down at least 30 to 40 times to fully dissociate the tissue. The mixture was subsequently centrifuged for 20 minutes at 300 *x g* at 4°C. The supernatant was discarded, and the pellet resuspended in fresh ice-cold DEPC-PBS.

2.8 Single Cell Drop Sequencing

The methodologies for the Single-Cell Drop Sequencing technique (Drop-Seq) can be separated into the Drop-seq procedure and subsequent alignment and Bioinformatic analysis.

2.8.1 Drop-Seq Procedure

The *Drosophila* intestinal cells were dissociated using the protocol described in 2.7. The Drop-seq procedure was originally described in (225) with an adapted version of the protocol undertaken, in collaboration with Vincent Croset, Christoph Treiber and Scott Waddell described in (226). Using a Dolomite Bio (Royston, UK) Single-Cell RNA-Seq system. Dissociated cells were diluted to a concentration of 200 cells per microlitre into a PBS and BSA (0.01%) solution. The cells were subsequently passed through a 100 µl filter. Barcoded beads (SeqB), were diluted to a concentration of 200 beads per microlitre into a solution consisting of 200 mM Tris pH 7.5, 6% Ficoll PM-400, 0.2% Sarkosyl, 20 mM EDTA and 50 mM DTT.

For each Drop-Seq run, 700µl of the diluted cells were loaded into a microcentrifuge tube inside of a reservoir connected to a Mitos P-Pump (Dolomite microfluidics, 3200016), providing a constant and regulated flow of 30µl per minute. This reservoir was placed into a stirring plate containing a stir bar which provides agitation to the solution. Importantly, the stir bar was placed inside of the reservoir but outside of the tube containing the cells in suspension, to avoid damage to the cells. 600µl of the

solution containing the beads were loaded into a 50cm sample loop connected to the second Mitos P-Pump, providing a constant and regulated flow of 30µl per minute. This loop was required to avoid the sedimentation of the beads, in addition to eliminating the requirement for stirring, thus avoiding damage to the beads. QX200 Droplet Generation Oil for EvaGreen (BioRad, 1864006) was loaded inside the third Mitos P-Pump set to provide a regulated and constant flow of 200 microlitres per minute. All three flows from the cells, beads and oil were connected to a Single Cell RNA Seq Droplet Chip (Dolomite Bio) as recommended by the manufactures' instructions. This configuration allows the formation and paring of single-cells, with single-beads, within the 357pl droplets of oil containing aqueous cell/bead solution. To assess and facilitate the formation of the oil, the chip was positioned under a Meros High-Speed Digital Microscope and Camera with an HLB M Plan Apo 5X objective. Droplets of cells, beads and oil were collected in 50 ml Falcon tubes. Reagents were reloaded, and falcon tubes were replaced at intervals of every 15 minutes (226).

Subsequently, droplets in the Falcon tubes were broken, and the beads with captured mRNA were washed as described in (225). mRNA bound to beads was reverse transcribed immediately using Template Switch Oligo (5' – AAG CAG TGG TAT CAA CGC AGA GTG AAT rGrGrG – 3') and Maxima H Minus Reverse Transcriptase (Thermo Scientific, EP0753). The consequent cDNA was treated with Exonuclease I (NEB, M0293L) and amplified using multiple 50µl PCR reactions completed on aliquots of around 2000 beads, using a SMART PCR primer (5' – AAG CAG TGG TAT CAA CGC AGA GT – 3') and Hifi HotStart Readymix (Kapa Biosystems, KK2602). A total of 17 cycles was

used, and 10µl from each of the PCR reactions was pooled. The amplified cDNA was purified twice with 0.6X and 1.0X volumes of Agencourt AMPure XP beads (Beckman Coulter, A63880). The cDNA was then quantified on a Bioanalyzer, utilising a High-Sensitivity DNA kit (Agilent, 5067–4626). From each of the samples, two aliquots of 600pg of amplified cDNA were tagged using the Nextera XT DNA Sample Preparation Kit (Illumina, FC-131–1024), with New-P5-SMART PCR hybrid (5' – AAT GAT ACG GCG ACC ACC GAG ATC TAC ACG CCT GTC CGC GGA AGC AGT GGT ATC AAC GCA GAG T*A*C – 3') and one of the Nextera N701 to N706 oligos. The cDNA libraries were purified twice, with 0.6X and 1.0X volumes of Agencourt AMPure XP beads (Beckman Coulter, A63880) and quantified again using a Bioanalyzer, utilising the High-Sensitivity DNA kit (Agilent, 5067–4626). The libraries were pooled and sequenced using an Illumina HiSeq2500 sequencer using a custom Read1 primer (5' – GCC TGT CCG CGG AAG CAG TGG TAT CAA CGC AGA GTA C – 3') and standard Illumina Read2 primers. All of the oligos were ordered and produced by Sigma and were HPLC purified.

2.8.2 Data Processing and Bioinformatics

The following processing of the Drop-seq data was achieved using the Drop-seq software tools and Seurat tools, and the protocols described in (225-227). The sequencer gathers data from both reads of the read pair. Read one, termed the barcoded read, contains the cellular and molecular barcodes, which will later classify the read as originating from a transcript of a particular cell. Read two denotes the biological read, containing a proportion of the sequence pertaining to the transcript.

As a first step, both of these files were combined into one file. Following the Drop-seq computational protocol v1.0.1 (<http://mccarrolllab.org/wp-content/uploads/2016/03/Drop-seqAlignmentCookbookv1.2Jan2016.pdf>) and the Drop-seq software tools (v1.13) from the McCarroll lab, I proceeded to process the data. Initially, the first 12 bases of the barcoded read containing the cell barcode were tagged for future reference. The next 8 bases contained the molecular barcode which were also tagged for subsequent analysis. The information was then converted into single-ended reads, where barcoded reads with low-quality bases were purged, in addition to their corresponding genome reads. Following this, the 5' adapter sequence and the 3' poly-A tails were detected and trimmed from the sequence, this resulted in a cleaned but unaligned file.

Next, these sequencing reads were aligned to the genome in order to determine which genes the reads belonged to using STAR (228). The genome file contains the sequences for all genes in the *Drosophila* genome (*Drosophila melanogaster* genome release 6.13 from Flybase.org) in addition to reference sequences pertaining to *Gal4*, *CD8-GFP*, *HistoneRFP*, *TubG80ts*, *FLP Dronc-Suntag-HA-Cherry* and *Suntag-HA-Cherry* transgenes. Following alignment, the reads were merged with the information from the previously described cell barcode and molecular barcode tags, before being converted into a file compatible for the next stage of processing (225). Both the control (*Dronc* heterozygous) and the *esg*-induced *Dronc* knockout intestines were processed separately using this method.

The data was subsequently analysed using Seurat, an R-based toolkit for single-cell genomics (227, 229). Both the *Dronc* heterozygous and the *esg*-induced *Dronc* knockout datasets were imported into this toolkit. As a next step, the data for cells which contained less than 5 genes, or more than 1000 genes were purged, to remove low-quality data or discard droplets which may have contained multiple cells, respectively. The data was then normalised and scaled to ensure comparability between the two data sets. Next, I ran canonical correlation analysis (CCA) to identify the common sources of variation between the two samples; this also has the advantage of merging both data sets into a single file. Next, I aligned the CCA subspaces, reducing the dimensionality of the data allowing for downstream analysis, such as clustering. Subsequently, I ran t-SNE analysis to reduce the dimensions of the data into a 2D plot of the identified clusters within the data. The FindConservedMarkers function was used to identify canonical cell marker genes which were conserved across the two experimental conditions. The conserved markers from the FindConservedMarkers function was inserted into Fly-Gut seq tool (150) (<http://flygutseq.buchonlab.com/>) in order to assign cluster identity. To identify differential expression of genes between the two conditions, I used the FindMarker function and analysed genes which appeared to exhibit a log-fold change of 1.5 or above.

2.8.3 Metataxonomic analysis

Using the Kraken2 metagenomic sequence classification tool (230), I first built the database, including bacterial, plasmid, viral, human, fungi, plant and protozoa libraries.

Subsequently, I classified the sequences of the unaligned but trimmed drop-seq datasets, pertaining to the *Dronc* heterozygous and the progenitor cell *Dronc* knockout conditions. The resultant taxa with their corresponding read count were converted to a comma-separated value (.csv) file for manipulation in Microsoft Excel, before figure production in Graphpad Prism.

2.9 FACS sorting of Intestinal Progenitor Cells.

Intestinal cells were dissociated using the protocol outlined in section 2.2. The cells were diluted in PBS; before FACS sorting using a BD FACS Aria III in the Dunn School Flow Cytometry Facility by Michal Maj, using a 4-way purity mask, 100 µm Nozzle and 20 psi sheath pressure. Cells which were positive for GFP but DAPI negative (to exclude dead cells) were collected in a fresh Eppendorf tube for later processing.

2.10 Quantitative PCR

Adult mated female *Drosophila* intestines were dissected in ice-cold PBS using the protocol outlined in 2.2. Subsequently, the intestines were transferred to autoclaved Eppendorf tubes containing 350 µl of RLT Buffer Plus supplied from the RNeasy Plus Micro Kit (QIAGEN Cat. No 74034), supplemented with 2-mercaptoethanol (Sigma-Aldrich M6250) at a final concentration of 1% v/v. Using a new 1.5 ml pestle (Kimble 749521-1590) for each sample, I homogenised the samples within the Eppendorf tube. Next, the RNA was extracted using the protocol and solutions supplied with the RNeasy

Plus Micro Kit. The quantity of RNA obtained from each of the samples were assessed twice using the Nanodrop Lite Spectrophotometer (Thermo Fisher Scientific) and the average quantity determined. 500ng of the RNA was synthesised to cDNA (Thermo Fisher Scientific Maxima First Strand cDNA Synthesis Kit – K1671).

For qPCR of FACS sorted Intestinal progenitor cells, I first dissected the adult mated female *Drosophila* intestines following the protocols outlined in 2.2. The cells were dissociated, and FACS sorted following the methods described in sections 2.7 and 2.9. Following sorting, the collected cells were centrifuged for 20 minutes at 300 x *g* at 4°C, and the supernatant removed. Subsequently, 350µl of RLT Buffer Plus supplied from the RNeasy Plus Micro Kit (QIAGEN Cat. No 74034) supplemented with 2-mercaptoethanol (Sigma-Aldrich M6250) at a final concentration of 1% v/v, was added to the pelleted cells. The cells were resuspended and lysed through pipetting up and down using a P1000 pipette. The quantity of RNA in each of the samples was assessed twice using the Nanodrop Lite Spectrophotometer (Thermo Fisher Scientific) and the average quantity determined. 1ng of the RNA was synthesised to cDNA (Thermo Fisher Scientific Maxima First Strand cDNA Synthesis Kit – K1671) and the cDNA amplified using SSO Advanced Pre-Amp Supermix (BIORAD, 172-5160), using qPCR primers for the genes of interest and following the manufacturers' instructions.

qPCR was performed using reagents and protocols from the QIAGEN QuantiTect SYBR Green PCR Kit (Cat No./ID: 204145) and using the QIAGEN Rotor-Gene Q. Each of the reactions were completed in duplicate. Following the end of the qPCR reaction, the

results were quantified. For biological replicates included within the same qPCR run, the average of the duplicates were taken. The Ct of the internal control (e.g Rpl32) was subtracted from the condition tested (ΔCt). These results were then exported for statistical analysis.

For biological replicates tested over different qPCR runs, a dilution curve was included to account for variances within each run. Dilution curves were established through an initial 1/10 dilution of the cDNA, followed by 4 subsequent 1/5 dilutions. The Ct values obtained were plotted against the $\log_{10}$ of the dilutions and the slope of the curve established. The efficiency of the reaction was calculated using the ThermoFisher Scientific qPCR efficiency tool (<https://www.thermofisher.com/uk/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/qpcr-efficiency-calculator.html>). To account for these variances, the primer efficiency was multiplied by the power of the corresponding Ct, for each condition ($\text{Primer efficiency}^{Ct}$). Subsequently, the Ct of the internal control was subtracted from the condition tested ($\text{Primer efficiency}^{\Delta Ct}$).

The following qPCR primers were used in this thesis:

Table 4: qPCR primers used in this thesis

Gene	Forward Primer (5' – 3')	Reverse Primer (5' – 3')	Reference
<i>Rpl32</i>	ATGCTAAGCTGTCGCACA AAT	GTTTCGATCCGTAACCGAT GT	(231)
<i>FAD2</i>	TCTTCGCTATATGGAGTC TGGC	GTGCGATGAGCCCACAAA C	PP36363 (232)
<i>E(spl)α</i>	GCAGGAGGACGAGGAG GATG	GATCCTGGAATTGCATGG AG	(231)
<i>E(spl)β</i>	AGCCCACCCACCTCAAC	AGAAGTGAGCAGCAGCC	(231)
<i>E(spl)m3</i>	AGCCCACCCACCTCAAC	GTCTGCAGCTCCATTAGT C	(231)
<i>Cyp4g1</i>	TGGAAGTAGTTCAGGAG ACGC	GGTGCCCACTAAGGTGGT T	PP10878 (232)
<i>αTry</i>	ATGGTCAACGACATCGCT GT	CTGGCTCTGGCTAACGAT GT	PD44223 (232)
<i>Amy-p</i>	GCTGCAGTACCTGACCAA CT	TTGTAGGTCAGAACGTCG GC	PD40007 (232)
<i>βTry</i>	ATGGTCAACGACATCGCT GT	TGACATAACGCAGCTGGG AG	PD44297 (232)
<i>Ehbp1</i>	CAGTGCCCGACAATCACA C	TTCGATGACAAACGTCCA GTC	PP31286 (232)
<i>Drs</i>	CTGGGACAACGAGACCT GTC	ATCCTTCGCACCAGCACTT C	PD40133 (232)
<i>Mal-A1</i>	AGGACTTTGAGCGAATG ATCG	ACTTGAGTGGTTTGGTAC GAAG	PP35697 (232)
<i>Mal-A6</i>	GCAGGGCATCATTTCCAA GTT	TCCGGCTGAATATCGAAG AAGT	PP18770 (232)
<i>Mal-A7</i>	ATCCTAGCTGGTCATGTG AAACC	GCTGCCGTAACCTCCAGA T	PP25826 (232)
<i>Yp1</i>	CAACTCCGTCAACCAGGC ATT	GACAGGTGGTAGACTTGC TGC	PP16750 (232)
<i>Yp2</i>	GCACCCTTTGCGTTATGG C	TAGAGCTTGTCCAACAGC GTA	PP11504 (232)
<i>Yp3</i>	AATGACCGACTGAAGCC GAC	TGGACTTGATAATCCAGA CGGG	PP13290 (232)
<i>Amy-d</i>	GCATAGTGTGCCTCTCCC TC	TACGACCGGATGCGTAGT TG	PD40005 (232)
<i>Jvl</i>	CTTTTCCCGACCTCAAGC ACA	GCCACCTACCGAGTGACT G	PP14864 (232)

<i>Jon65Aiii</i>	AACACCTGGGTTCTCACT GC	TCAGGGAAATGTCGTTCC TC	(233)
<i>Nora Virus</i>	AACCTCGTAGCAATCCTC TCAAG	TTCTTGTCCGGTGTATCCT GTATC	(234)
<i>Actin5c</i>	GGCGCAGAGCAAGCGTG GTA	GGGTGCCACACGCAGCTC AT	(235)
<i>Lactobacillus fructivorans</i>	AACCTGCCCAGAAGAAG GGGA	GCGCCGCGGATCCATCCA AA	(236)
<i>Lactobacillus brevis</i>	GCAGTTGCCGAGGTCCA A	CCAACGCATTTTCAGCAT CA	(237)
<i>Lactobacillus plantarum</i>	TGTCTCAGTCCCAATGTG GCCG	GGCTATCACTTTTGGATG GTCCCGC	(236)
<i>Acetobacter tropicalis</i>	TAGCTAACGCGATAAGC ACA	ACAGCCTACCCATACAAG CC	(236)
<i>Acetobacter pomorum</i>	TGTTTCCCGCAAGGGACC TCT	AGAGTGCCCAGCCCAACC TGA	(236)

2.11 Statistical Analysis

Microsoft Excel was used for the basic preparation of the data. Collated data was subsequently entered into Graph Pad Prism (Version 8). The “identify outliers” analysis was used, using the ROUT method and a Q value of 1% (All N numbers presented in this thesis are prior to this analysis). The “cleaned” data was subsequently tested for normality using the D’Agostino-Pearson omnibus normality test, excluding qPCR data which was analysed using the Shapiro-Wilk normality test. The statistical analysis test used for each of the experiments can be found in the figure legends. The P-Value format used is as follows: ns = $P > 0.05$, * = $P \leq 0.05$, ** = $P \leq 0.01$, *** = $P \leq 0.001$ and **** = $P \leq 0.0001$.

2.12 Thesis & Figure Production

The confocal images displayed in this thesis were first prepared using the Fiji imaging software as described in 2.5.1. The graphs displayed in this thesis were prepared using Graph Pad Prism (Version 8). All subsequent preparations of the figures were completed using Adobe Illustrator CC 2017 or PerkinElmer ChemDraw 18.1. The thesis was written using the Microsoft Word with the references managed by Endnote X9.

3. Non-apoptotic caspase activation occurs within in the *Drosophila* Intestinal System.

3.1 Results

Reproducible apical-caspase activity is observed using the DBS-S-QF reporter

Using the model organism: *Drosophila melanogaster*, I attempted to gain mechanistic insights into the role of the apical-caspases in the regulation of the *Drosophila* intestinal system. In addition to discovering the possible deregulated signalling process relating to the loss of *caspase-9* observed in some colon carcinomas (109).

Initially, before investigating a functional role for the *Drosophila* homologue of caspase-9/2, Dronc, I sought to determine whether I could observe activation of Dronc within the *Drosophila* Intestinal System. To that end, I used a Dronc activity reporter, DBS-S-QF, previously developed in the laboratory (219).

The DBS-S-QF reporter makes use of the downstream processing steps of the effector caspase, drICE, following Dronc activation (219). Before becoming fully activated, drICE undergoes two successive stages of enzymatic processing; the first cleavage event is predominantly mediated by initiator caspases, such as Dronc (57). Following this initial cleavage event, the resulting two subunits (termed large and short) remain bound, establishing its enzymatic activity (57). The DBS-S-QF reporter exploits these processing

steps, producing a reporter of initiator caspase activity, using a truncated template of drICE as a backbone. This truncated template contains a Cysteine to Alanine substitution at position 211, rendering the protein enzymatically inactive (and thus unable to initiate the apoptosis process). The protein, however, remains cleavable by apical-caspases. Furthermore, all but 16 of the amino acids were removed downstream from the Dronc cleavage site, and replaced with the sequence for the *QF* transcriptional activator. Thus preventing the physiological binding of the small and large subunits (57, 219, 238). Upstream, the CD8 transmembrane domain was appended to the N-terminus. The construct was then subcloned downstream of the ubiquitous *Actin* promoter (219) [Figure 3-1].

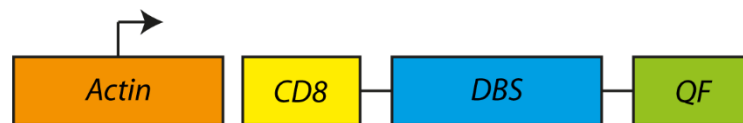


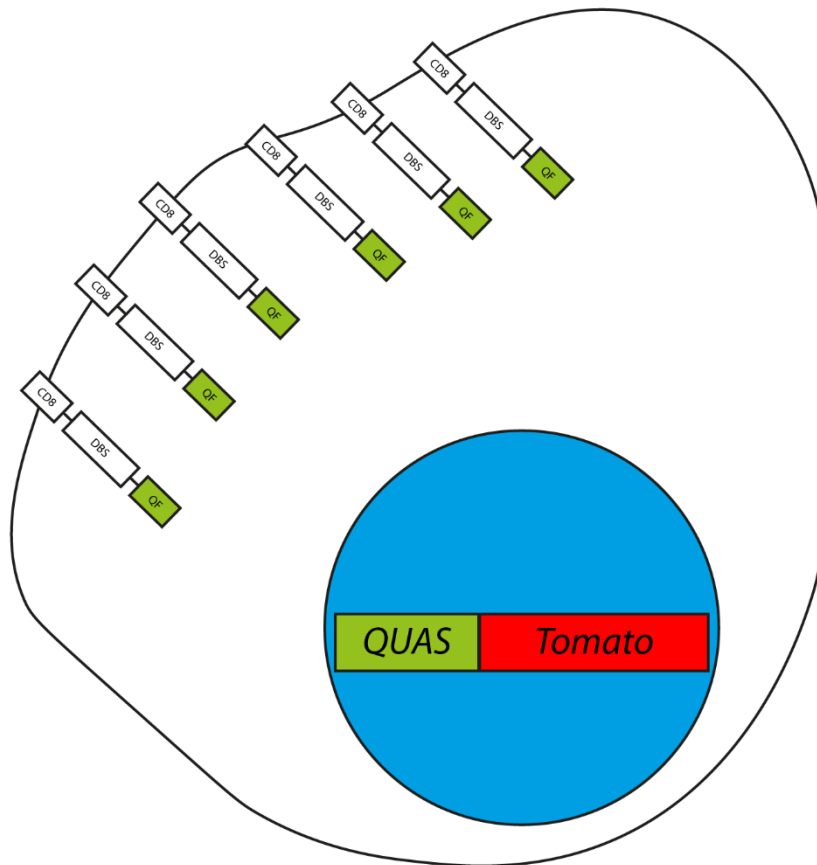
Figure 3-1: Rational Design of the DBS-S-QF Reporter

The catalytically inactive, but still cleavable truncated template of the drICE protein (DBS) is flanked at the C-terminus by the QF transcriptional activator, and at the N-terminus by the CD8 transmembrane domain. The construct was subcloned downstream of the *Actin* promoter resulting in its ubiquitous expression throughout the fly.

In the same genetic background, a HA-tagged Tomato fluorescent protein was included under the regulation of the *QUAS* promoter (109, 239). When apical-caspases are not active, the CD8 transmembrane domain tethers the construct to the cellular membranes of the cell, impeding the QF translocation to the nucleus. Thus the QUAS

promoter remains inactive, and the tomato fluorescent protein is not expressed (239) [Figure 3-2 A]. Following activation of the apical-caspases, the DBS fragment of the construct is cleaved. This cleavage event releases the QF fragment, allowing its translocation to the nucleus, where it binds to the QUAS promoter and activates the expression of the HA-tagged tomato fluorescent protein (219, 240) [Figure 3-2 B].

A



B

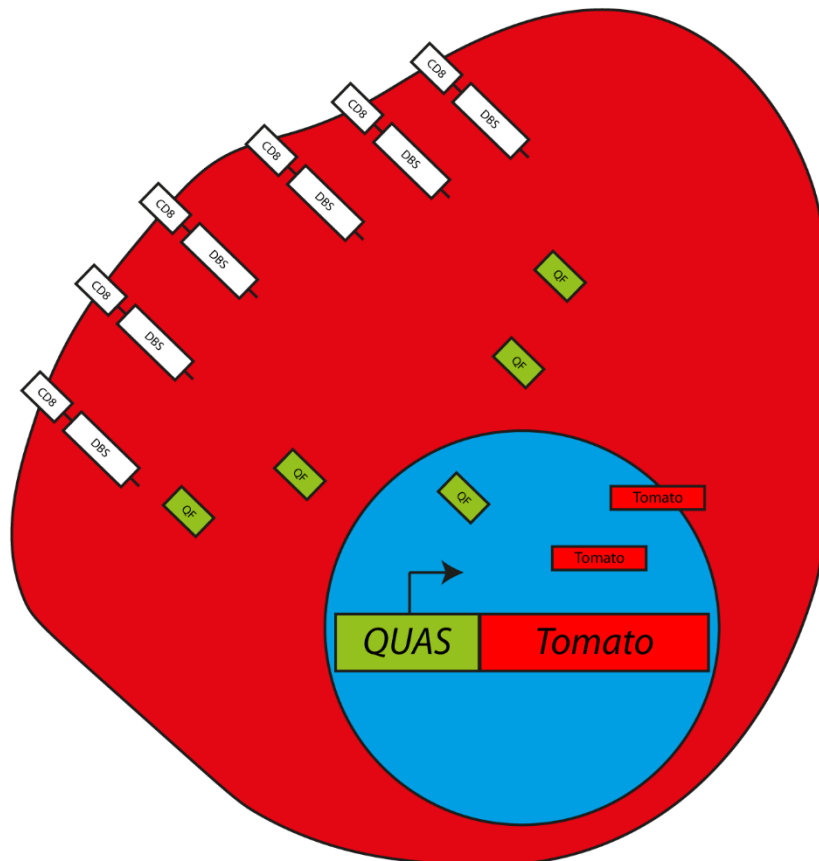


Figure 3-2: Schematic of the DBS-S-QF in apical-caspase inactive and activated conditions.

(A) When apical-caspases are inactive, the CD8 domain tethers the reporter to the inner cellular membranes, preventing the QF translocating to the nucleus and activating the expression of tomato. (B) When apical-caspases are active, they cleave the DBS segment of the construct. Thus, allowing the QF transcription factor (green square) to translocate to the nucleus (blue circle) where it binds to the QUAS promoter, activating the expression of the HA-tagged tomato fluorescent protein. The tomato fluorescent protein following translation in the ribosome, diffuses into the cytoplasm, allowing detection via fluorescent microscopy (219, 239).

As a first step, using the DBS-S-QF reporter, I sought to determine whether I could observe activation of the apical-caspases within the *Drosophila* intestinal system. I elected to use adult female *Drosophila* rather than males, as studies have shown that female Intestinal Stem Cells divide more readily than males, thus facilitating future experiments into their dynamics (190). Using the R4/R5 region of the posterior midgut, a well-studied and easily locatable area analogous to the mammalian small intestine (137), I was able to observe widespread caspase activation [Figure 3-3 A]. Using a cell membrane marker (Armadillo) and a nuclear marker for Enteroendocrine Cells (Prospero) (241), I was able to observe activation of the apical-caspases in all of the cell types present in the intestinal system (Intestinal Stem Cells, Enteroblasts, Enteroendocrine cells and Enterocytes) [Figure 3-3 B]. To establish whether there was preferential activation of the DBS-S-QF reporter (and thus apical-caspase activation), in any of the aforementioned cell types, I quantified the number of the cell types activating the reporter within the Intestine. Divergent from previously published effector caspase reporters where caspase activity is observed almost exclusively in enterocytes (242, 243); the DBS-S-QF reporter appeared most activate in the Enterocyte and Enteroblast

cell populations, with substantially less Intestinal Stem Cells and the Enteroendocrine Cells being labelled with the reporter. Furthermore, this difference in the subpopulations of cells activating the reporter appeared statistically significant [Figure 3-3 C].

As the DBS-S-QF reporter activation within the Enteroblast population was not observed with other effector caspase reporters (242, 243), I sought to confirm this observation using the Enteroblast specific marker, Su(H) (165, 179). Using the transcriptional reporter, *Su(H)*-LacZ, in combination with the DBS-S-QF reporter, I observed a 60.96% overlap between cells positive for both reporters, confirming the previous observations [Figure 3-3 E-G].

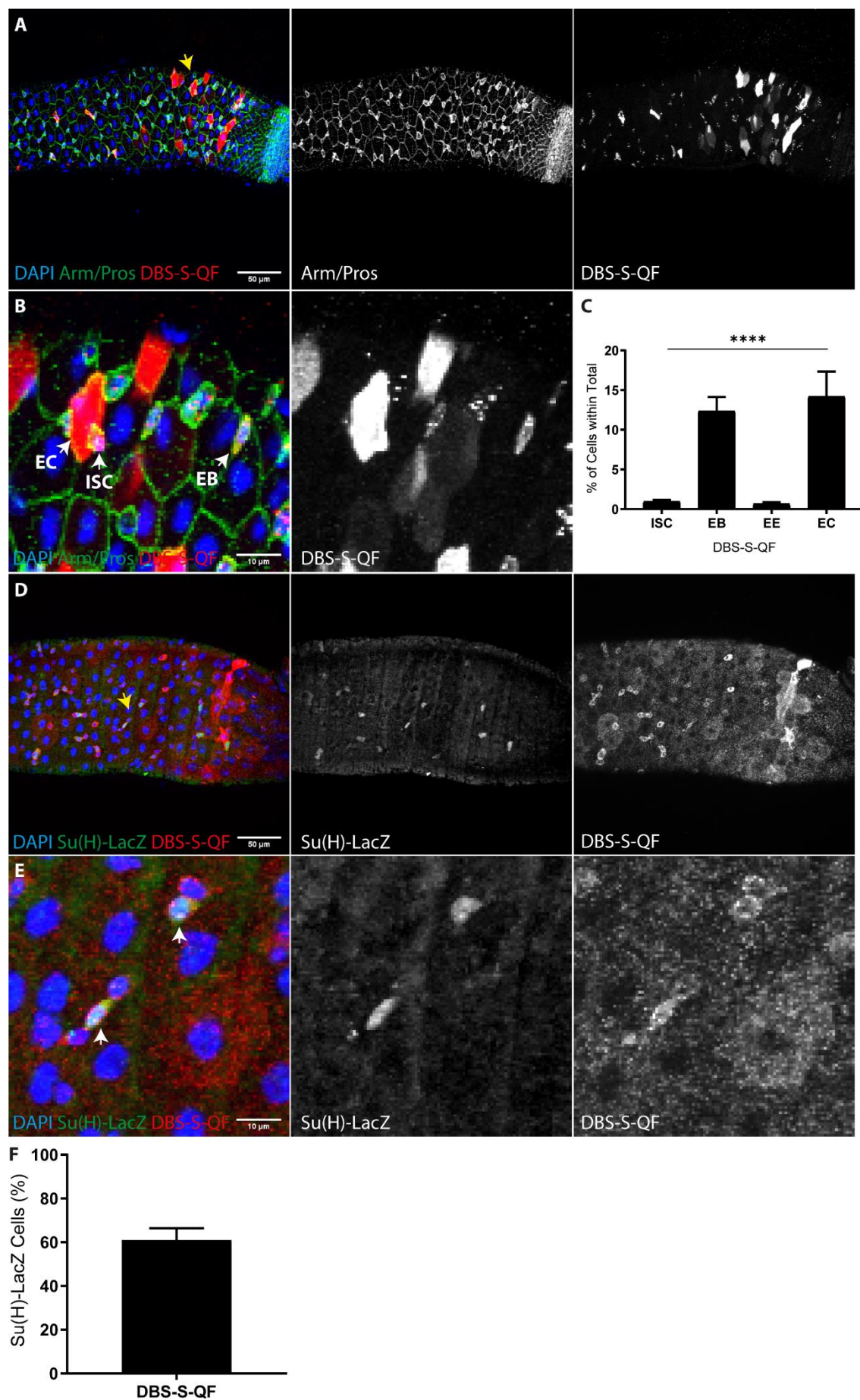


Figure 3-3: DBS-S-QF reporter activity is observed in the *Drosophila* intestinal system

(A) Transient activation of the DBS-S-QF reporter in the R4/R5 region of the intestinal system of 7-9d mated female *Drosophila* (QUAS-tomato-HA anti-HA in red), Armadillo (green) labels the cellular membranes, and Prospero (green) labels the nuclei of Enteroendocrine Cells. (B) Enlargement of the area depicted by the yellow Arrow in (A). White arrows denote Intestinal Stem cells, Enteroblasts and Enterocytes positively labelled with the DBS-S-QF reporter. (C) Quantification of the intestinal cell subtypes activating the DBS-S-QF reporter relative to total cell number, using DAPI staining as a proxy of total cell number. There is a statistically significant difference in the number of intestinal cell types activating the reporter as a proportion of total cells (One-way ANOVA, N = 8, P = <0.0001). The DBS-S-QF reporter appears preferentially activated in Enteroblasts and Enterocytes. (D) Expression of the *Su(H)-LacZ* reporter (green) appears to be active in some of the same cells as the DBS-S-QF reporter (Red). (E) Enlargement of the area depicted by the yellow arrow in (D). White arrows indicate the co-localisation between the *Su(H)-LacZ* and the DBS-S-QF reporter. (F) Quantification of the percentage of *Su(H)-LacZ* cells activating the DBS-S-QF reporter (Mean = 60.96). DAPI (blue) labels the nuclei of all cells. Genotype of the *Drosophila* in panels A-E is $w^{1118} \text{ DBS-S-QF UAS-CD8-GFP QUAS-dTomato-HA}$ and in D-F is $w^{1118} \text{ DBS-S-QF UAS-CD8-GFP QUAS-dTomato-HA} / w^{1118} \text{ Su(H)-LacZ}$.

Following the observation that the reporter was preferentially activated in Enterocytes and Enteroblasts, I sought to ascertain whether the apical-caspase activation was stochastic and apoptotic, or regulated across the whole of the R4/R5 region of the posterior midgut. Using the immunofluorescent channel of the DBS-S-QF reporter of multiple intestines at 3-5, 7-9 and 14-16 days post eclosion, I generated heatmaps of the DBS-S-QF activation. At 3-5 days the activation of the reporter appears concentrated in the posterior of the R4/R5 region [Figure 3-4 A], however, as time progresses the

activation appears to spread anteriorly at 7-9 days [Figure 3-4 B] and progressively more so at 14-16 days [Figure 3-4 C]. Collectively, suggesting that the activation of the apical-caspases within this region could be regulated and not merely stochastic and apoptotic.

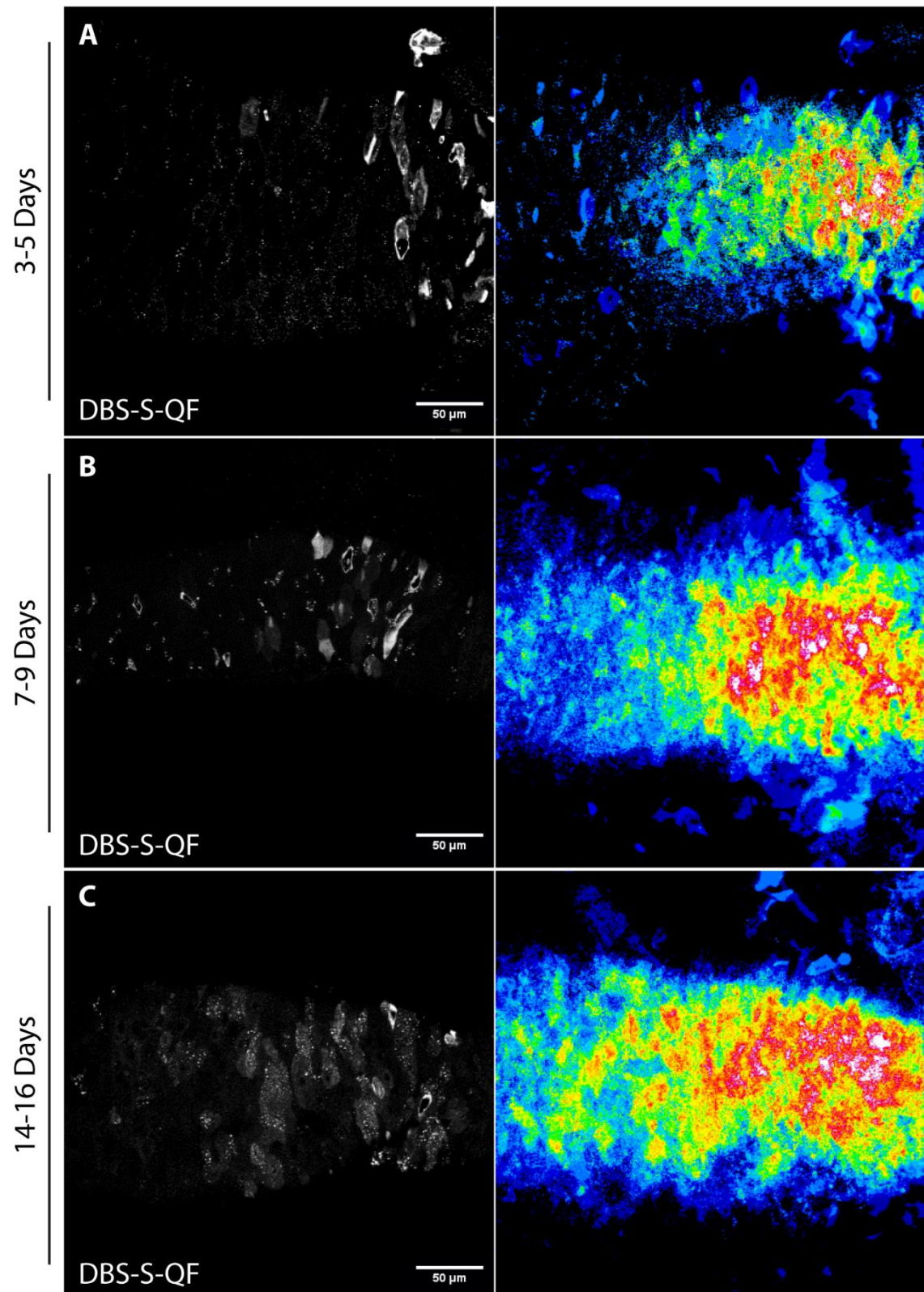


Figure 3-4: Activation of the reporter spreads anteriorly over time.

Left panels show DBS-S-QF activation with *QUAS*-tomato-HA anti-HA in white. The right panels depict the overlay of multiple DBS-S-QF intestines of the same age. White/red areas indicate a higher level of cells activating the reporter in this area, conversely, in blue areas fewer cells are activating the reporter. (A) Activation of the reporter at 3-5 days appears concentrated predominantly in the posterior region of the R4/R5 (N=13). (B) Activation of the reporter appears to have spread more anteriorly. Note the increase in blue anteriorly where previously was black in (A) (N= 25). (C) Activation of the reporter appears to have spread more anteriorly still. Note the increase in yellow and red when compared to (B) (N = 24). Genotype of the *Drosophila* in the figure is *w¹¹¹⁸ DBS-S-QF UAS-CD8-GFP QUAS-dTomato-HA*. All images in this figure were acquired using the same magnification and settings.

The apical-caspase activity observed with the DBS-S-QF reporter appears non-apoptotic

Next, I attempted to establish whether the apical-caspase activation observed using the DBS-S-QF reporter was apoptotic or non-apoptotic. Interestingly, cells activating the reporter did not demonstrate visible signs of apoptosis, such as nuclear condensation, collapse and/or fragmentation (244) [Figure 3-3]. Additionally, the visualisation of the fluorescent tomato protein in the previous figures would suggest that apoptotic, caspase-mediated proteolysis of proteins was not occurring (245), a common occurrence in dying cells. Furthermore, I observed progressive temporal activation of the reporter posteriorly to anteriorly, suggesting that the apical-caspases were regulated, and the activation was not a consequence of apoptosis [Figure 3-4]. Despite these arguments however, I sought to establish a more unambiguous method to

determine the nature of this caspase activation and its apoptotic or non-apoptotic nature.

In order to achieve a more unbiased method of detecting non-apoptotic apical-caspase activity, I incorporated two additional transgenes into the DBS-S-QF reporter background, a *QUAS-Flippase (Flp)* and an *Actin-FRT-STOP-FRT-nLacZ*. When the DBS-S-QF reporter is not cleaved by apical-caspases, translocation of the QF fragment to the nucleus does not occur [Figure 3-2 A]; thus, the QUAS-Flp is not expressed [Figure 3-5 A]. Upon cleavage, the QF translocates to the nucleus and activates the expression of the fluorescent tomato protein [Figure 3-2B], and the flippase recombinase protein [Figure 3-2 B]. The flipase then mediates the excision of the STOP cassette flanked by two FRT sites, thus, permitting the permanent expression of nLacZ under the *Actin* promoter [Figure 3-5 B]. Note, in contrast to the previous LacZ, a nuclear localisation signal is attached to this version of the protein. This tool allows for the detection of historical apical-caspase activity through its permanent labelling, even when temporary labelling through the *QUAS*-tomato expression is terminated. Moreover, cells which appear labelled with LacZ, but no longer labelled with QUAS-tomato, must have activated apical-caspases in the past and subsequently terminated their activation. Thus, the activation of the apical caspases must have been non-apoptotic.

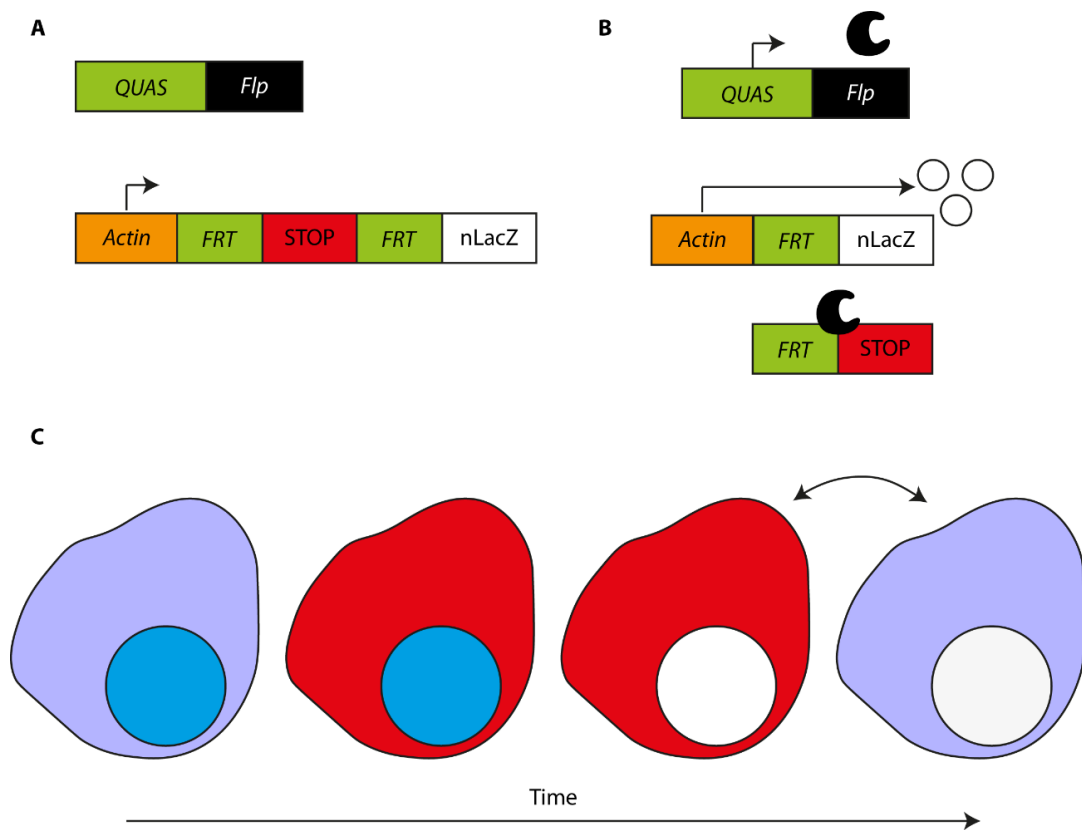


Figure 3-5: Schematic of the temporal detection of non-apoptotic caspase activity using the DBS-S-QF

(A) A flippase recombinase (*Flp*) under the regulation of QUAS, was combined with a construct containing the sequence for nuclear-localised LacZ, under the regulation of the *Actin* promoter. The expression of the nLacZ is impeded by the inclusion of an FRT flanked STOP sequence, before the nLacZ coding sequence. (B) Following cleavage of the DBS-S-QF reporter by apical-caspases, the QF fragment translocates to the nucleus activating expression of the Flp (Black pincer object). The Flp then mediates the flip-out recombination of the STOP cassette (246, 247), permitting the expression of nLacZ (small white circles). Note the expression of the LacZ under the *Actin* promoter is a permanent labelling. (C) Various temporal states of the DBS-S-QF labelling system. When there is no activity of the reporter, the cells remain unlabelled (leftmost cell). Activation of the reporter initially leads to tomato labelling (red, middle left). Subsequently, the excision of the STOP cassette triggers the expression of LacZ labelling (white) of the nucleus

(middle right cell). The nLacZ permanently labels the cell even when the activation of the DBS-S-QF reporter ceases (right cell). Note it is possible to subsequently reactivate the reporter and tomato labelling (middle right cell).

In 7-day old female *Drosophila* intestines, I observed widespread current and historical activation of the DBS-S-QF reporter throughout the whole of the R4/R5 region [Figure 3-6 A]. Strikingly, more cells appeared labelled with nuclear LacZ staining than those which were currently activating the reporter, suggesting that non-apoptotic apical-caspase activation is common throughout this region of the midgut [Figure 3-6 A]. Next, I sought to determine whether I could observe non-apoptotic apical-caspase activation in all of the subtypes of cells within the *Drosophila* intestinal system, as observed previously using the DBS-S-QF reporter alone [Figure 3-3 A]. I was able to observe historical labelling in Intestinal Stem Cells, Enteroblasts, Enteroendocrine and Enterocyte cells [Figure 3-6 B]. However, due to this method of permanent labelling, it is impossible to determine whether the nuclear lacZ observed in differentiated cells is due to DBS-S-QF activation in this cellular subtype, or a previous activation in progenitor cells. Collectively nevertheless, I was able to observe evidence of widespread non-apoptotic apical-caspase activation throughout the R4/R5 region of the posterior midgut, in all of the cells highlighted with the DBS-S-QF reporter.

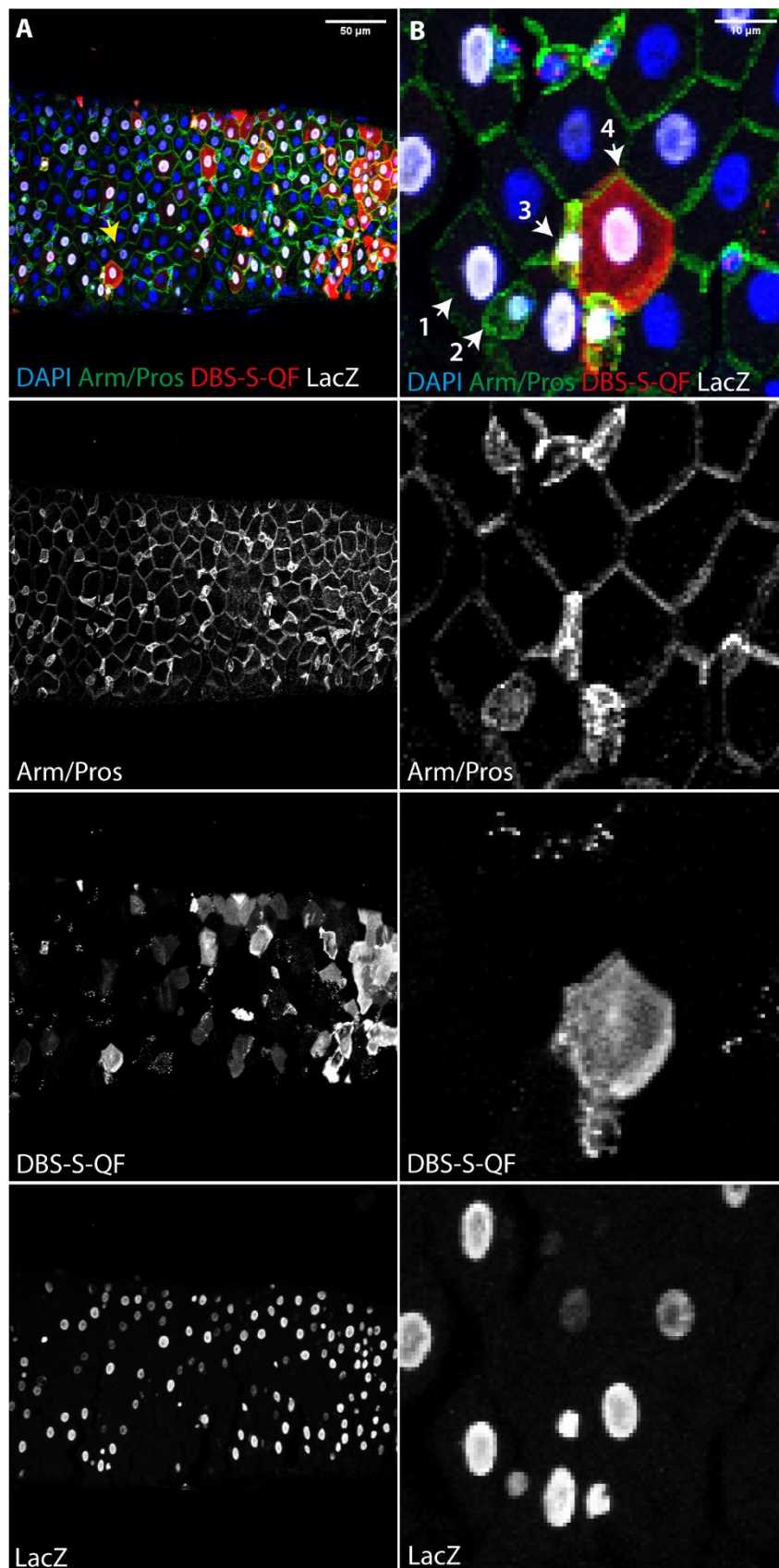


Figure 3-6: Non-apoptotic apical-caspase activation is observed using the DBS-S-QF reporter.

(A) Representative image of transient activation of the DBS-S-QF reporter (red) with historical/permanent labelling of caspase activation in white. Armadillo (green) labels the cellular membranes, and Prospero (green) labels the nuclei of Enteroendocrine cells. DAPI labels all cell nuclei. (B) Enlargement of the area depicted by the yellow arrow in (A). White arrow 1. & 2. indicate an Enterocyte and Enteroendocrine cell respectively, which have historically activated apical-caspases but have since terminated their activity. 3. & 4. denote an Enteroblast and Enterocyte respectively, which have both DBS-S-QF and nuclear LacZ labelling, suggesting long-term or recurrent apical-caspase activation. Genotype of the *Drosophila* in the figure is w^{1118} DBS-S-QF UAS-CD8-GFP QUAS-dTomato-HA; QUAS-Flp / Cyo; Actin > Stop > LacZ / TM6B.

Intestinal regeneration does not occur for at least 7 days post-activation of the ReDDM reporter

These initial observations of a regulated pattern of non-apoptotic caspase activation within the intestinal system suggested the potential for a functional role. Before investigating this however, I sought to determine the basal proliferation and differentiation dynamics of the intestine, in addition to the apoptotic vs non-apoptotic activation of the caspase pathway. This is an essential step, as numerous studies have previously demonstrated intestinal defects in proliferation and differentiation following inhibition of apoptosis (109, 248). Thus accordingly, I used the “Repressible Dual Differential stability marker” (ReDDM) cell lineage tracing tool, which allows the study of precursor cells with spatial and temporal resolution (249)

This tool exploits two fluorescent proteins of differing half-lives; a short-lived Green Fluorescent Protein (GFP) and a persistent Histone 2B tagged RFP (HistoneRFP). These two fluorescent proteins are under the regulation of a *UAS* promoter [Figure 3-7 A & B]. In combination with a construct expressing a GAL4 under the regulation of a cell marker gene, it is possible to observe the proliferation and differentiation of precursor cells in many different tissues. Temporal regulation is achieved through the incorporation of a *Tubulin* expressed thermosensitive Gal4 repressor (TubG80^{ts}), repressing the activity of the Gal4 transcription factor at 18°C, and permitting its activity at 29°C [Figure 3-7 A & B] (249).

To observe the basal cellular dynamics of the intestinal progenitors (Intestinal Stem Cells and Enteroblasts), I utilised a transgene containing the GAL4 coding sequence under the regulation of the *escargot* (*esg*) promoter. *Esg* is a zinc-finger, Snail family transcription factor essential for maintaining stem cell identity, with its loss promoting differentiation of these cells into terminally differentiated Enterocyte and Enteroendocrine cells (177).

Using the ReDDM tool in combination with the *esg*-GAL4 driver in non-damaging conditions (where there is no differentiation to replace apoptotic or necrotic cells). I would expect to observe a perfect overlap between the short-lived membrane localised GFP and the long-lived HistoneRFP, in Intestinal Stem Cells and Enteroblasts [Figure 3-7 C]. In damaging conditions, where cell differentiation into Enterocytes or Enteroendocrine cells is required to replace damaged cells; the *esg* promoter is no longer active, and thus the membrane GFP is no longer expressed. The HistoneRFP signal

however, persists for at least 28 days within the differentiated cell, thus allowing for detection of this differentiation event (249) [Figure 3-7 D].

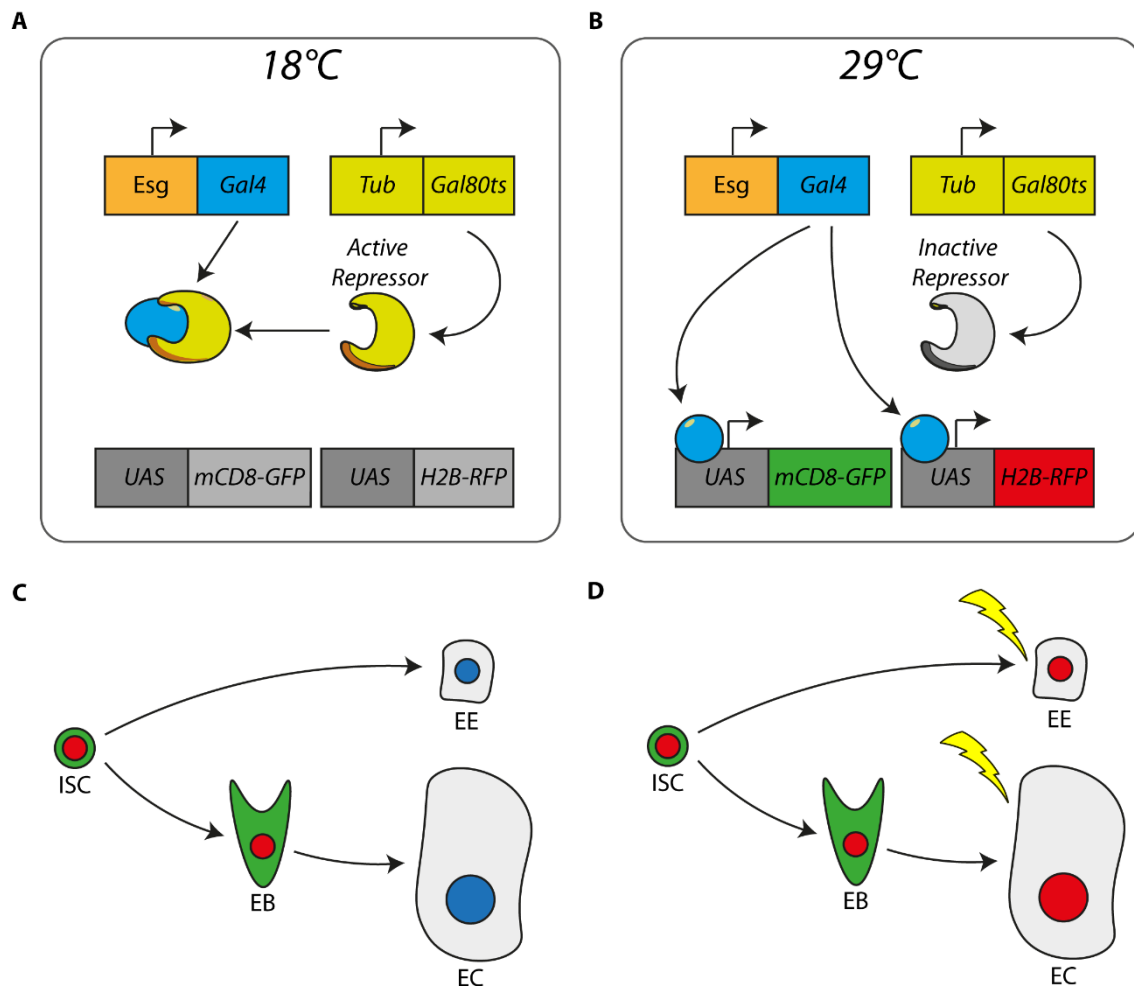


Figure 3-7: Schematic of the ReDDM tool

(A) Illustration of the inactive ReDDM tool at 18°C, GAL80^{ts} (yellow pincer) under the regulation of the *Tubulin* promoter, represses GAL4 (blue circle engulfed by yellow pincer) and thus inhibits the expression of mCD8-GFP (GFP) and the H2B-RFP (HistoneRFP). (B) At 29°C the GAL80^{ts} (grey pincer) is inactive allowing the GAL4 (blue circle) to activate the expression of the mCD8-GFP and the HistoneRFP. (C) In non-damaging conditions where no differentiation occurs, there is a perfect overlap between the mCD8-GFP and HistoneRFP in the Intestinal Stem Cells (ISC) and Enteroblasts (EB). (D) When damage (yellow lightning) occurs in the intestine and differentiation

occurs, the mCD8-GFP is no longer expressed, however, the HistoneRFP is retained (see the red nucleus of the Enterocyte (EC) and Enteroendocrine cell (EE)). (A) and (B) have been adapted and redrawn from (249).

In my experimental conditions (see methods 2.1.3), I observed activation of the DBS-S-QF reporter [Figure 3-8 A & Figure 3-3 A]; however, I did not observe any signs of regeneration in the R4/R5 region of the posterior midgut during the first seven days post-activation of the ReDDM reporter [Figure 3-8 B]. This was evidenced by the almost exclusive overlap of the GFP and the HistoneRFP markers, and thus the absence of differentiation into Enterocytes or Enteroendocrine cells [Figure 3-8 B]. I subsequently quantified the total GFP and HistoneRFP cells at 3- & 7- days post-activation of the ReDDM reporter and did not observe any statistical significance between these populations of cells; confirming the previous observations [Figure 3-8 C]. Furthermore, no significant difference was observed between the GFP and RFP populations of cells at 7-days post activation of the ReDDM reporter [Figure 3-8 C]. Further experiments determined that only after 14 days post activation of the ReDDM reporter was a full turnover of the epithelial tissue observed.

In order to further validate these findings, we analysed the behaviour of both the DBS-S-QF and the ReDDM reporters in non-specific tissue-damaging conditions, triggered by Paraquat treatment. Paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride) is a highly toxic member of the bipyridyl group of herbicides, which generates oxidative stress through the generation of reactive oxygen species. Thus, it is a widely used damaging agent for this purpose in *Drosophila* (250-255). As expected, following a 16-hour

treatment of paraquat, I observed more activation of the DBS-S-QF reporter, suggesting an increase in apical-caspase activity and apoptosis [Figure 3-8 D]. Additionally, using the ReDDM reporter, I observed an increased number of HistoneRFP cells following paraquat treatment, indicative of differentiation [Figure 3-8 E]. As before, I quantified both of the GFP and HistoneRFP markers before and after paraquat treatment (3- and 7- days), observing a statistically significant increase in HistoneRFP following paraquat treatment [Figure 3-8 F]. However, no significant difference was observed between the GFP and HistoneRFP markers at 7d post activation of the ReDDM reporter, suggesting despite an increase in proliferation, little differentiation is occurring [Figure 3-8 F].

Next, I sought to determine whether the non-regenerative nature of our dietary conditions was unique or observed in other dietary media. To this end, I used the commercially available *Drosophila* Quick Mix Medium (Blue). Similar to the Paraquat treatments [Figure 3-8 D-F], I observed increased activity of the DBS-S-QF reporter in these dietary conditions [Figure 3-8 G]. Furthermore, this correlated as observed previously, with statistically increased numbers of HistoneRFP and additionally GFP ReDDM markers at 7-days, suggestive of regenerative conditions [Figure 3-8 H & I]. Accordingly, no significant difference was observed between the GFP and HistoneRFP markers at 7d post activation of the ReDDM reporter.

Collectively, these results confirmed the non-regenerative nature of our *Drosophila* medium and experimental protocol, for at least the first 7-9 days following eclosion. Moreover, this data supports the previous observation of non-apoptotic caspase

activity, demonstrated using the DBS-S-QF reporter and lineage tracing tools [Figure 3-6].

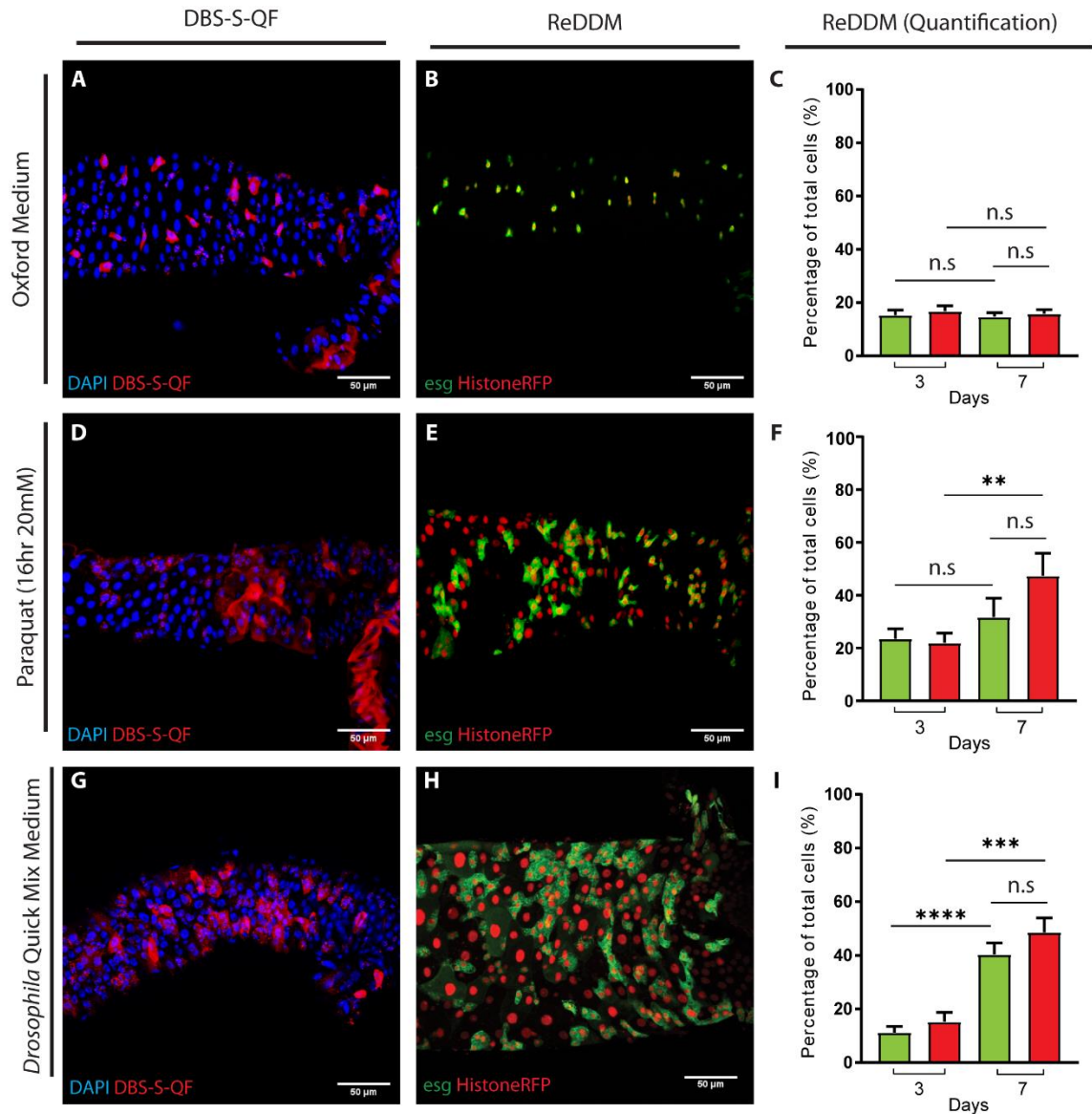


Figure 3-8: DBS-S-QF & ReDDM reporters demonstrate caspase activation in non-regenerative conditions.

(A) DBS-S-QF in Oxford Food. (B) ReDDM in Oxford Food, note the strong co-localisation between the GFP and the HistoneRFP markers indicative of non-regenerative conditions. (C) ReDDM quantifications, there is no significant difference between the number of GFP ($P = 0.5267$) and HistoneRFP ($P = 0.2752$) cells as a percentage of total cells, between 3- and 7-days (unpaired

Mann-Whitney test, 3d N = 34, 7d N = 45). (D) DBS-S-QF following a 16 hour 6.5mM Paraquat treatment. Note the increased DBS-S-QF reporter signal compared to (A). (E) ReDDM following Paraquat treatment, note the increase in the number of HistoneRFP labelled cells, indicative of regeneration. (F) Quantification of GFP and HistoneRFP labelled cells before and after paraquat treatment. I observed a statistically significant increase in HistoneRFP labelled cells following Paraquat treatment ($P = 0.0099$, unpaired two-tailed t-test, 3d n = 9, 7d N = 7). (G) DBS-S-QF in *Drosophila* Quick Mix (Blue). Note the increased DBS-S-QF activation compared to (A). (H) ReDDM in *Drosophila* Quick Mix (Blue). Similar to Paraquat treatment, I observed evidence of regeneration, i.e. increased numbers of HistoneRFP and GFP markers. (I) Quantification of (H) there is a statistically significant increase observed in both GFP ($P = <0.0001$) and HistoneRFP ($P=0.0004$) between 3- and 7-days (unpaired two-tailed t-test, 3d N=14, 7d N=34). All of the intestines were dissected from adult *Drosophila* mated females. Left panels demonstrate the DBS-S-QF reporter in various dietary conditions. DAPI (Blue) labels the nuclei of all cells and DBS-S-QF is labelled in red. Middle panels demonstrate the ReDDM reporter, with the short-lived GFP (green) and the long-lived HistoneRFP (red). Right panels demonstrate the quantification of the ReDDM reporter at 3- and 7- days post activation of the reporter. Error bars represent Standard Error of the Mean. Genotype of the *Drosophila* in panels A, D and G is $w^{1118} DBS-S-QF UAS-CD8-GFP QUAS-dTomato-HA$; in all other panels the genotype is $w^{1118}; esg-Gal4 UAS-mCD8-GFP / Cyo; UAS-HistoneRFP TubG80ts / TM6B$. All DBS-S-QF and ReDDM images were acquired using the same magnification and settings.

In order to further validate these findings, I overexpressed two copies of the baculoviral effector-caspase inhibitor, P35 (57). The overexpression of this viral protein inhibits the activity of both drICE and DCP-1 (and thus apoptosis) (256). Confirming previous observations, overexpressing two copies of P35 in *esg* cells did not increase the number of progenitor or differentiated cells, which would be expected following inhibition of

apoptosis in progenitor cells destined to die (257) [Figure 3-9 A-C]. Additionally, this genetic manipulation did not affect the morphology of either the intestine or progenitor cells. Collectively, this unambiguously confirmed that in the dietary conditions used in these experiments, there was negligible cell death, and thus any caspase activation observed with the DBS-S-QF reporter is likely to be non-apoptotic. However, these results raised the question of whether there was a functional requirement for the caspase activity, as blocking the downstream activity appeared inconsequential.

B

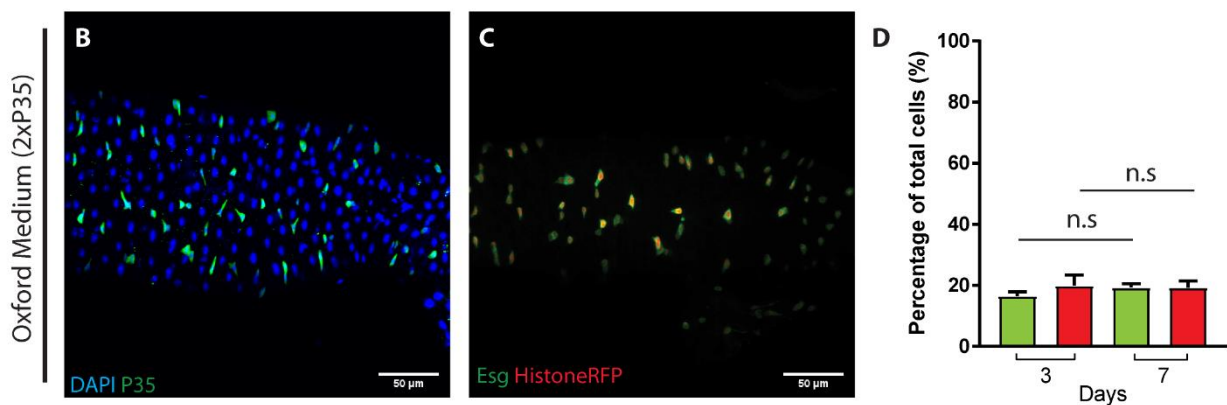
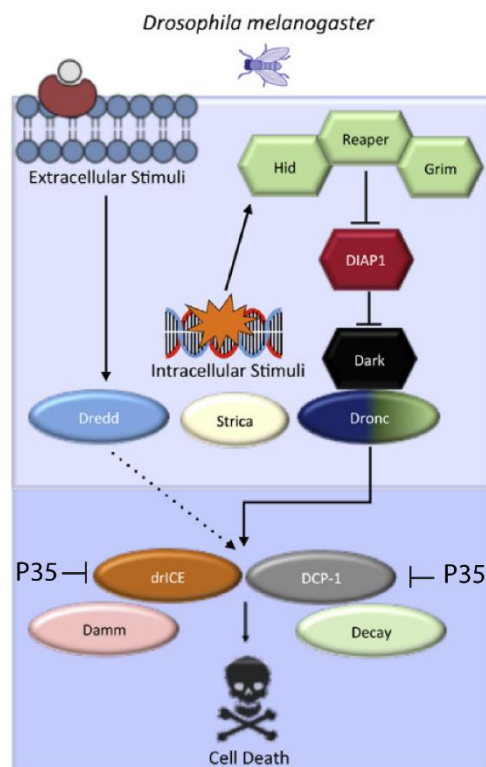


Figure 3-9: Overexpression of 2X P35 in Oxford Medium does not result in phenotypes.

(A) Apoptotic pathway demonstrating P35 inhibition. Dronc activates drICE and DCP-1 through cleavage. Once activated, the activity of drICE and DCP-1 is inhibited by the suicide substrate: P35, thus inhibiting apoptosis. (B) Representative image of a mated adult female *Drosophila* intestine, overexpressing P35 under the *esg* promoter in Oxford Media. DAPI (blue) labels all cell nuclei, and *esg* cells expressing P35 can be visualised through anti-p35 staining (green). Note there does not appear to be any morphological changes in the cells through the overexpression of P35. (C) Overexpression of P35 and the ReDDM reporter. There appears perfect co-localisation of the GFP and HistoneRFP markers, suggestive of non-regenerative conditions. (D) Quantification of ReDDM intestines overexpressing two copies of P35 under the regulation of the *esg* promoter. There is no statistical increase in GFP ($P = 0.1352$) or HistoneRFP ($P = 0.9801$) labelled cells between 3- and 7- days post activation of ReDDM and overexpression of P35. (unpaired two-tailed t-test, 3d $N=12$, 7d $N=11$). The genotype of the *Drosophila* in this figure is *w¹¹¹⁸; esg-Gal4 UAS-mCD8-GFP / UAS-P35; UAS-HistoneRFP TubG80^{ts} / UAS-P35*.

3.2 Discussion

In this section, using the DBS-S-QF sensor, I have demonstrated the presence of apical-caspase activity throughout the whole of the R4/R5 region of the intestinal system. Importantly, using the DBS-S-QF sensor in combination with a permanent labelling approach, the observed activation appears non-apoptotic. Furthermore, this non-apoptotic activity appears regulated as opposed to stochastic as we observe its progression posteriorly to anteriorly over time.

The non-apoptotic nature of the apical-caspase activity can be further confirmed using the “Repressible Dual Differential stability marker” (ReDDM). Using Oxford Media and the experimental protocols outlined in the Materials and Methods section 2.1.3, I observed no evidence of regeneration of the intestine. This was confirmed using known damaging agents, such as paraquat, to induce widespread and non-specific intestinal damage; in addition to commercially available food (*Drosophila* Quick Mix Media (Blue)), which do not replicate the non-regenerative conditions observed using my experimental approach. I was able to further confirm the non-regenerative nature of my experimental approach by overexpressing two copies of the effector caspase and apoptosis inhibitor, P35. Using this experimental manipulation, I was unable to observe any increase in cell number or differentiation, confirming that the observed apical-caspase activation using the DBS-S-QF was non-apoptotic.

The DBS-S-QF reporter activation differs in part from previously published effector caspase reporters.

The similarities between the activation of the two sensors, such as activation in Enterocytes, are not surprising. The DBS-S-QF sensor is based on a truncated form of the drICE protein (219), and the effector-caspase sensors incorporate a small consensus sequence (DQVD), known to preferentially be cleaved by the effector caspases (242, 243). Thus there is likely to be overlapping activation. Interestingly however, we did not entirely recapitulate the results obtained from previously published reporters of effector caspase activity (242, 243). The disparities, such as the preferential activation of the DBS-S-QF in progenitors, could arise from a specific requirement of the apical-caspases to maintain progenitor homeostasis. This also correlates with the observation that overexpressing two copies of the effector caspase inhibitor, P35, did not produce any noticeable intestinal or cellular morphological phenotypes.

In the canonical caspase-cascade of apoptosis, upregulation of the initiator of apoptosis proteins: Reaper, Hid and Grim, precedes the inhibition of the inhibitor of apoptosis protein, DIAP1. Dronc, the main initiator caspase, in collaboration with Dark, form the apoptosome. This structure leads to the activation of the effector caspases (drICE, DCP-1, Damm and Decay) resulting in apoptosis (39). Owing to the apparent regulated activation of the DBS-S-QF sensor, and the evidence of permanent labelling, without a functional requirement for effector caspase activity; collectively, this would suggest that

if there is a functional requirement for apical-caspase activation, it must be effector-caspase independent.

Basal turnover of intestinal cells does not occur for 7-9 days post eclosion, in non-damaging conditions.

In addition to the P35 experiments indicating effector caspase independent activity of the apical-caspases, it also suggests an absence of homeostatic cell turnover of the progenitor cells. This was further confirmed using the ReDDM tool where I observed a strong co-localisation between the GFP and the HistoneRFP, indicative of an absence of regeneration and thus cellular death, in my dietary conditions. Temporal homeostatic turnover of the *Drosophila* midgut has been previously documented in the literature, with one study suggesting complete turnover of epithelial cells at a rate of about once every 1-2 weeks (166), however other studies have suggested a slower turnover rate of 2-3 weeks (208). Problematically, there do appear to be regional variances to the turnover rate, with the R4ab or P1–2 region undergoing complete renewal in as little as 4-8 days (258). Moreover, this turnover rate appears to be in part sex-related, with female intestines undergoing faster turnover than their male counterparts (190, 208).

Interestingly, while the rate of turnover in our dietary conditions (over 7 days) is still in accordance with previously published literature, previous studies have reported a progressive cell turnover during this period (208). Conversely, I rarely observe evidence

of this turnover occurring in my dietary conditions throughout the 7-days of ReDDM activation. The origins of this phenomenon remain unclear, and while I cannot exclude a genetic component, these observations could in part result from the composition of the media and the regular flipping regime undertaken in my experiments.

Numerous studies have noted a dietary contribution to intestinal integrity dysfunction in *Drosophila*. For example, a high sugar diet has been demonstrated to contribute to loss of intestinal integrity and early onset death in *w¹¹¹⁸ Drosophila* (259). Furthermore, intestinal barrier defects were also observed in diets high in salt (NaCl), and emulsifiers such as TWEEN 20 (259). In addition to diet directly effecting intestinal integrity, it has also been demonstrated to contribute to microbiota changes in *Drosophila* (259), although this is still debated (260). While unclear in *Drosophila*, the link between diet and changes in the microbiota of humans have been observed (261), and its deregulation linked to a multitude of diseases such as Inflammatory bowel disease or *H. pylori*-mediated gastric carcinoma (262).

Whilst diet-mediated microbiome changes in *Drosophila* remain controversial, the converse microbiome regulated manipulations of host metabolism and immunity are widely reported (263). Furthermore, it has been demonstrated that high diversity in the microbiome reduces the chances of intestinal colonisation by pathogenic organisms (264). Thus reducing the likelihood of pathogenic bacteria from triggering intestinal damage and the requirement for the regeneration of damaged tissue (167).

The experimental conditions used for these experiments may, therefore, contain all of the essential components required to sustain *Drosophila*, but in the optimum proportions as to maintain intestinal integrity and limit damage (i.e low sugar). Alternatively, the main contributing factor to the limited regeneration observed in my experimental conditions, could stem from the acquisition of a diverse and healthy microbiota, thus limiting the likelihood of pathogenic organisms colonising the intestinal system. Additionally, as many pathogenic organisms such as *S. marcescens* infect their host enterically (265), the regular flipping of the *Drosophila* into new vials every couple of days may reduce the probability of the flies encountering pathogenic organisms. In reality however, the non-regenerative conditions observed in my experimental conditions is likely to be a combination of all of these factors.

Whilst understanding the experimental conditions which are protective against intestinal damage is undoubtedly an exciting avenue of research, and advantageous for the study of the non-apoptotic roles of the caspases in this context; the mechanistic intricacies of how this manifests is beyond the scope of this thesis, and thus will not be commented on any further.

Caspase activity within the intestinal system is unlikely to be related to the anastasis phenomena

Anastasis is classified as the process by which a cell recovers from the brink of apoptotic cell death, following activation of the caspases (266). Canonically, this phenomenon is usually correlated with a cellularly lethal dose of a damaging agent, capable of activating the apoptotic caspase-cascade. Importantly, using the experimental conditions documented within this thesis, I failed to observe any signs of apoptotic cell death or regeneration of the intestinal epithelium. This suggests that the apical-caspase activity observed using the DBS-S-QF reporter is unlikely to result from anastasis, and is more likely to be related to an undiscovered non-apoptotic role for the caspases in this context.

Caspase activation within the *Drosophila* intestinal system correlates with clinical colon cancer data.

The observation of apical-caspase activity within the posterior midgut is in agreement with the colon cancer clinical data, suggesting that *caspase-9* downregulation is more commonly observed in poorly differentiated tumours, with its overexpression resulting in poor colony formation and reduced proliferation (109). Whilst currently the experimental data is preliminary, the correlation between these observations suggests possible functional conservation of the caspases within the intestinal system.

4. The catalytic activity of Dronc regulates progenitor differentiation independently of the apoptotic pathway

4.1. Results

Dronc prevents the excessive proliferation and differentiation of intestinal precursors

Following the observation of regulated non-apoptotic, apical-caspase activation in the R4/5 region of the adult posterior midgut, using the DBS-S-QF and ReDDM reporters, I next sought to determine whether there was a functional requirement for this activity.

In the *Drosophila* apoptotic pathway, Dronc (caspase-9/2), is the main initiator caspase driving the apoptotic process, in addition to the non-immune related non-apoptotic functions (39, 267-270). Additionally, many *Dronc* homozygous loss of function alleles report developmental lethality, suggestive of an indispensable role for Dronc in this process. (271). Thus, the previously discussed data, in combination with clinical studies suggesting a potential role for caspase-9 in tumorigenesis (109), lead me to study the function of Dronc within the *Drosophila* intestinal system.

As a first step, I utilised a *Dronc* loss of function allele generated by the Baena-Lopez laboratory, *Dronc*^{KO}. This allele was produced using CRISPR/Cas9 and homologous recombination, replacing the first exon of *Dronc* with an attP-integration site, resulting

in a knockout allele [Figure 4-1]. Previous literature has demonstrated that *Dronc* heterozygotes develop normally, maintaining a functional apoptotic pathway (54). Thus initially, I sought to establish whether ubiquitous heterogeneity of *Dronc* using this allele, exhibited any visible phenotypes within the *Drosophila* intestinal system.

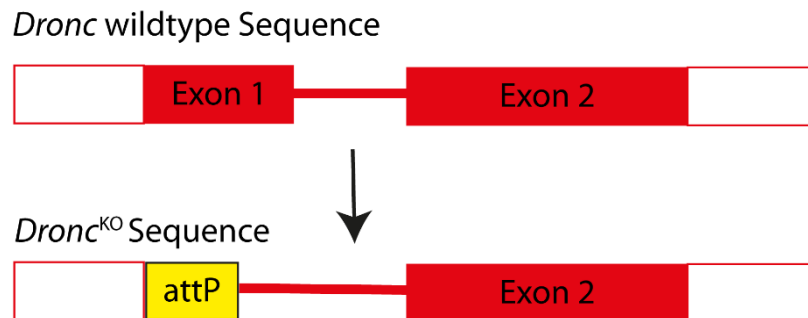


Figure 4-1: Schematic of the *Dronc*^{KO} allele.

White Boxes indicate untranslated regions (UTRs). Red lines indicate introns and red boxes denote exons. The genomic sequence for *Dronc* consists of two exons and one intron. In the *Dronc*^{KO} allele, the first exon is replaced by an attP landing site for the later reintegration of transgenes into this locus. The removal of the first exon also produces a knockout allele for *Dronc*.

Using a *Drosophila* line with the *Dronc*^{KO} allele recombined with the ReDDM tool, I investigated the dynamics (i.e. proliferation and differentiation) of the *Drosophila* intestinal system in *Dronc* heterozygous conditions. Upon initial inspection, there appeared to be no morphological or cellular defects resulting from the loss of function allele [Figure 4-1 A&B]. However, I observed a small but statistically significant increase in the number of *esg*-positive cells (GFP-positive cells) in *Dronc*^{KO} heterozygous intestines compared to wild-type controls [Figure 4-1 C]. At the same time, I also detected a minor increase in the number of HistoneRFP without GFP cells, suggesting a

slight increase in the rate of differentiation. Despite being statistically significant, the biological relevance of this slight increase is however debatable.

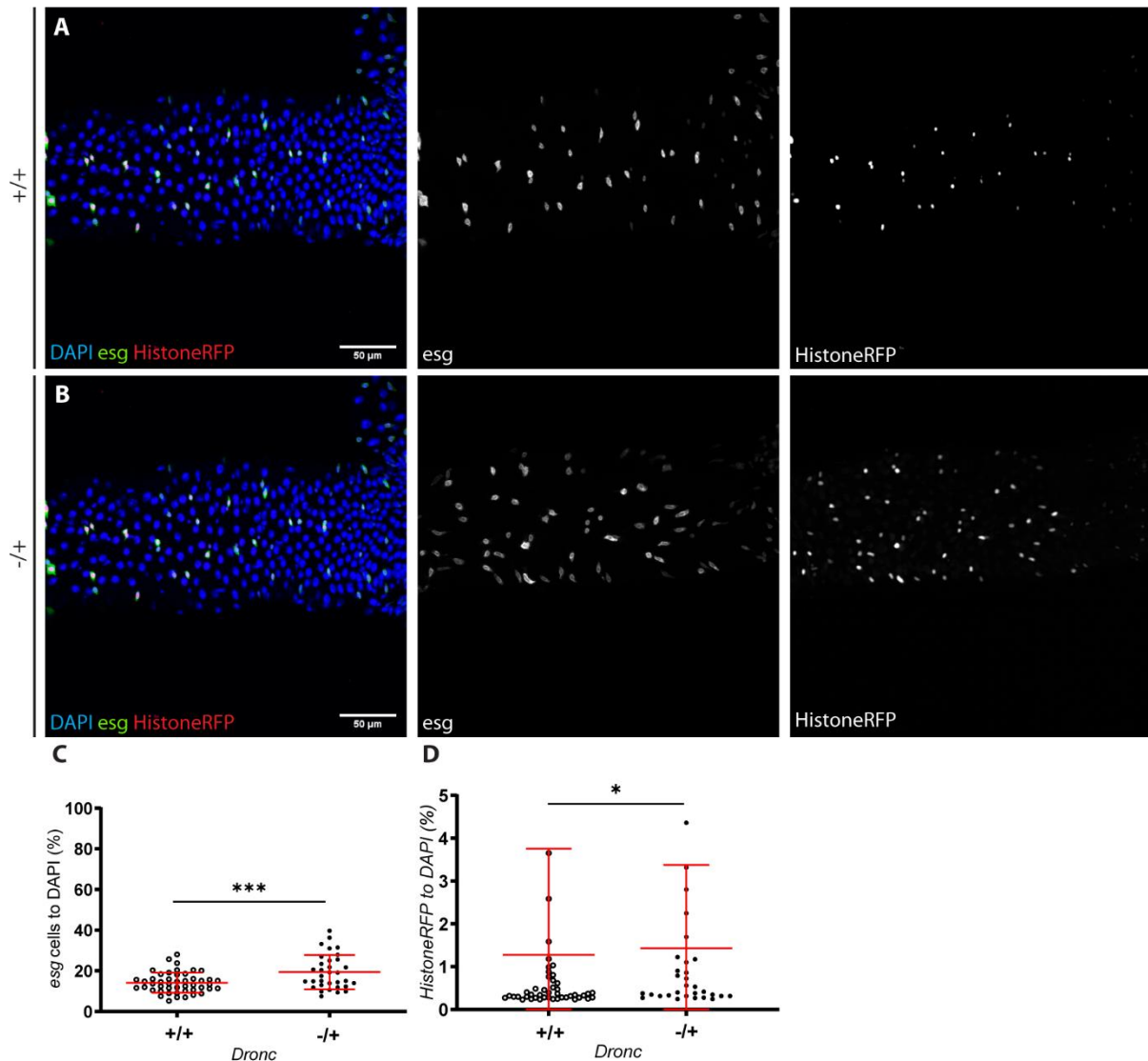


Figure 4-2: *Dronc*^{KO} heterozygous intestines demonstrate mild proliferation and differentiation defects.

(A) *Dronc* Wildtype. The ReDDM tool, *esg*-Gal4 UAS-mCD8-GFP (green) labels Intestinal Stem Cells and Enteroblasts. HistoneRFP (*UAS-HistoneRFP*) acts as a semi-permanent label allowing the tracking of progenitor differentiation. (B) *Dronc* heterozygous intestines appear morphologically similar to (A). (C) Quantification of the number of *esg* labelled cells relative to DAPI. There is a slight increase in *esg* cell number in *Dronc* heterozygous intestines in comparison to wildtype (P

= 0.0008, unpaired t-test, +/+ N = 47, +/- N = 33). (D) Quantification of the number of HistoneRFP cells without GFP labelling. There is a slight increase in this population of cells in the *Dronc* heterozygous intestines when compared to the wildtype (P = 0.0211, unpaired Mann-Whitney, +/+ N = 48, +/- *Dronc*^{KO} N = 33). DAPI labels all cell nuclei. Error bars represent Standard deviation of the Mean in all panels. All of the experiments described in the figure were performed in Oxford medium with an experimental regime which protects epithelial integrity. Scale Bars represent 50 µm. The genotype of the +/+ *Drosophila* is *w*¹¹¹⁸; *esg-Gal4* UAS-*mCD8-GFP* / *Cyo*; UAS-*HistoneRFP TubG80ts* / *TM6B*. The genotype of the +/- *Drosophila* is *w*¹¹¹⁸; *esg-Gal4* UAS-*mCD8-GFP* ; UAS-*HistoneRFP TubG80ts Dronc*^{KO} / *SM6A-TM6B*

After observing mild defects in proliferation and differentiation of progenitor cells in *Dronc* heterozygous intestines, I sought to further investigate its functional role through inducing its complete loss of function specifically in Intestinal Stem Cells and Enteroblasts. To achieve this, I used a conditional knockout allele for *Dronc* generated by the laboratory, based on the *Dronc*^{KO} allele. Initially, the wildtype *Dronc* construct for reintegration was modified through the inclusion of the sequences for GFP and Apex before the stop codon at the C-terminus. Furthermore the sequence was modified to include two FRT recombination sequences flanking the *Dronc*-GFP-APEX, allowing the excision of the cassette in the presence of the flippase recombinase protein (Flp), induced by any Gal4 fly strain. This enables the efficient elimination of *Dronc* expression in specific subsets of cells, including those with low rates of proliferation such as Enteroblasts. The sequence for QF was cloned downstream of the FRT flanked *Dronc*-GFP-Apex sequence. Importantly, flies expressing the *Dronc*-GFP-Apex in heterozygous

conditions are viable and only show minor morphological defects, such as a slight increase in wing opacity.

Through the inclusion of the ubiquitously expressed thermosensitive Gal4-repressor (TubG80^{ts}), I can obtain temporal, in addition to spatial specificity of excision. This is especially important owing to the developmental lethality observed in *Dronc* knockout larvae (271). Finally, the induced expression of the transcriptional activator QF, driven by the physiological regulation of the *Dronc* promoter upon excision of the *Dronc-GFP-APEX* FRT cassette from the genome, allows the activation of any gene of interest placed after the *QUAS* binding sequences (e.g. *QUAS-LacZ*). This enables the identification of FRT-excision events (i.e. when the *Dronc* rescue cassette is excised) and the expression of any gene of interest in *Dronc*-transcribing cells [Figure 4-3].

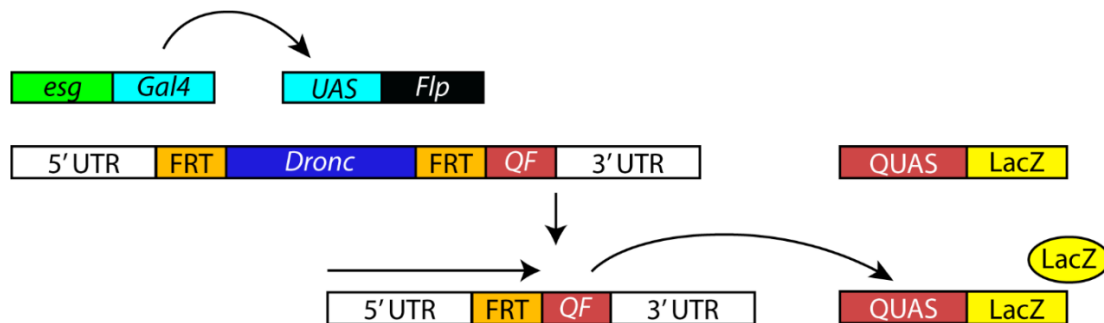


Figure 4-3: Schematic of *Dronc* excision efficiency tool.

The *Gal4* transcription factor sequence inserted into the *esg* locus, ensures its expression in the Intestinal Stem Cell and Enteroblast populations. The *Gal4* induces expression of a Flippase recombinase protein which mediates the FRT-excision of the *Dronc* rescue cassette including the *GFP* and *APEX2* tagged to the protein (not shown). This excision event generates a *Dronc* loss of function allele. The removal of the *Dronc* cassette also enables the expression of the *QF*

transcription factor. This factor in combination with the expression of nuclear localised LacZ, under the regulation of the *QUAS* promoter, enables the labelling of the excision event in cells which express *Dronc*.

As a first step, I attempted to determine the excision efficiency of the allele, through inducing the expression of the Flp protein under the regulation of *esg*, and subsequently the expression of LacZ (QF > *QUAS*-LacZ), upon removal of the *Dronc*-rescue cassette from the genome. Thus, allowing the identification and quantification of excision events. Additionally, in the same genetic background, I included the UAS-GFP construct to enable identification of the *esg* cell population [Figure 4-4].

Following *esg*-induced *Dronc* KO, I observed widespread co-localisation between the *esg*-Gal4 UAS-GFP, and the LacZ labelled nuclei [Figure 4-4 A]. Upon quantifying this co-localisation, I was able to determine an average 91.35% excision efficiency of the *Dronc* allele in *esg*-expressing cells [Figure 4-4 B]. On closer inspection, I also perceived morphological defects within the LacZ-positive cell populations, with cells appearing vastly larger in size when compared to those observed in previous experiments [Figure 4-4 B and Figure 4-2]. Additionally, I noticed signs of hyperplasia, with large cells appearing LacZ-positive without GFP labelling [Figure 4-4 B]. These results confirmed the suitability of our allele to assess the function of *Dronc* in progenitor cells and suggested a possible role for *Dronc* in the regulation of intestinal homeostasis.

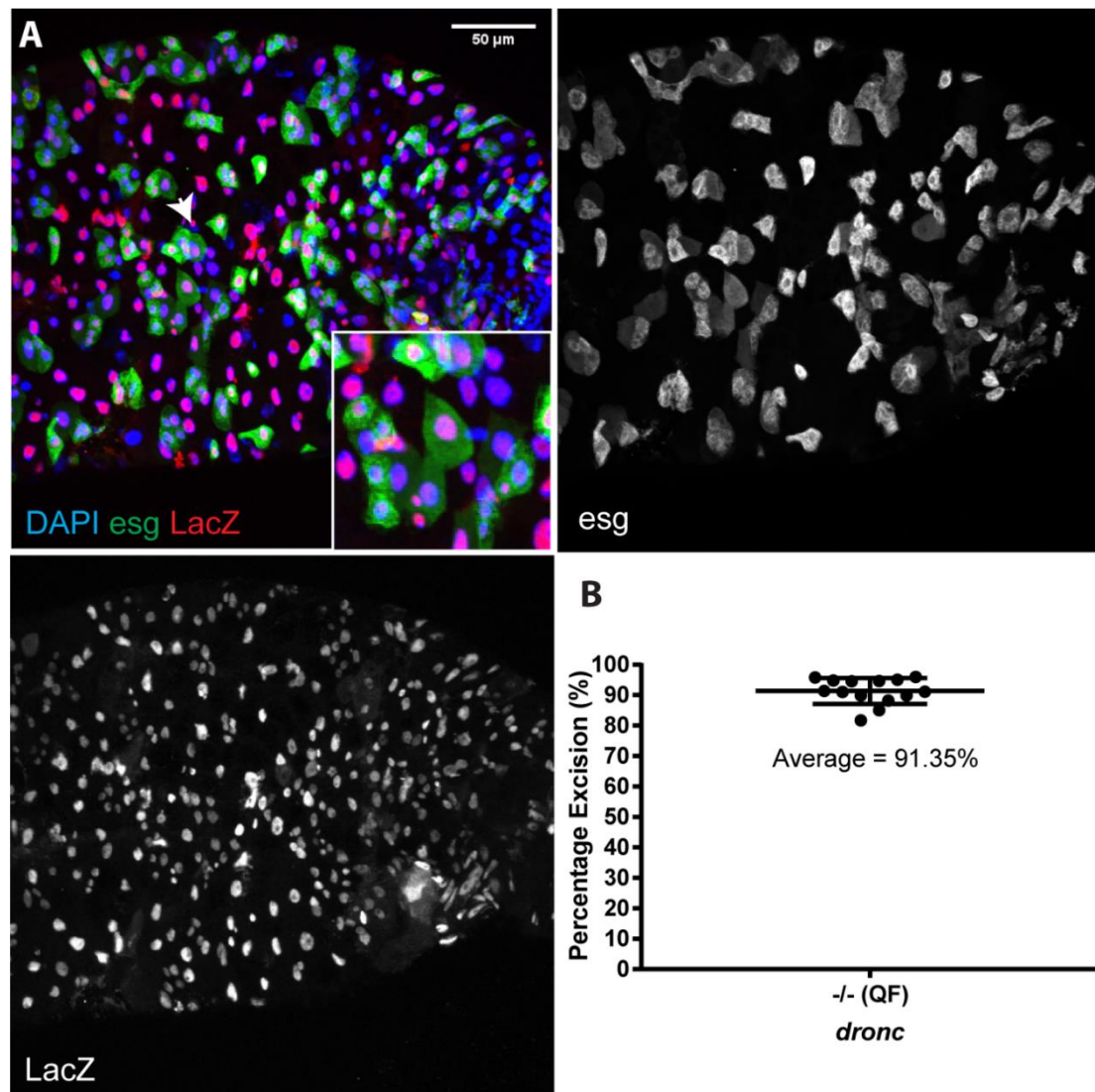


Figure 4-4: *Dronc* is efficiently excised under the regulation of *esg* and results in hyperplastic defects.

(A) Representative image of the R4/5 region of a mated female *Drosophila*, following the loss of *Dronc* expression in the progenitor cells for 3 days. Esg (green) labels the Intestinal Stem Cells and Enteroblasts. LacZ (red) labels the nuclei of cells which have excised the *Dronc* rescue cassette and DAPI (blue) labels the nuclei of all cells. There appears to be widespread colocalisation between the *esg* and LacZ markers; these cells also appear enlarged. Furthermore, the labelling of cells positive for LacZ, but negative for *esg*, suggests an increased rate of differentiation and hyperplasia. Inset depicts enlargement of the area indicated by the white

arrow. (B) Quantification of the co-localisation of *esg* and LacZ. There is a 91.35% co-localisation and thus efficiency of *Dronc* excision (N = 14). Error bars represent Standard deviation of the Mean in all panels. All of the experiments described in the figure were performed in Oxford medium with an experimental regime which protects epithelial integrity. Scale Bars represent 50 μ m. The genotype of the *Drosophila* in this figure is *w¹¹¹⁸; esg-Gal4 UAS-mCD8-GFP / QUAS-LacZ; UAS-HistoneRFP TubG80ts Dronc^{KO} / UAS-FLP FRT Dronc-GFP-APEX FRT QF*

Despite initial indications of a non-apoptotic role for Dronc in regulating intestinal homeostasis, I was unable to rule out the possibility of these phenotypes originating through the induced expression of the QF transcription factor; as previous studies have demonstrated its toxicity in some tissue contexts (132, 133, 240). To discriminate between these two possibilities, I used an alternative conditional allele. This allele generated by the laboratory contains a chimeric Suntag-HA-Cherry polypeptide, after the FRT-flanked Dronc-rescue cassette. In contrast to the QF protein, there is no evidence suggesting potential toxicity of the Suntag-HA-Cherry peptide [Figure 4-5]



Figure 4-5: Representation of the Dronc-GFP-APEX2 to Suntag-HA-Cherry allele

The sequence for *Cherry* fluorescent protein, *HA* & *Suntag* were subcloned into the *Dronc* open reading frame, after the *Dronc-GFP-APEX2* rescue cassette. The *Dronc* rescue cassette sequence is flanked by two FRT recombinase sites. Upon excision of the cassette, the downstream *Suntag-HA-Cherry* becomes expressed.

To confirm the observations from the previous experiment, I combined this allele with the ReDDM lineage labelling system [Figure 4-6 A]. Importantly, this was achievable as the *Dronc*-GFP-Apex construct was undetectable using immunofluorescence. Following excision of the *Dronc* rescue cassette and expression of the neutral Suntag-HA-Cherry, I was also unable to detect the expression of the Suntag-HA-Cherry. However, I observed striking differences in the intestinal morphology compared to *Dronc* heterozygous intestines [Figure 4-6 B]. Firstly, I observed an increase in the number of *esg* labelled cells at 3- and 7-days post loss of *Dronc* function [Figure 4-6 C]. Furthermore, this phenotype appeared progressive rather than an instantaneous response to *Dronc* loss of function, as the total number of *esg*-labelled cells appears further increased at 7d when compared to equivalent 3d intestines [Figure 4-6 C]. Additionally, *esg*-labelled cells appeared to increase in size when compared to the heterozygous *Dronc* intestines [Figure 4-6 A & B middle panel], indicating a possible propensity to differentiate into Enterocyte fate; as before this phenotype appeared to worsen from 3- to 7-days post excision [Figure 4-6 D]. Additionally, the intestines exhibited signs of hyperplasia [Figure 4-6 A & B right panel], with a significant increase in the number of differentiated cells (HistoneRFP without GFP) [Figure 4-6 E]. Conversely to the aforementioned parameters, the number of differentiated cells present at 3-days post-*Dronc* loss of function is not statistically significant and only significant at 7-days [Figure 4-6 E]. Thus, suggesting that the hyperplasia is a consequence of the earlier proliferative events and not a direct result of the loss of *Dronc* protein.

As previous observations demonstrated an increase in the average cell size of *esg*-labelled *Dronc* KO cells [Figure 4-7 F], suggesting differentiation into Enterocyte fate; I sought to confirm this using staining for the enterocyte marker, Pdm1. During Enteroblast differentiation into Enterocyte, the downregulation of *esg* facilitates the upregulation of differentiation genes, such as the POU-domain transcription factor, Pdm1. Importantly, Pdm1 is permanently expressed in enterocytes, thus acting as a marker of cell fate (177, 216). As expected in physiological conditions, *esg* and Pdm1 do not co-localise, mimicked in my *Dronc* heterozygous experimental conditions [Figure 4-6 A middle panel]. However, following the loss of *Dronc*, I observed co-localisation between the *esg* and Pdm1 markers [Figure 4-6 B middle panel]. Furthermore, the proportion of *esg* labels labelled with this marker appears significantly increased compared to control [Figure 4-6 F]. In addition to acquiring the Pdm1 marker during differentiation into Enterocytes, these cells endoreplicate their genetic material, achieving maximum ploidies of 64 chromatin copy numbers per cell (272), thus increasing their nuclear size (273, 274). Reassuringly, I observed an increase in the nuclear size of *esg* labelled cells at 7-days post-*Dronc* excision [Figure 4-6 G], confirming the Pdm1 data.

In addition to the Enterocytes, the *Drosophila* intestinal system contains a second population of terminally differentiated cells, referred to as Enteroendocrine cells. As previously I had demonstrated an increase in the number of Enterocyte cells following *Dronc* LOF; I attempted to ascertain whether there was any differentiation into Enteroendocrine cells, by quantifying the number of small HistoneRFP labelled cells

without Pdm1 or *esg* labelling. In these experiments, I observed a statistically significant increase in the number of possible Enteroendocrine cells [Figure 4-6 H]. However, the increase observed is approximately 0.71% of the total cells, and therefore the contribution of these cells to the hyperplastic phenotypes was negligible in comparison to the Enterocytes [Figure 4-6 E & H].

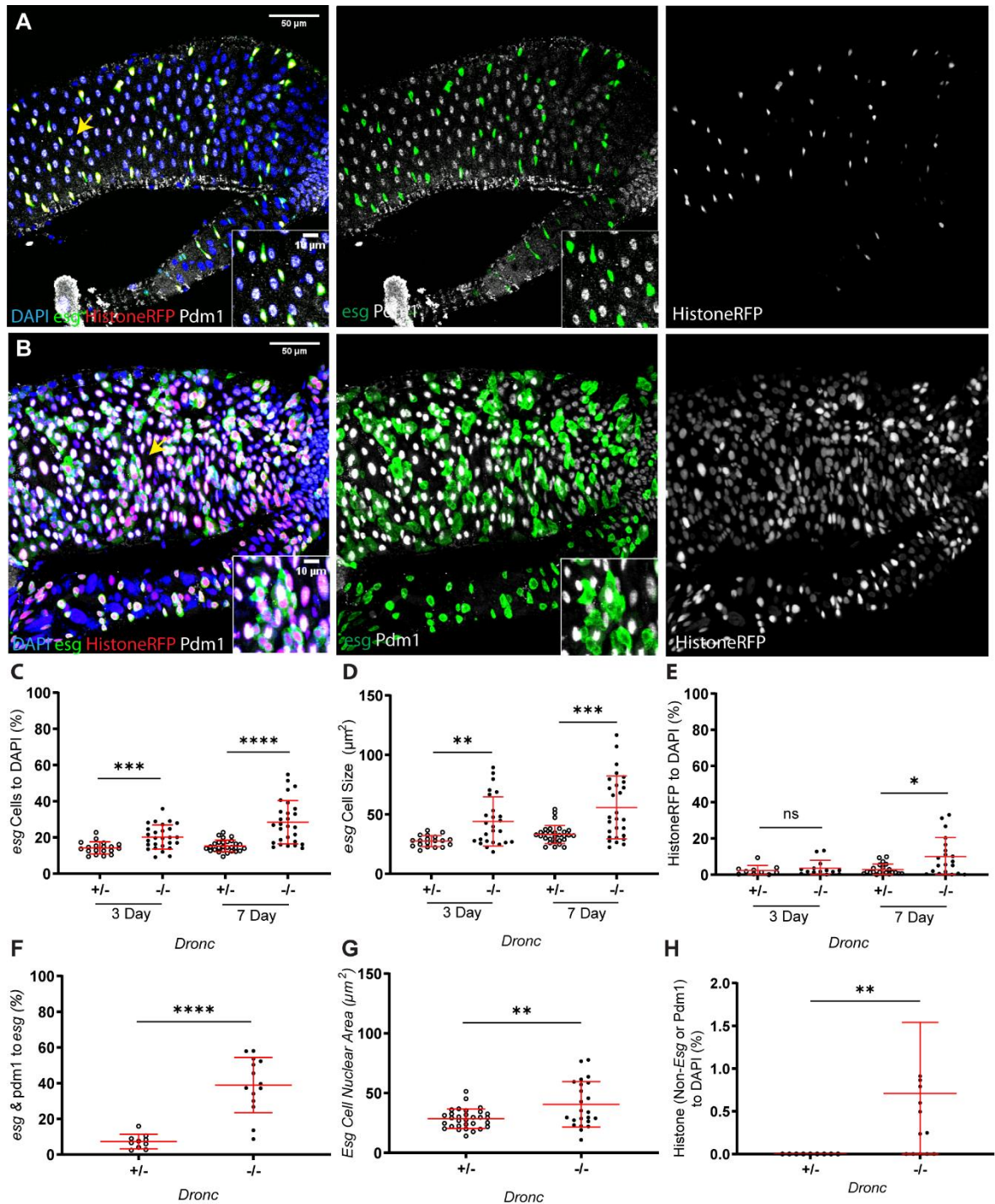


Figure 4-6: Dronc predominantly regulates the differentiation of Enteroblasts to Enterocytes.

(A) Representative image of a *Dronc* heterozygous intestine, which has activated the ReDDM tool for 7 days. *esg*-Gal4 *UAS*-mCD8-GFP (green) labels the Intestinal Stem Cells and Enteroblast cell populations. HistoneRFP acts a semi-permanent marker, allowing the tracking of progenitor cell differentiation. DAPI (blue) labels the nuclei of all cells. Pdm1 (grey) labels the nuclei of

Enterocyte cells. Enlarged inset area depicted by the yellow arrow (B) Representative image of a 7d *esg*-induced, *Dronc* KO intestine in combination with the ReDDM tool. *Esg* labelled cells appear larger than (A), and there is co-localisation between the *esg* and Pdm1 marker (see inset middle panel – compared with (A)). Furthermore, there is increased HistoneRFP labelling (see right panel and compare with (A)). (C) Quantification of *esg* labelled cells normalised to DAPI. The percentage of *esg* labelled cells is significantly higher in *Dronc* KO progenitors at 3d ($P = 0.0007$) and 7d ($p = <0.0001$) (unpaired two-tailed t-test, +/- N=32, -/- N=25). (D) Quantification of average *esg* cell size (μm^2). *Esg* labelled cell size appears significantly increased in progenitor *Dronc* KO intestines at 3d ($P = 0.0014$) and 7d ($P = <0.0001$), suggesting differentiation into Enterocyte fate (unpaired two-tailed parametric t-test, 3d +/- N = 19, 3d -/- N = 28, 7d +/- N = 33, 7d -/- N = 28). (E) Quantification of the number of cells labelled with HistoneRFP without *esg*, normalised to DAPI. The number of HistoneRFP labelled cells is significantly increased in progenitor *Dronc* KO intestines, indicating increased differentiation at 7d ($P = 0.0046$) but not 3d (Mann-Whitney test, +/- N = 24, -/- N = 20). (F) Quantification of the percentage of *esg*-positive cells labelled with Pdm1, normalised to total *esg* cells at 7d. Pdm1 labelled *esg* cells appear significantly increased in progenitor *Dronc* KO intestines ($P = < 0.0001$, unpaired t-test, +/- N=11, -/- N=14). (G) Average *esg* cell nuclei size (μm^2) is increased in *esg* knockout cells compared to heterozygotes at 7d ($P = 0.0383$, Mann-Whitney test, +/- N = 33, -/- N = 23). Quantification of small HistoneRFP cells without *esg* or Pdm1 labelling (Enteroendocrine cells) at 7d, there is a statistically significant increase ($P = 0.0028$) in this population of cells (Mann-Whitney test, +/- N = 13, -/- N = 15). Error bars represent Standard deviation of the Mean in all panels. All of the experiments described in this figure were performed in Oxford medium with an experimental regime which protects epithelial integrity. Scale Bars represent 50 μm . The genotype of the +/- *Drosophila* is $w^{1118}; \text{esg-Gal4 UAS-mCD8-GFP}; \text{UAS-HistoneRFP TubG80ts Dronc}^{KO} / \text{SM6A-TM6B}$. The genotype of the -/- *Drosophila* is $w^{1118}; \text{esg-Gal4 UAS-mCD8-GFP} / +; \text{UAS-HistoneRFP TubG80ts Dronc}^{KO} / \text{UAS-Flp FRT Dronc-GFP-APEX FRT Suntag-HA-Cherry}$.

Despite ruling out the possibility of these phenotypes being the result of QF toxicity, there remained several explanations to these phenotypes which could be independent of *Dronc* LOF. The first is that the excision of *Dronc* from the genome could result in genetic stress, which could lead these cells to behave as previously described. The second explanation could be that the Suntag-HA-Cherry, similar to the QF, is cytotoxic, and the third explanation could be that these phenotypes originate as a result of an off-target effect which occurred during the preparation of this knockout allele through CRISPR/Cas9. To rule out these possibilities, I used an alternative allele generated by the laboratory. As before, this allele consists of an FRT flanked *Dronc* sequence tagged with *GFP* and *Apex2* before the stop codon. However, in this allele upon excision, the *Dronc* wildtype protein is expressed, tagged with Suntag, HA and Cherry [Figure 4-7].



Figure 4-7: Representation of the *Dronc*-GFP-APEX2 to *Dronc*-Suntag-HA-Cherry allele

The sequence for wildtype *Dronc* tagged with *Cherry* fluorescent protein, *HA* & *Suntag* were subcloned into the *Dronc* open reading frame, after the *Dronc*-GFP-APEX2 rescue cassette. The *Dronc* rescue cassette sequence is flanked by two FRT recombinase sites. Upon excision of the cassette, the downstream *Dronc*-*Suntag*-*HA*-*Cherry* becomes expressed.

Following *esg*-induced excision of the rescue cassette and subsequent expression of *Dronc*-Suntag-HA-Cherry under the regulation of the endogenous *Dronc* locus; I was unable to observe any obvious morphological defects following expression of the tagged

Dronc protein [Figure 4-8]. Furthermore, I was unable to recapitulate the excessive proliferation and hyperplastic phenotypes observed with the previous *Dronc* KO alleles [Figure 4-6 B & Figure 4-8]. Importantly, as before I was unable to detect the expression of both the Dronc-GFP-Apex and Dronc-Suntag-HA-Cherry constructs. These experiments successfully confirmed that the phenotypes observed from the previous alleles were a consequence of *Dronc* loss of function within the progenitor cells and not alternative factors.

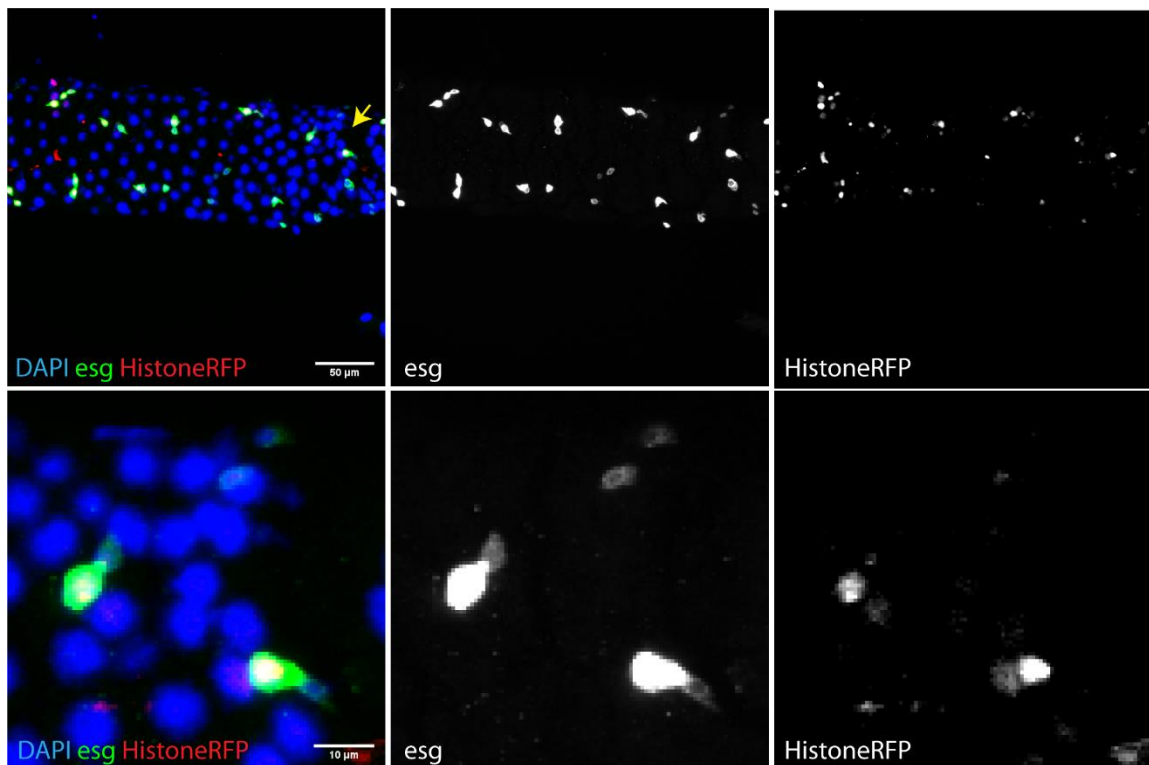


Figure 4-8: Expression of a wildtype tagged version of Dronc does not recapitulate the *Dronc* LOF phenotypes.

(A) Representative image of an intestine expressing a Suntag-HA-Cherry tagged version of Dronc, in addition to activating the ReDDM labelling system, for 7d. *Esg*-Gal4 *UAS*-mCD8-GFP (green) labels the Intestinal Stem Cell and Enteroblast cell populations. HistoneRFP acts a semi-permanent labelling, allowing tracking of progenitor cell differentiation. DAPI (blue) labels the nuclei of all cells. No intestinal defects are observed from the expression of Dronc-Suntag-HA-

Cherry. (B) Arrow in (A) indicates the enlarged area depicted. The experiment described in this figure was performed in Oxford medium with an experimental regime which protects epithelial integrity. Scale Bars represent 50 μm . The genotype of the *Drosophila* in this figure is *w¹¹¹⁸; esg-Gal4 UAS-mCD8-GFP / +; UAS-HistoneRFP TubG80ts / UAS-Flp FRT Dronc-GFP-APEX FRT Dronc-Suntag-HA-Cherry*.

The catalytic activity of Dronc is required to prevent hyperplasia

Previous studies have demonstrated a role for Dronc in regulating signalling events independently of its enzymatic activity, i.e. through protein-protein interactions (89). Thus as a next step, I investigated whether the catalytic activity of Dronc was required for maintaining intestinal homeostasis, or merely the presence of the protein. To discriminate between these possibilities, I utilised a further two conditional alleles. The first allele upon rescue-cassette excision expresses a catalytically inactive and uncleavable version of the protein, through amino acid substitutions at positions 318 and 352 respectively (FLCAEA) (55) [Figure 4-9]. The second allele contains the previous two substitutions, but also contains a truncated form of the caspase recruitment domain, CARD (Δ CAEA) (54, 55) [Figure 4-9]. Importantly, both versions of the protein are expressed under the endogenous *Dronc* promoter.

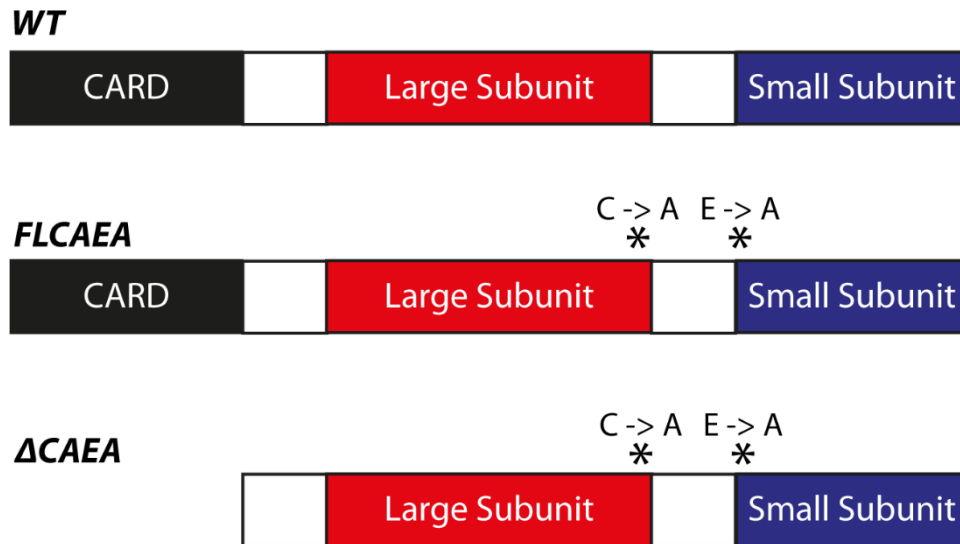


Figure 4-9: Schematic of Dronc activity alleles.

The WT Dronc protein contains the activation and caspase recruitment domain (CARD), a large subunit and a small subunit. The FLCAEA contains all of these components, but has a Cysteine to Alanine mutation at the C-terminus of the Large subunit (position 318) and a Glutamic acid to Alanine substitution at 352, rendering it catalytically inactive and uncleavable. The ΔCAEA contains these mutations, in addition to the deletion of the CARD domain. This figure is redrawn and adapted from (54).

Following, *esg*-mediated excision of the *Dronc*-rescue cassette and the expression of the FLCAEA version of Dronc, I observed a recapitulation of the *Dronc* LOF phenotypes; i.e. increased proliferation and average size of *esg* labelled cells, and an increased number of HistoneRFP labelled cells [Figure 4-10 B, D-F]. However, the expression of this protein appeared to have a slightly less penetrant phenotype when compared to those resulting from *Dronc* loss of function, in regards to proliferation [Figure 4-10 E], but equivalent in terms of cell size increase and differentiation [Figure 4-10 F-G]. To further validate these findings, I used the ΔCAEA allele. As expected, these results strongly correlated with the

FLCAEA allele [Figure 4-10 C-F]. Collectively suggesting, that the catalytic activity of *Dronc* is required to regulate the process of proliferation and differentiation to prevent hyperplasia, and the presence of Dronc alone is insufficient to prevent premature differentiation of intestinal precursors. Furthermore, this data also suggests that the CARD domain does not have a prominent regulatory role in these processes, owing to the similarities in phenotypes between the two alleles [Figure 4-10 B-E].

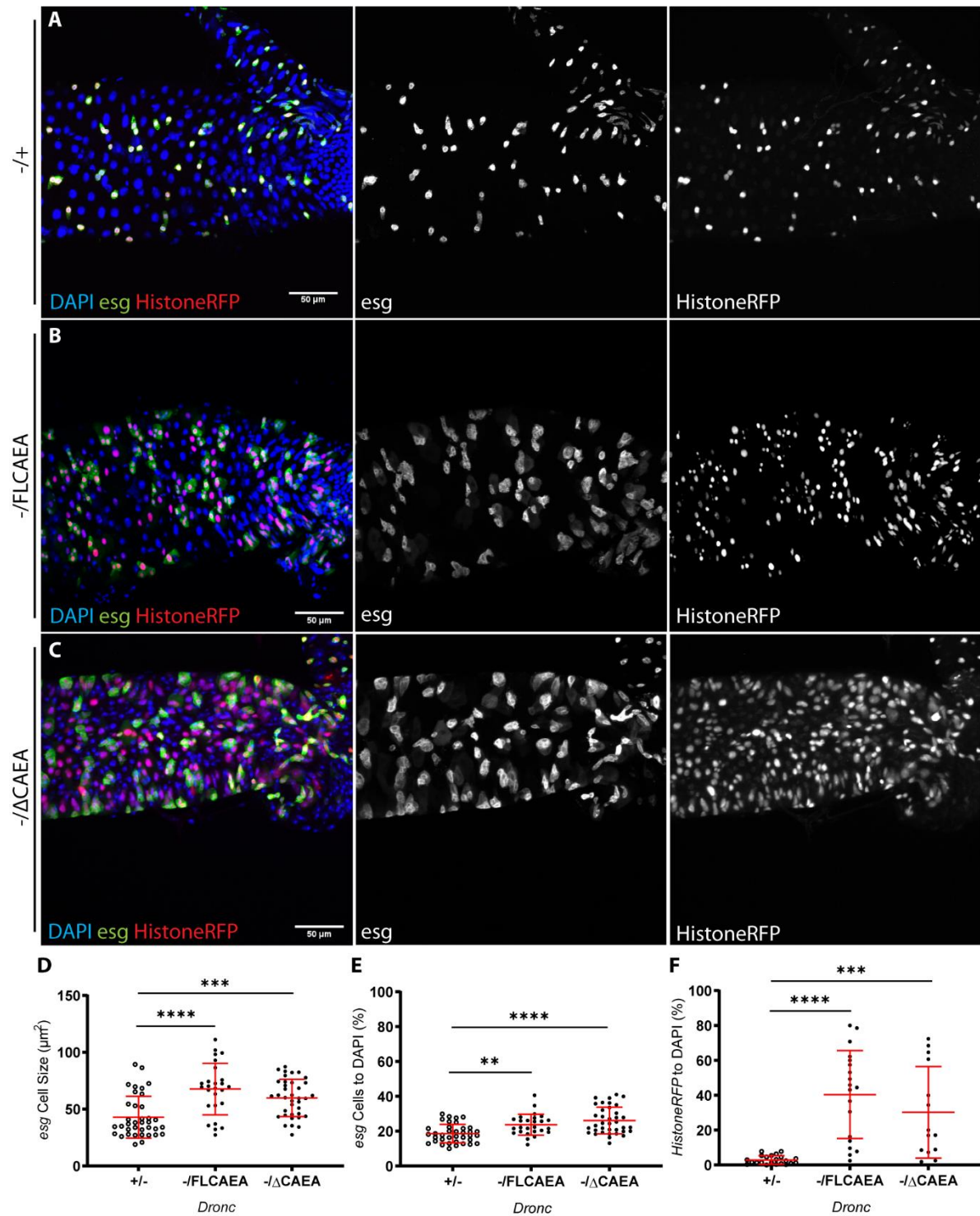


Figure 4-10: The catalytic activity is required for non-apoptotic Dronc function.

(A) Representative image of a *Dronc* heterozygous intestine activating the ReDDM reporter for 7d. *Esg*-Gal4 UAS-CD8-GFP (green) labels Intestinal Stem Cells and Enteroblasts; UAS-HistoneRFP (red) labels *esg* cells, but is also retained in differentiated cells. DAPI (blue) labels the nucleus of all cells (B) A representative image of an intestine expressing the catalytically inactive and

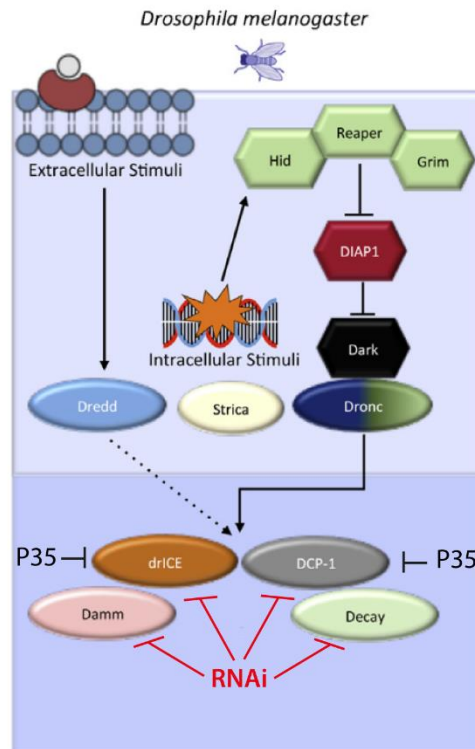
uncleavable form of Dronc (FLCAEA) for 7d, in combination with the ReDDM reporter. The *Dronc* FLCAEA recapitulates similar phenotypes to those observed in the KO. *Esg* labelled cells appear enlarged and guts appear hyperplastic compared to (A). (C) A catalytically inactive and uncleavable form of Dronc without the CARD domain (Δ CAEA), recapitulates similar phenotypes to those observed in the KO and (B). (D) Quantification of the percentage of *esg* cells relative to DAPI. *Esg* cell number appears to increase in FLCAEA ($P = 0.0054$) and Δ CAEA ($P = <0.0001$) expressing intestines compared to controls (Dunnett's multiple comparisons test, +/- N = 39, -/FLCAEA N = 26, -/ Δ CAEA N = 37). (E) Quantification of the average *esg* cell size (μm^2). *Esg* labelled cell size is significantly increased in the FLCAEA ($P = <0.0001$) and Δ CAEA ($P = 0.0004$) genetic backgrounds (Dunnett's multiple comparisons test, +/- N = 39, -/FLCAEA N = 26, -/ Δ CAEA N = 37). (F) Quantification of the percentage of cells Histone-RFP positive without *esg* labelling relative to DAPI. HistoneRFP alone cells are significantly increased in the FLCAEA ($P = <0.0001$) and Δ CAEA ($P = 0.0004$) genetic backgrounds (Dunnett's multiple comparisons test, +/- N = 39, -/FLCAEA N = 26, -/ Δ CAEA N = 37). Error bars represent Standard Deviation of the Mean in all panels. All of the experiments described in this figure were performed in Oxford medium with an experimental regime which protects epithelial integrity. Scale Bars represent 50 μm . The genotype of the -/+ *Drosophila* is $w^{1118}; \text{esg-Gal4 UAS-mCD8-GFP}; \text{UAS-HistoneRFP TubG80ts Dronc}^{KO} / \text{SM6A-TM6B}$. The genotype of the -/FLCAEA *Drosophila* is $w^{1118}; \text{esg-Gal4 UAS-mCD8-GFP} / +; \text{UAS-HistoneRFP TubG80ts Dronc}^{KO} / \text{UAS-Flp FRT Dronc-GFP-APEX FRT Dronc-FLCAEA-Suntag-HA-Cherry}$. The genotype of the -/ Δ CAEA *Drosophila* is $w^{1118}; \text{esg-Gal4 UAS-mCD8-GFP} / +; \text{UAS-HistoneRFP TubG80ts Dronc}^{KO} / \text{UAS-Flp FRT Dronc-GFP-APEX FRT Dronc-}\Delta\text{CAEA-Suntag-HA-Cherry}$.

Dronc functions independently of the canonical apoptosis pathway

Following demonstrating that the catalytic activity of Dronc was required to regulate proliferation, cell size and differentiation within the *Drosophila* intestinal system, I sought to determine the cleavage substrate of Dronc which could be orchestrating this regulation. As the substrates of Dronc in many contexts, including apoptosis, are the effector caspases (Damm, drICE, DCP-1 & Decay) [Figure 4-11 A], I hypothesised that these could be the mediators of the Dronc activity. Moreover, previous studies in the *Drosophila* intestine have demonstrated that the Hippo-induced cleavage of Brahma by the caspases, was inhibited through application of caspase-3 and caspase-10 inhibitors, implying a partial requirement for the effector caspases in this process (104).

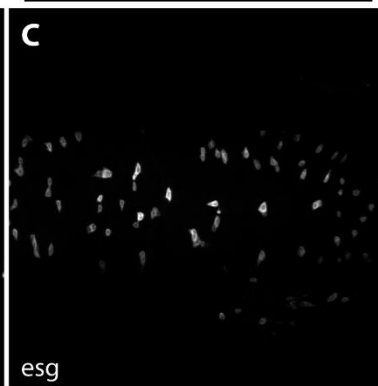
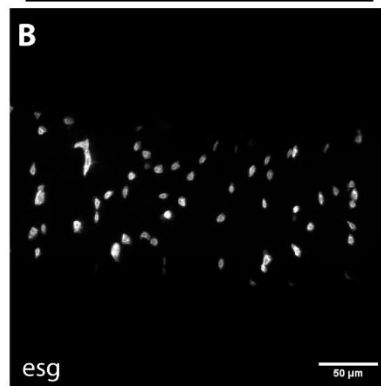
In a previous experiment, I had already excluded the enzymatic requirement for the effector caspases, since overexpression of two copies of P35 failed to alter any cellular or morphological features of the intestine [Chapter 3 + 4-11 C]. Nevertheless, I could not rule out the possibility of the effector caspases acting as scaffolding proteins. To circumvent this possibility, I simultaneously overexpressed validated RNAis against all of the effector caspase members (Damm, drICE, DCP-1 & Decay) [Figure 4-11 A] (275). This also prevented possible functional redundancy between the effector caspases, as seen in some cellular contexts (256, 276). Intriguingly, this set of experiments failed to recapitulate the excessive proliferation and differentiation phenotypes observed during the loss of *Dronc* function [Figure 4-11 D].

A



-/+

esg > 2 x P35



esg > Effector Caspase RNAi

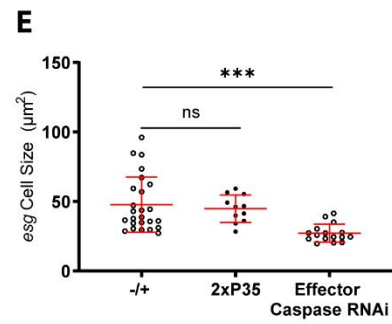
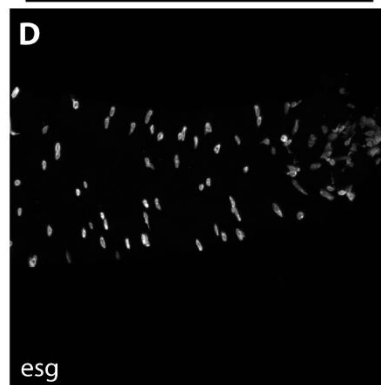


Figure 4-11: Dronc regulation of progenitors is independent of the effector caspases.

(A) The *Drosophila* apoptosis pathway. Canonically, upregulation of the pro-apoptosis proteins, Hid, Grim and Reaper, degrade the inhibitor of apoptosis proteins (DIAP1). The loss of DIAP1 precedes the activation and formation of the apoptosome by Dark and Dronc. The apoptosome subsequently activates the effector caspases (Damm, drICE, DCP1-1 & Decay), resulting in apoptosis (39). P35 targets the catalytic activity of the drICE and DCP-1 rendering them inactive (277). Validated RNAis knockdown the protein levels of all effector caspases (275). (B) Representative image of a 7d *Dronc* heterozygous intestine. Esg (grey) labels Intestinal Stem Cells and Enteroblasts. (C) *Dronc* heterozygous intestine overexpressing 2 x P35 under the regulation of the *esg* promoter. As before overexpression of two copies of P35 does not cause any cellular defects. (D) *Dronc* heterozygous intestine overexpressing RNAis against Damm, drICE, DCP-1 and Decay under the regulation of the *esg* promoter. Similar to (C), RNAi knockdown does not produce any obvious phenotypes. (E) Quantification of the average *esg* cell size (μm^2). There is no significant change in cell size when overexpressing two copies of P35 ($P = > 0.9999$) and a statistically significant decrease when overexpressing RNAi ($P = 0.0003$), under the regulation of the *esg* promoter (Dunn's multiple comparisons test, +/- N = 25, 2 x P35 N = 11, Effector Caspase RNAi N = 16). Error bars represent Standard Deviation of the Mean in all panels. All of the experiments described in this figure were performed in Oxford medium with an experimental regime which protects epithelial integrity. Scale Bars represent 50 μm . The Genotype of the +/- *Drosophila* is $w^{1118}; \text{esg-Gal4 UAS-mCD8-GFP}; \text{UAS-HistoneRFP TubG80ts Dronc}^{KO} / \text{SM6A-TM6B}$. The genotype of the *esg* < 2 x P35 *Drosophila* is $w^{1118}; \text{esg-Gal4 UAS-mCD8-GFP} / \text{UAS-P35}; \text{UAS-HistoneRFP TubG80ts Dronc}^{KO} / \text{UAS-P35}$. The genotype of the *esg* > Effector Caspase RNAi *Drosophila* is $w^{1118}; \text{esg-Gal4 UAS-mCD8-GFP} / \text{UAS-Drice RNAi UAS-Decay RNAi}; \text{UAS-HistoneRFP TubG80ts Dronc}^{KO} / \text{UAS-Damm RNAi UAS-DCP1 RNAi}$.

Following the observation that the effector caspases were not required for Dronc regulation of intestinal progenitors, I attempted to establish whether the upstream activators of Dronc were essential for this process. Using a validated micro-RNA line, I targeted the expression of the major pro-apoptotic factors, Reaper, Hid and Grim (RHG), in *esg*-expressing cells (278) [Figure 4-11 A]. I was unable to detect any morphological or cellular changes upon overexpression of the micro-RNA line [Figure 4-12]. Collectively, these results suggested that the Dronc regulation of progenitor cell properties is reliant on its catalytic activity, but is entirely independent of the effector caspases, the upstream activators and consequently the apoptosis program.

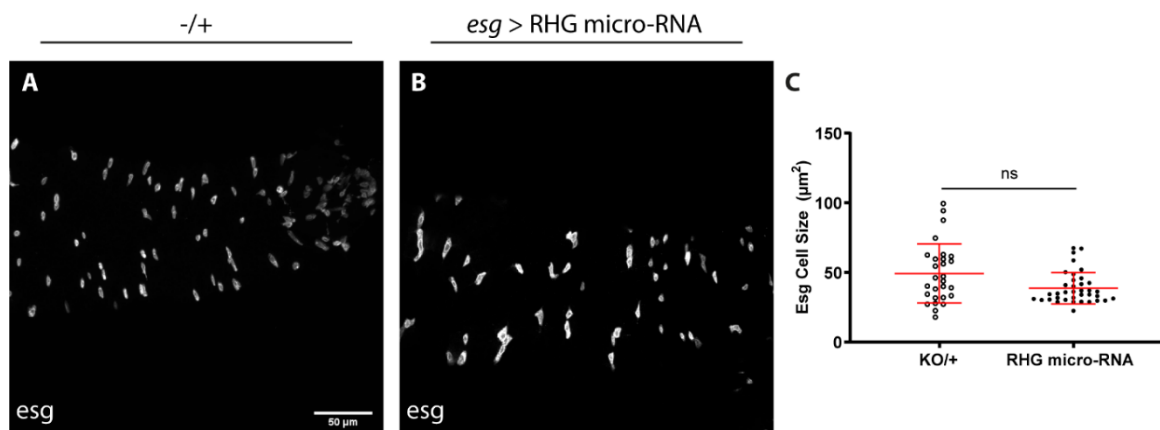


Figure 4-12: Dronc regulation of progenitors is independent of the pro-apoptotic proteins.

A) Representative image of a 7d *Dronc* heterozygous intestine. Esg (grey) labels all of the Intestinal Stem Cells and Enteroblasts. (B) Representative image of a 7d *Dronc* heterozygous intestine, overexpressing a micro-RNA for Reaper, Hid and Grim under the regulation of the *esg* promoter. There are no apparent phenotypes. (C) Quantification of the average *esg* cell size. There is no significant change in cell size when overexpressing a micro-RNA against Reaper, Hid and Grim ($P = 0.502$, Mann-Whitney, $+/- N = 28$, RHG micro-RNA $N = 41$). Error bars represent Standard Deviation of the Mean in all panels. All of the experiments described in this figure were

performed in Oxford medium with an experimental regime which protects epithelial integrity. Scale Bars represent 50 μm . The genotype of the $-/+$ *Drosophila* is $w^{1118}; \text{esg-Gal4 UAS-mCD8-GFP}; \text{UAS-HistoneRFP TubG80ts Dronc}^{KO} / \text{SM6A-TM6B}$. The genotype of the *esg* > RHG micro-RNA *Drosophila* is $w^{1118}; \text{esg-Gal4 UAS-mCD8-GFP} / \text{UAS-RHG miRNA}; \text{UAS-HistoneRFP TubG80ts Dronc}^{KO} / +$.

4.2 Discussion

In this section, I have demonstrated that the elimination of *Dronc* in *esg*-expressing cells increased the proliferation and differentiation of intestinal progenitors, promoting the onset of hyperplasia. Subsequently, I was able to demonstrate that the catalytic activity of *Dronc* was required to prevent the appearance of these phenotypes. The CARD domain appeared to have a negligible role in this process however, as the expressed ΔCAEA allele is additionally both proteolytically inactive and uncleavable, it is difficult to fully ascertain its role in this context. Using the caspase activity inhibitor, P35 and an RNAi line against all of the known effector caspases, I was unable to recapitulate the phenotypes observed during *Dronc* LOF conditions. This suggests that the effector caspases and the apoptotic machinery are not required for *Dronc* regulation of progenitor function. Finally, when overexpressing a micro-RNA line against the pro-apoptotic factors, I was also unable to recapitulate the *Dronc* LOF phenotypes, suggesting the upstream canonical apoptosis pathway was also not involved in the *Dronc* regulation of intestinal progenitors.

Dronc is required in progenitor cells to prevent hyperplasia

Loss of *Dronc* within the Intestinal Stem Cells and Enteroblasts produces striking phenotypes within the midgut, namely excessive proliferation and differentiation, resulting in hyperplasia. Aberrant caspase signalling is frequently observed in tumorigenesis and is considered a requirement for the formation of cancer, however, canonically these defects have been attributed to evasion of the apoptotic process (71, 279-282). Conversely, as previously demonstrated in my experimental conditions, I observe no evidence of apoptosis. Furthermore, inhibition of the effector caspases does not result in increased cell number as would be expected if the apoptosis process was impeded. Recent data has demonstrated that the caspases can regulate the proliferation and differentiation of embryonic and adult stem cells, independently of the apoptosis process (39). Furthermore, their deregulation has been attributed to differentiation and proliferation defects in colon tumours (109). Thus, the data presented in this chapter would imply a non-apoptotic function for Dronc within the *Drosophila* intestinal system in preventing hyperplasia, rather than a consequence of defects in the apoptosis pathway.

The catalytic activity of Dronc is required independently of the apoptotic pathway

Previous studies have demonstrated that Dronc is able to regulate the proliferation and differentiation of neuroblasts, through a protein-protein interaction with Numb. Conversely, other studies have demonstrated that the proteolytic activity of the caspases is necessary for the cleavage of shaggy (GSK-3 β), in the regulation of neural

precursors (87). The data presented in this thesis suggests that within the intestinal system, the presence of Dronc itself is insufficient to prevent hyperplasia, and thus the proteolytic activity of the Dronc is required. However, the canonical substrates of Dronc proteolytic activity, the effector caspases, cannot be involved as their inhibition does not produce any morphological defects. The substrate must, therefore, be independent of the apoptosis pathway. Furthermore, inhibition of the initiator of apoptosis proteins (hid, reaper and grim) also failed to recapitulate any Dronc loss of function phenotypes. Collectively suggesting, that Dronc regulation of the intestinal system occurs entirely independently of the apoptosis pathway.

5. Dronc specifically modulates the differentiation of Enteroblasts through the fine-tuning of the Notch and Insulin/mTOR signalling pathways

5.1 Results

Dronc is specifically required within the Enteroblasts

In the previous chapter, I demonstrated that loss of *Dronc* leads to an increase in proliferation and differentiation of intestinal progenitors, resulting in hyperplastic phenotypes. Furthermore, the regulation of these processes required the catalytic activity of Dronc, but was independent of the effector caspases and the apoptotic pathway. As a next step, I attempted to establish which of the progenitor cells were responsible for the phenotypes, as *esg-Gal4* is expressed in both the Intestinal Stem Cells and the Enteroblasts.

To better untangle the functional requirement of Dronc in intestinal progenitors, I used a different driver to knockout *Dronc*, which is specifically expressed in the Intestinal Stem Cells (*Delta-Gal4*) [Figure 5-1] (128, 283).

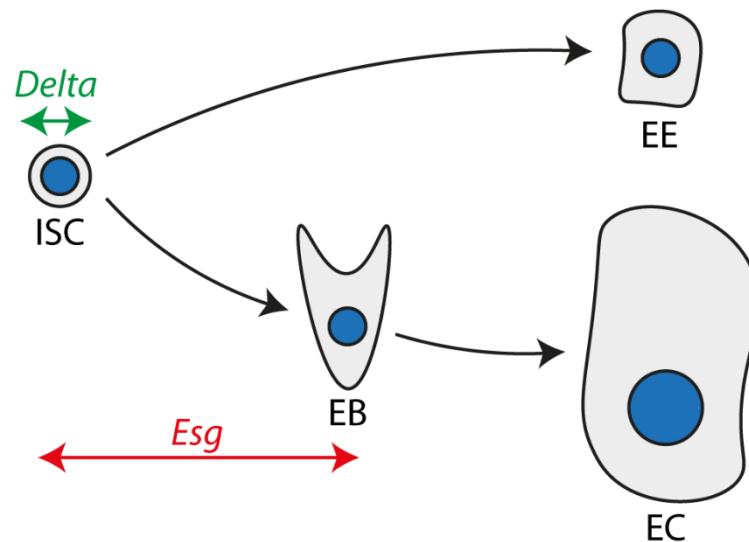


Figure 5-1: Expression pattern of the Delta and esg drivers.

Delta-Gal4 is solely expressed in the Intestinal Stem Cells, with its expression lost during the transition from Intestinal Stem Cell (ISC) to Enteroblast (EB). The stemness marker *esg*, is expressed in both Intestinal Stem Cells and Enteroblasts.

Before establishing the functional relevance of the loss of *Dronc* in Intestinal Stem Cells, I quantified the excision efficiency of the *Dronc* rescue cassette, using the *Delta*-Gal4 driver. Similar to the data obtained with the *esg*-Gal4 driver, I obtained a high rate of efficiency, with 81.15% [Figure 4-2 A&B] of Intestinal Stem Cells positive for LacZ labelling (please see explanations relating to the calculation of the excision efficiency in Chapter 4). Interestingly however, loss of *Dronc* within this population of cells failed to demonstrate any cellular or morphological alterations of the Intestinal tissue [Figure 4-2 A].

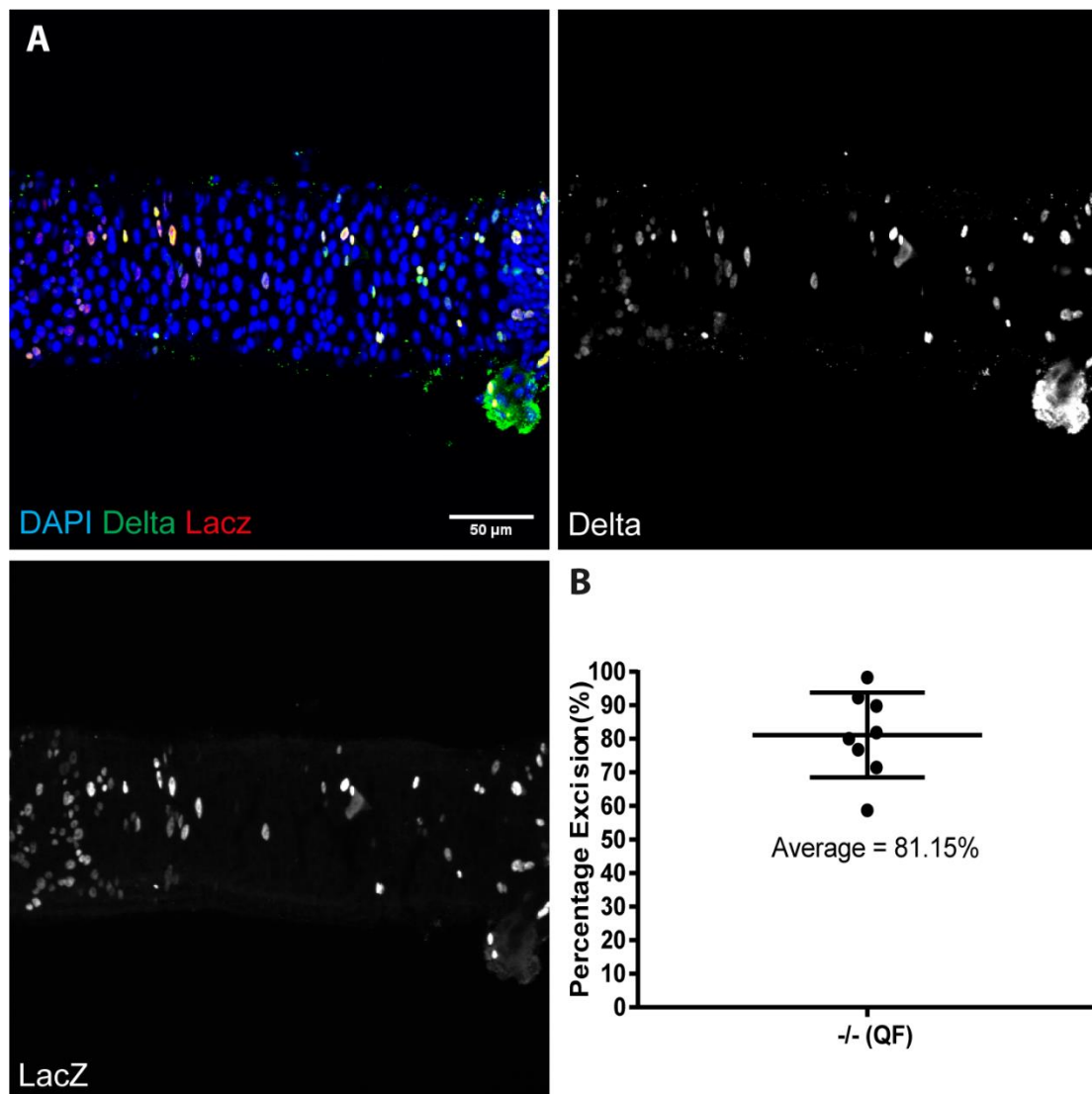


Figure 5-2: Delta-Gal4 induced *Dronc* excision demonstrates high efficiency.

(A) 3d post-activation of the fippase recombinase machinery under the regulation of *Delta*-GAL4, in the R4/5 region of the *Drosophila* posterior midgut. DAPI (blue) labels the nuclei of all cells. *Delta*-Gal4 UAS-GFP (green) labels all of the Intestinal Stem Cells, and LacZ (red) labels the *Dronc* rescue-cassette excision in *Dronc* expressing tissues. There is strong co-localisation between the two markers, suggestive of a high rate of excision. (B) 3d following activation of the recombinase machinery I observe an average excision efficiency of 81.15% (N = 14). Error bars represent Standard deviation of the Mean. The experiment described in the figure was performed in Oxford medium with an experimental regime which protects epithelial integrity. Scale Bars represent 50

µm. The genotype of the *Drosophila* in this figure is W¹¹¹⁸; + / QUAS-LacZ; TubG80ts *Dronc*^{KO} Delta-Gal4 / UAS-FLP FRT *Dronc*-GFP-APEX FRT QF.

To confirm these observations, I utilised the *Dronc* Suntag-HA-Cherry rescue-cassette allele. In *Delta* mediated excision of the rescue cassette, I did not observe an increase in cell number [Figure 5-3 A-C] or cellular size [Figure 5-3 A,B & D], following *Dronc* KO. As Intestinal Stem Cells have been observed to differentiate directly into Enteroendocrine cells, I quantified the number of small nuclei expressing the Enteroendocrine marker, Prospero. Contradictory to the *esg*-Gal4 driven excision of the *Dronc* rescue cassette, I did not observe any increase in cell number in the Enteroendocrine cell population (See 5.2 Discussion) [Figure 5-3 A,B & E]. The data unambiguously indicated a specific requirement for the catalytic activity of *Dronc* in the Enteroblasts. Furthermore, it explained the differentiation preference of *Dronc* KO progenitors towards Enterocyte fate.

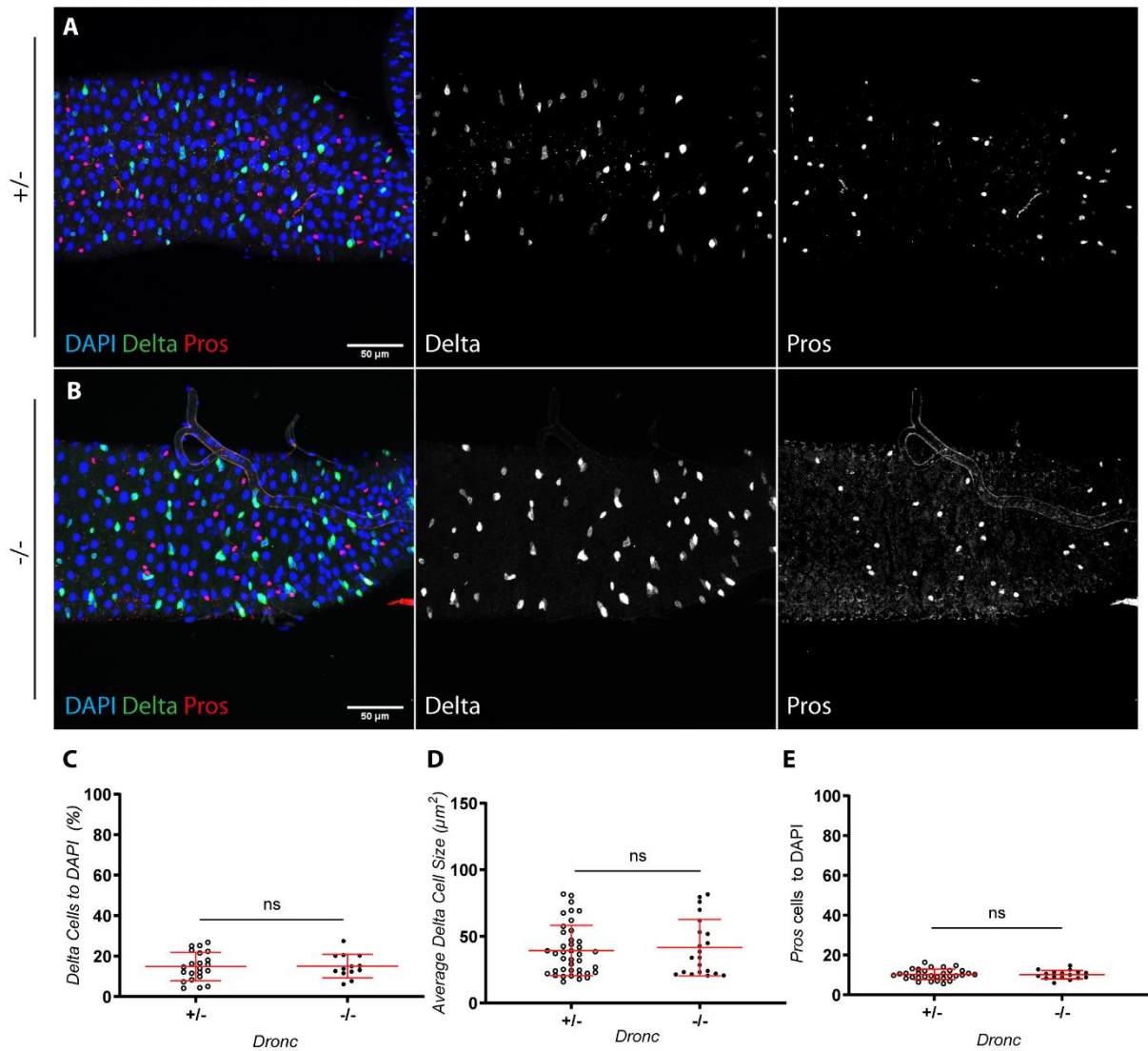


Figure 5-3: *Delta*-induced *Dronc* loss of function produces no noticeable phenotypes.

(A) 7d *Dronc* heterozygous Intestines. DAPI (blue) labels the nucleus of all cells, *Delta*-Gal4 UAS-GFP (green) labels Intestinal Stem Cells, and Prospero (red) labels the nucleus of Enteroendocrine cells (B) *Dronc* knockout Intestinal Stem Cells do not display clear morphological or numerical differences to those observed in (A). (C) Quantification of the number of *Delta* labelled cells normalised to DAPI. There is no significant increase in cell number following Intestinal Stem Cell *Dronc* KO ($P = 0.9231$, unpaired two-tailed t-test, +/- $N = 22$, -/- $N = 13$). (D) Quantification of the average *Delta* cell size (μ m²). There is no significant increase in Intestinal Stem Cell size following *Dronc* KO ($P = 0.9694$, unpaired two-tailed t-test, +/- $N = 42$, -/- $N = 21$). Quantification of the number of Prospero labelled cells relative to DAPI. There is no significant change in Prospero cell

number following *Dronc* KO in the Intestinal Stem Cells ($P = 0.8193$, unpaired two-tailed t-test, +/- $N = 31$, -/- $N = 17$). The genotype of the +/- *Drosophila* is $W^{1118}; +/+; TubG80ts Dronc^{KO} Delta-Gal4 / TM6B$. The genotype of the -/- *Drosophila* is *Dronc*^{KO} & *Dronc*-GFP-APEX to *Suntag*-HA-Cherry rescue cassette under the regulation of the *Delta* promoter.

The specificity of Dronc within Enteroblasts appears related to its protein stabilisation

The aforementioned data suggest that the non-apoptotic, functional role for Dronc was restricted to the Enteroblast cell population; however, the molecular origin of this specificity remained elusive. To elucidate this question, I attempted to explore whether the transcriptional regulation of *Dronc* was restricted to Enteroblasts, thus explaining its functional specificity. To test this hypothesis, I used a transgenic fly line generated by the Baena-Lopez laboratory, containing the sequence for the *Gal4* transcription factor inserted into *Dronc* locus. The *Gal4* becomes a de facto transcriptional readout of *Dronc* and can be visualised through the inclusion of a fluorescent protein (i.e. UAS-GFP).

Using the *Dronc*^{GAL4} transgenic line, I was able to observe widespread transcription of *Dronc* throughout the whole of the posterior midgut [Figure 5-4 A]. Furthermore, I also detected *Dronc* transcription within the innervating neurons of the intestinal system [Figure 5-4 A & B]. Additionally, both small cells (likely Intestinal Stem Cells, Enteroblasts or Enteroendocrine cells), in addition to large cells (Enterocytes), were positively labelled with the reporter, correlating with the apical-caspase activity observed in all cell types using DBS-S-QF reporter.

Using the *Dronc*^{Gal4} reporter in combination with the DBS-S-QF reporter, I was able to observe differences in the transcriptional activity of *Dronc* and the apical-caspase activity detected, using the DBS-S-QF reporter. On closer inspection of the intestine, I observed cells which were positive for DBS-S-QF activity, but negative for *Dronc* transcription; cells negative for DBS-S-QF activity, but transcriptionally expressing *Dronc*; or cells which were positive for both [Figure 5-4 C]. Collectively, the widespread nature of *Dronc* transcription throughout all cells of the posterior midgut, suggested that the functional specificity for *Dronc* in Enteroblasts was not regulated by transcription.

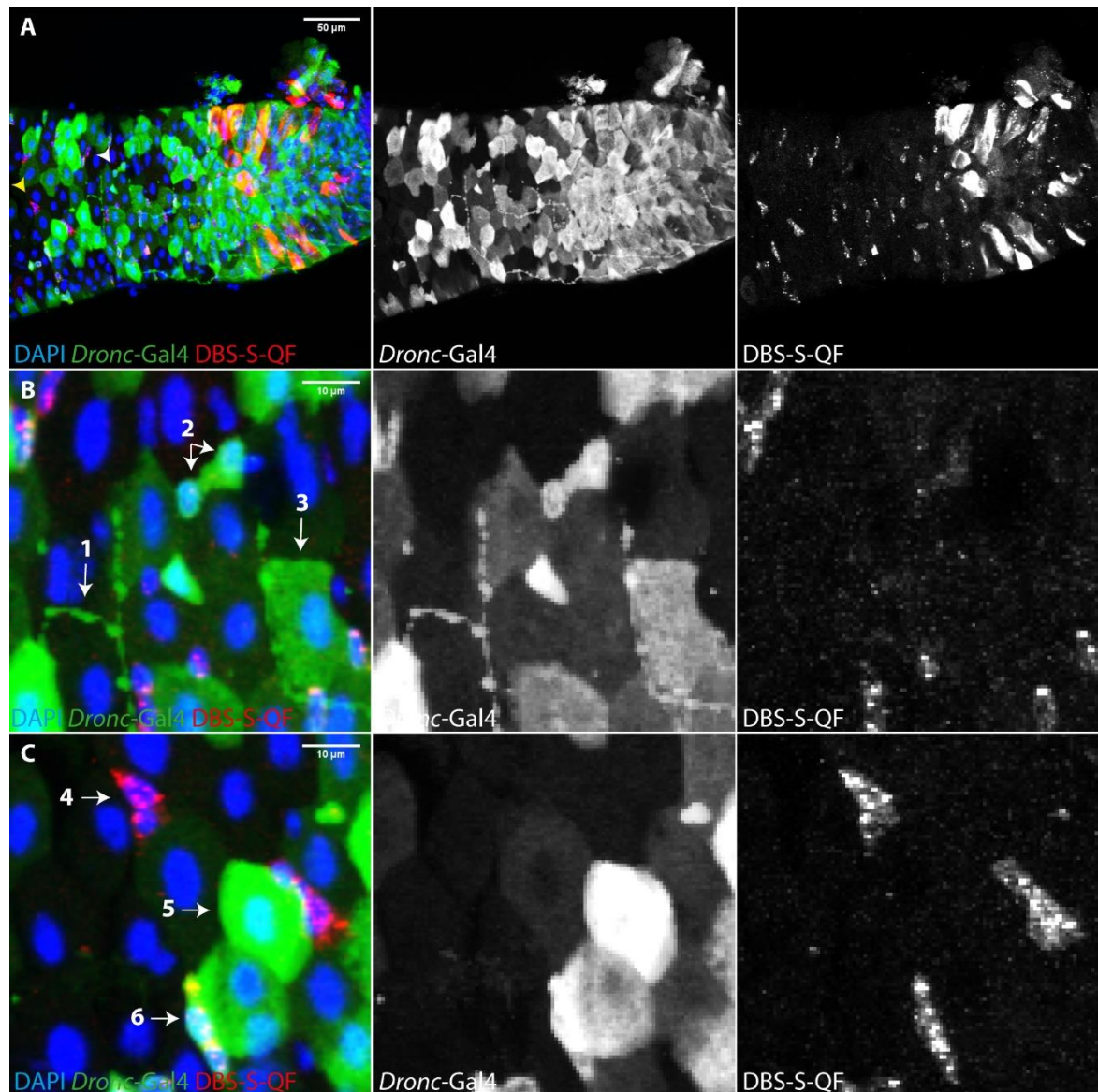


Figure 5-4 *Dronc* is widely expressed throughout the midgut

(A) Representative image of the R4/5 region of a 7d intestine with the DBS-S-QF and the *Dronc*^{Gal4} transcriptional reporter. DAPI labels all cell nuclei (blue). The transcriptional reporter of *Dronc* (green) can be observed throughout the whole of the posterior midgut. The apical-caspase reporter, DBS-S-QF, is labelled in red. (B) Enlargement of the area in (A) indicated by the white arrow. Transcription of *Dronc* can be observed in the intestinal neurons (1), in small cells likely to be intestinal precursors by morphology (2) and large enterocyte cells (3). (C) Enlargement of the area in (A) indicated by the yellow arrow. DBS-S-QF activation can be observed without labelling of the *Dronc* transcriptional reporter (4), or conversely, the transcriptional reporter of *Dronc* can

be active, without activation of the DBS-S-QF reporter (5), or the activity of both can be observed co-localising within the small cells (6). All of the experiments described in the figure were performed in Oxford medium with an experimental regime which protects epithelial integrity. Scale Bars represent 50 μ m. The genotype of the *Drosophila* in this figure is DBS-S-QF UAS-CD8-GFP QUAS-dTomato-HA / + ; + / +; *Dronc*^{Gal4} / TM6B.

Next, I sought to establish whether *Dronc* protein levels could be differentially regulated in Enteroblasts. Previously, the Baena-Lopez laboratory created several *Dronc* alleles tagged at the C-terminus with different fluorescent proteins and peptides, under the endogenous regulation of the *Dronc* promoter. Using these tools, I was unable to visualise the presence of these tagged versions of *Dronc* with immunofluorescence techniques. To circumvent this issue, the laboratory developed a new *Dronc*-protein sensor. This new sensor ubiquitously expresses a version of *Dronc* made catalytically inactive through a Cysteine to Alanine amino acid substitution at position 318, under the regulation of the *Actin* promoter. Thus, this allele of *Dronc* is unable to induce apoptosis. Furthermore, the protein is tagged at the C-terminus with a modified GFP and a Myc tag to facilitate its detection through immunofluorescence. The modified GFP (284) consists of two fragments of the GFP protein separated by an amino acid sequence, containing the *Dronc* preferential cleavage site, TETD. Importantly, the presence of this peptide inhibits the fluorescence of this protein. Upon cleavage of this sequence, by a wildtype version of *Dronc*, the two GFP fragments join and through a conformational change, becoming fluorescent [Figure 5-5]. Collectively, this new reporter enables the detection of *Dronc* activity, through the modified GFP fragment, in addition to its localisation within the cell. Importantly, the overexpression of this

construct generated fertile flies which demonstrated no evidence of developmental or morphological defects. Furthermore, Dr. Baena-Lopez was able to recapitulate the subcellular localisation of the Dronc sensor in other tissues, such as the wing imaginal discs and the salivary gland (268, 285).

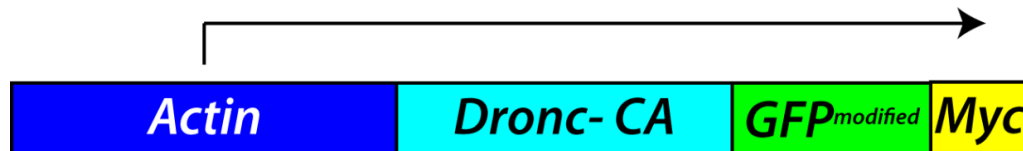


Figure 5-5 Rational Design of the Dronc-protein Reporter.

The sequence for a catalytically inactive form of *Dronc*, tagged with *GFP^{modified}* and *Myc*, was inserted downstream of the *Actin* promoter. The modified GFP only becomes activated following cleavage of the Dronc preferential cleavage site TETD, by a wildtype version of Dronc. Collectively, acting as a reporter for both Dronc activity and localisation.

I was unable to detect the activity of Dronc (through the fluorescence of the modified GFP) within the intestinal system, suggesting that as expected, the activation of Dronc is incredibly low, and only detectable through amplification of the activity (See DBS-S-QF reporter). Next, I sought to establish whether I could observe any localisation or accumulation of Dronc within any of the cells of the intestinal system. As although the *GFP^{modified}* is not fluorescent until caspase cleavage, the protein is still detectable through antibody labelling against GFP. Following immunolabeling, the Dronc-protein sensor appeared accumulated in the Intestinal Stem Cells and Enteroblasts [Figure 5-6 A]. Additionally, on closer inspection it appeared that the Dronc sensor was more highly accumulated in the Enteroblasts than the Intestinal Stem Cells. Furthermore, through

co-labelling experiments combining the Dronc sensor with the Enteroblast maker, Su(H), I detected a striking overlap between the two markers [Figure 5-6 B]. Complementing these findings, during Paraquat-induced damage to the intestinal epithelial tissue, the Enteroblast specific accumulation of Dronc disappeared, appearing widely upregulated and spread throughout all cell types [Figure 5-6 C]. Collectively, these results strongly correlated the non-apoptotic function of Dronc within Enteroblasts, with the specific stabilisation and/or accumulation of the protein.

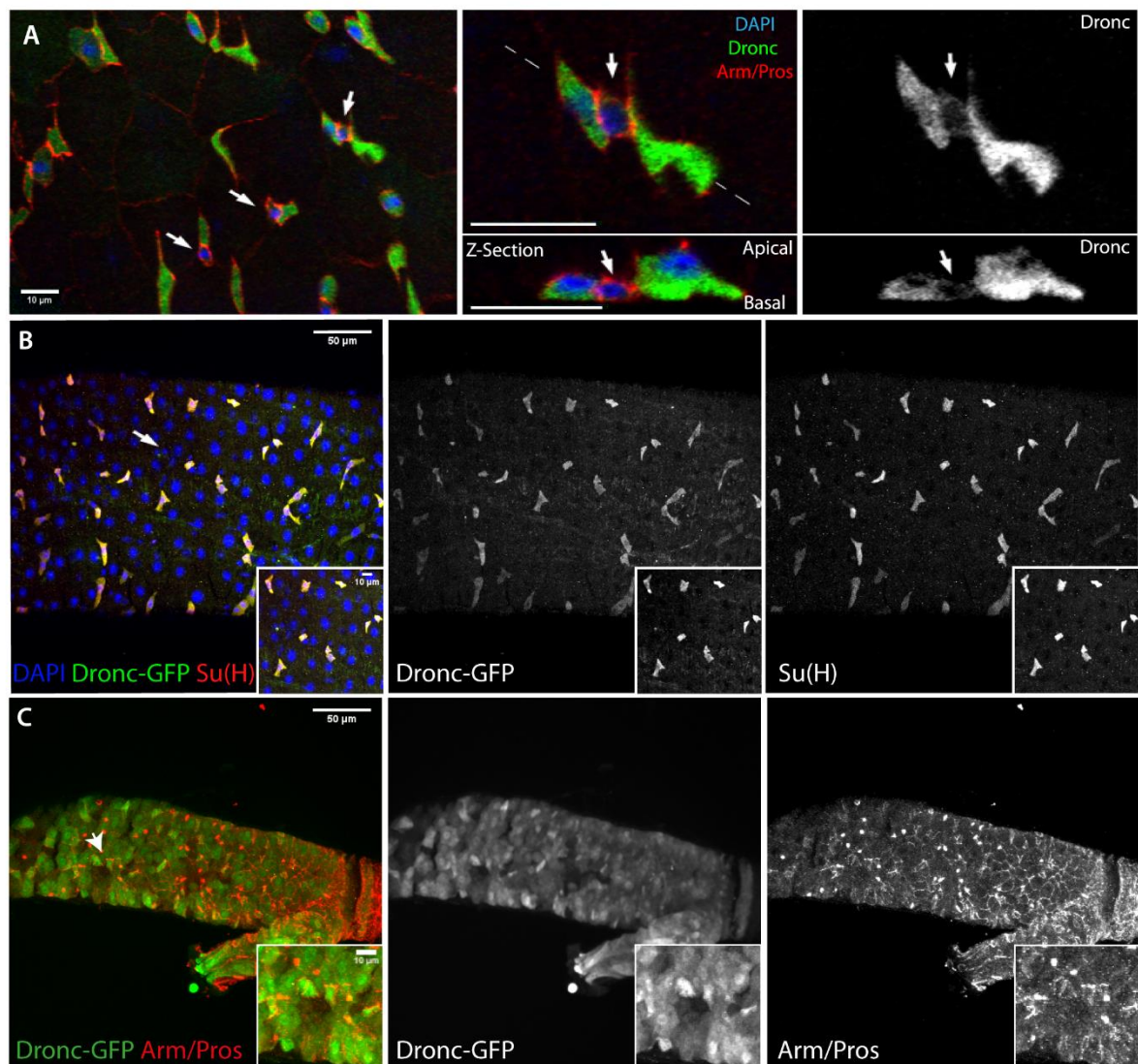


Figure 5-6: Dronc is accumulated in the Enteroblast cells.

(A) Representative confocal image of a 7d *Drosophila* posterior midgut, demonstrating the expression of Armadillo (red membrane), The Enteroendocrine cell marker Prospero (red nuclei) and Dronc (green, immunostaining against GFP). Dronc is enriched in Enteroblasts, with the white arrows indicating Intestinal Stem Cells with a lower accumulation of the Dronc protein. (B) The Enteroblast marker Su(H) (red, antibody staining against Beta-Galactosidase), strongly co-localises with the accumulation of the Dronc reporter (green, immunostaining against GFP). The white arrow indicates the area depicted in the insets. (C) Dronc-protein localisation (green, immunolabelling with anti-GFP) in the posterior midgut following a 16 hour 6.5mM treatment with paraquat; note that the Dronc specific accumulation in Enteroblasts is no longer present, the upregulation and accumulation of Dronc can instead be observed in all cell types. The genotype of the *Drosophila* in panels A and C is $w^{1118}; +/+; Actin-Dronc-GFP-MYC / TM6B$. The genotype of the *Drosophila* in panel B is $w^{1118} / Su(H)-LacZ; +/+; Actin-Dronc-GFP-MYC / TM6B$.

Dronc modulates the activity of the Notch pathway

Following the demonstration that Dronc appears to be accumulated in Enteroblasts, I next sought to establish the signalling mechanism by which Dronc mediates its non-apoptotic function. As previously stated, the Notch pathway is one of the main signalling cascades involved in the regulation of proliferation and differentiation in the *Drosophila* intestinal system (164, 286, 287). Furthermore, non-apoptotic protein-protein interactions, have been previously described between Dronc and the Notch pathway regulator, Numb. (89). Taking into account these precedents, I conducted classical

genetic epistasis to evaluate the potential genetic interplay between Dronc and the Notch pathway.

The interaction between the extracellular domain of the Notch receptor with its ligands, Serrate or Delta (Jagged in mammals), initiates the evolutionally conserved signalling cascade, through two proteolytic cleavage events. These cleavage events catalysed by various proteases, lead to the release of the Notch Intracellular domain (Notch^{Intra}) from the Notch receptor. The Notch^{Intra} fragment then translocates to the nucleus, where it interacts directly with the Suppressor of Hairless (Su(H)) DNA-binding protein, and the co-activator Mastermind to promote transcription of Notch responsive genes (288) [Figure 5-7].

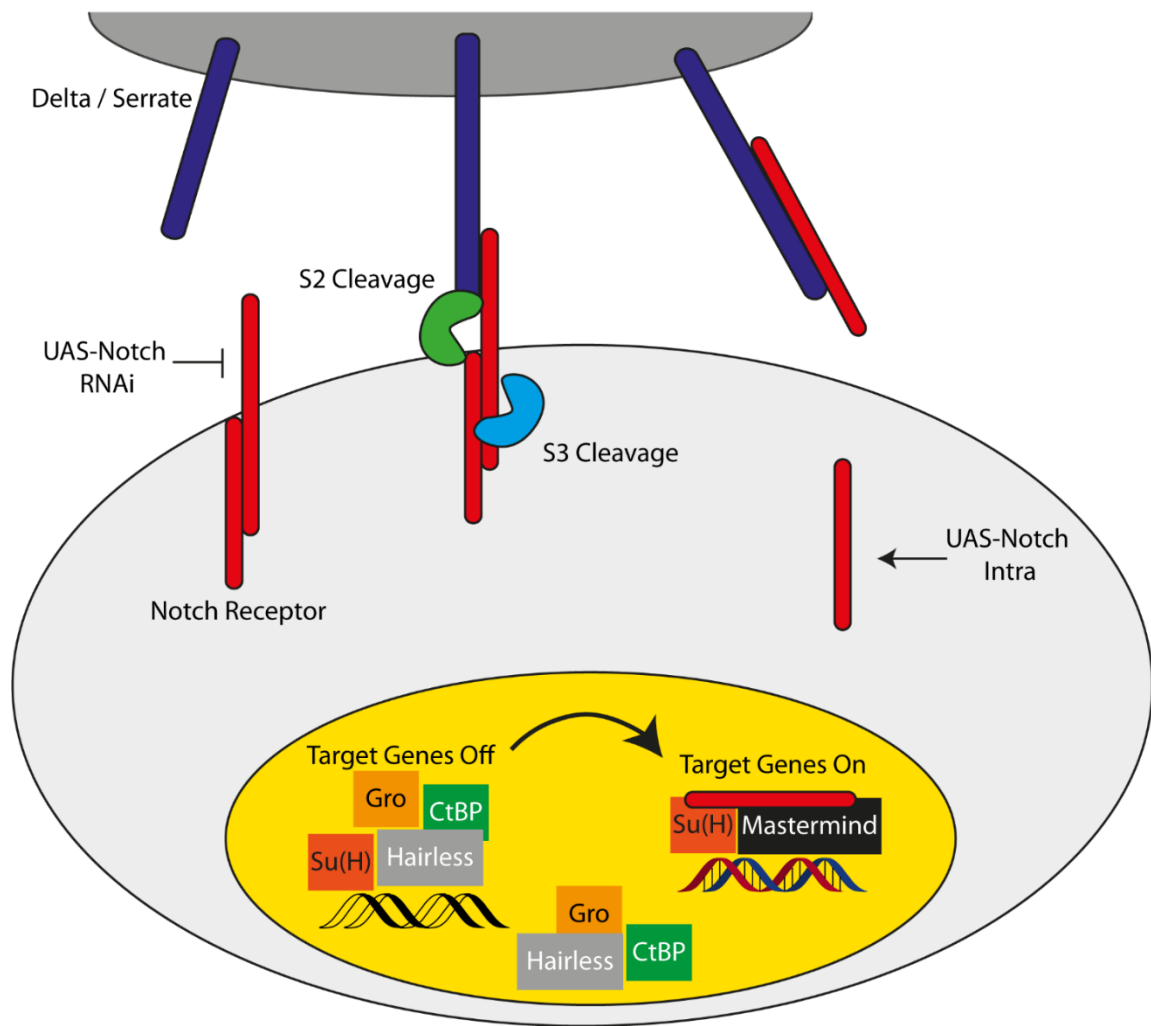


Figure 5-7: Notch pathway schematic and genetic epistatic targets

Neighbouring cells (dark grey semi-circle) express the Notch receptor ligands, Delta or Serrate (blue bar) on their cellular surface. These ligands bind to the extracellular domain of the Notch receptor on the recipient cell. The binding of the ligand to the Notch receptor induces two sequential proteolytic cleavages. The first S2 cleavage is catalysed by an ADAM-family metalloprotease (Kuzbanian/ADAM10 – green pacman) and the second S3 cleavage by a γ -secretase complex (consisting of Presenilin, Nicastrin, PEN2 and APH1 - blue pacman). This cleavage event releases the extracellular and Intracellular domain of the Notch receptor. The extracellular domain of Notch is endocytosed by the ligand presenting cell where it is targeted for lysosomal degradation (289, 290). The intracellular domain (red bar inside cell) translocates

to the nucleus where it directly binds and interacts with Su(H), and the co-activator Mastermind, promoting transcription of the Notch response genes. Using genetic epistasis, it is possible to modulate the activity of this pathway. Using an RNA interference construct targeting the Notch receptor under the regulation of the *UAS* promoter (UAS-Notch RNAi), it is possible to downregulate the Notch receptor, thus downregulating the pathway. Conversely, using a construct which overexpresses the Notch intracellular domain under the *UAS* promoter (UAS-Notch^{ntra}), it is possible to increase the amount of this fragment within the cell. Thus, increasing the number of translocation events to the nucleus and upregulating the transcription of the Notch Response Genes. Schematic was adapted and redrawn from (288).

In the *Drosophila* intestinal system, the loss of Notch signalling in *esg* expressing cells promotes the expansion of the Intestinal Stem Cells and the Enteroendocrine cells, while preventing the differentiation of Enteroblasts into Enterocytes. Since previous experiments have established that loss of *Dronc* in Enteroblasts facilitates their premature differentiation into Enterocytes, I sought to establish whether loss of Notch signalling could revert this phenotype. To that end, using the *esg*-Gal4 driver, I simultaneously targeted the expression of *Notch* (UAS-Notch RNAi) [Figure 5-8] and *Dronc* in progenitor cells. Upon simultaneous loss of function, the epithelial features of these intestines remained similar to those from *Notch* loss of function alone. Thus, the notable increase in Enteroblast cell size and differentiation relating to *Dronc* insufficiency were suppressed [Figure 5-8 A-B]. However, I did detect a slight increase

in the number of cells in the *Dronc* loss of functions intestines, perhaps relating to a potential rescue from apoptosis [Figure 5-8 C].

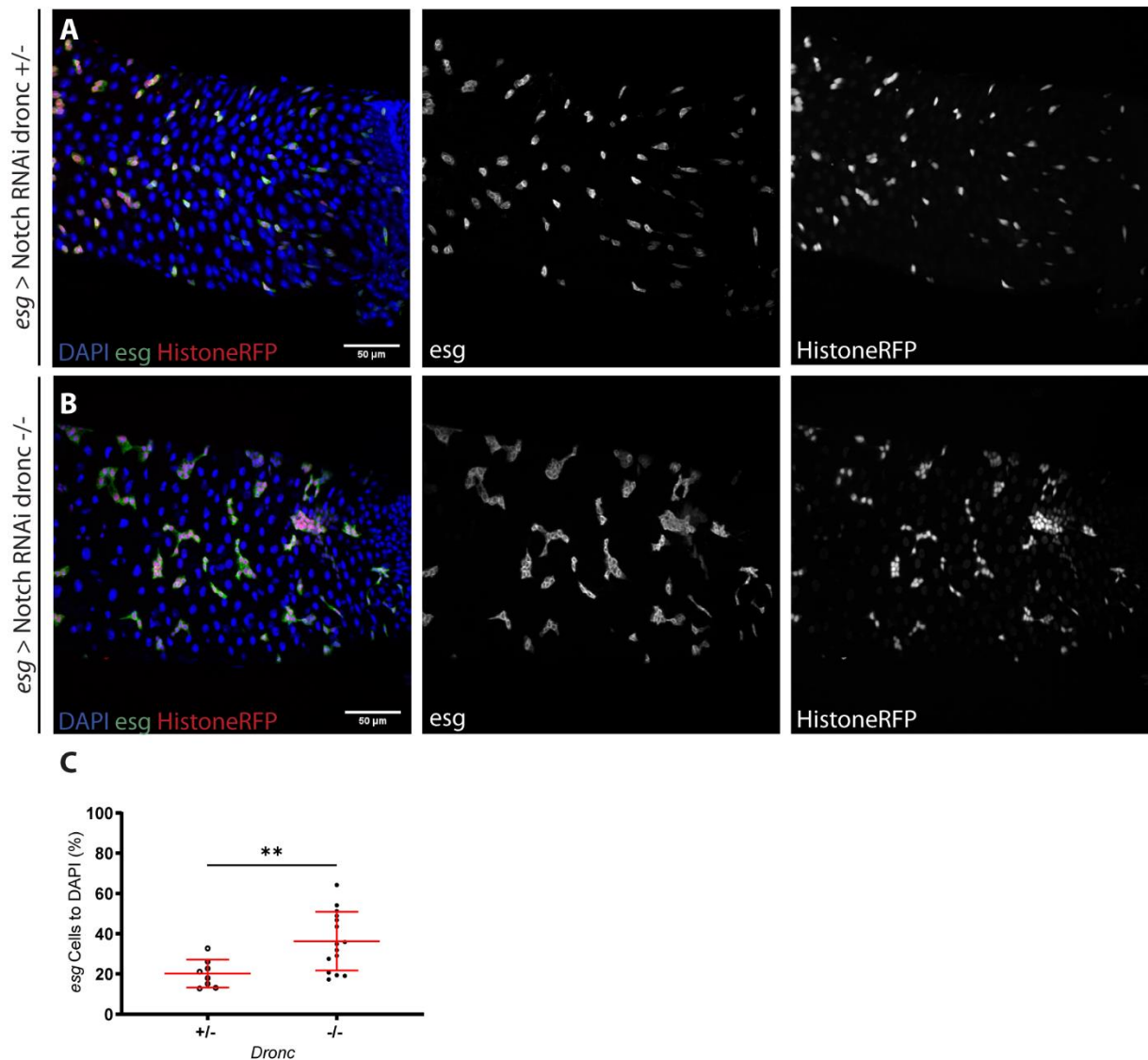


Figure 5-8: *Notch* loss of function rescues Enteroblast *Dronc* KO excessive differentiation into Enterocytes.

(A) Representative ReDDM labelling of a heterozygous *Dronc* intestine, overexpressing an RNAi construct against the Notch receptor for 3 days. Note, the absence of differentiated Enterocytes (HistoneRFP (red) labelling without *esg* expression (green, GFP). (B) Representative ReDDM labelling of an Intestinal Stem Cell and Enteroblast *Dronc* KO intestine, which has overexpressed an RNAi against the Notch Receptor for 3 days. Note the absence of differentiated Enterocytes (HistoneRFP (red) without *esg* GFP expression (green)), and the increase in GFP positive cells

present in (B) compared to (A). (C) Quantification of the total number of HistoneRFP cells (as a proxy of progenitor cell proliferation) normalised to DAPI, in the genotypes described in (A) and (B). There is a statistically significant increase in HistoneRFP cells in the *esg*-induced *Dronc* loss of function intestines compared to the heterozygous controls ($P = 0.0080$, unpaired two-tailed t-test, +/- $N = 8$, -/- $N = 15$). Error bars represent Standard deviation of the Mean in all panels. All of the experiments described in the figure were performed in Oxford medium with an experimental regime which protects epithelial integrity. Scale Bars represent 50 μm . The genotype of the *esg* > Notch RNAi -/+ *Drosophila* is w^{1118} UAS-Notch RNAi; *esg-Gal4* UAS-CD8-GFP / + ; *TubG80ts* UAS-Histone-RFP *DroncKO* / +. The genotype of the *esg* > Notch RNAi *dronc* -/- *Drosophila* is w^{1118} UAS-Notch RNAi; *esg-Gal4* UAS-CD8-GFP / + ; *TubG80ts* UAS-Histone-RFP *DroncKO* / UAS-FLP FRT *Dronc*-GFP-APEX FRT *Suntag*-HA-Cherry.

Following the demonstration that loss of *Notch* signalling in progenitor cells could suppress the hyperplastic phenotypes resulting from *Dronc* KO, I sought to establish if its ectopic activation could exacerbate the phenotypes. Simultaneous over-expression of the Notch pathway activator, UAS-Notch^{intra} [Figure 5-7], and loss of *Dronc* in *esg* expressing cells, increased the rate of conversion of the Enteroblasts into Enterocytes. This resulted in the subsequent elimination of the cells from the tissue [Figure 5-9 A-B] (291). Furthermore, this rapid depletion occurred faster in cells mutant for *Dronc* than the ectopic expression of Notch^{intra} alone [Figure 5-9 C]. Immunostaining with Pdm1 revealed that the remaining cells mostly consisted of Enterocytes. Collectively, these results genetically located the function of *Dronc* upstream of the Notch pathway, possibly acting as a negative regulator of the transition of Enteroblasts into the terminally differentiated Enterocytes.

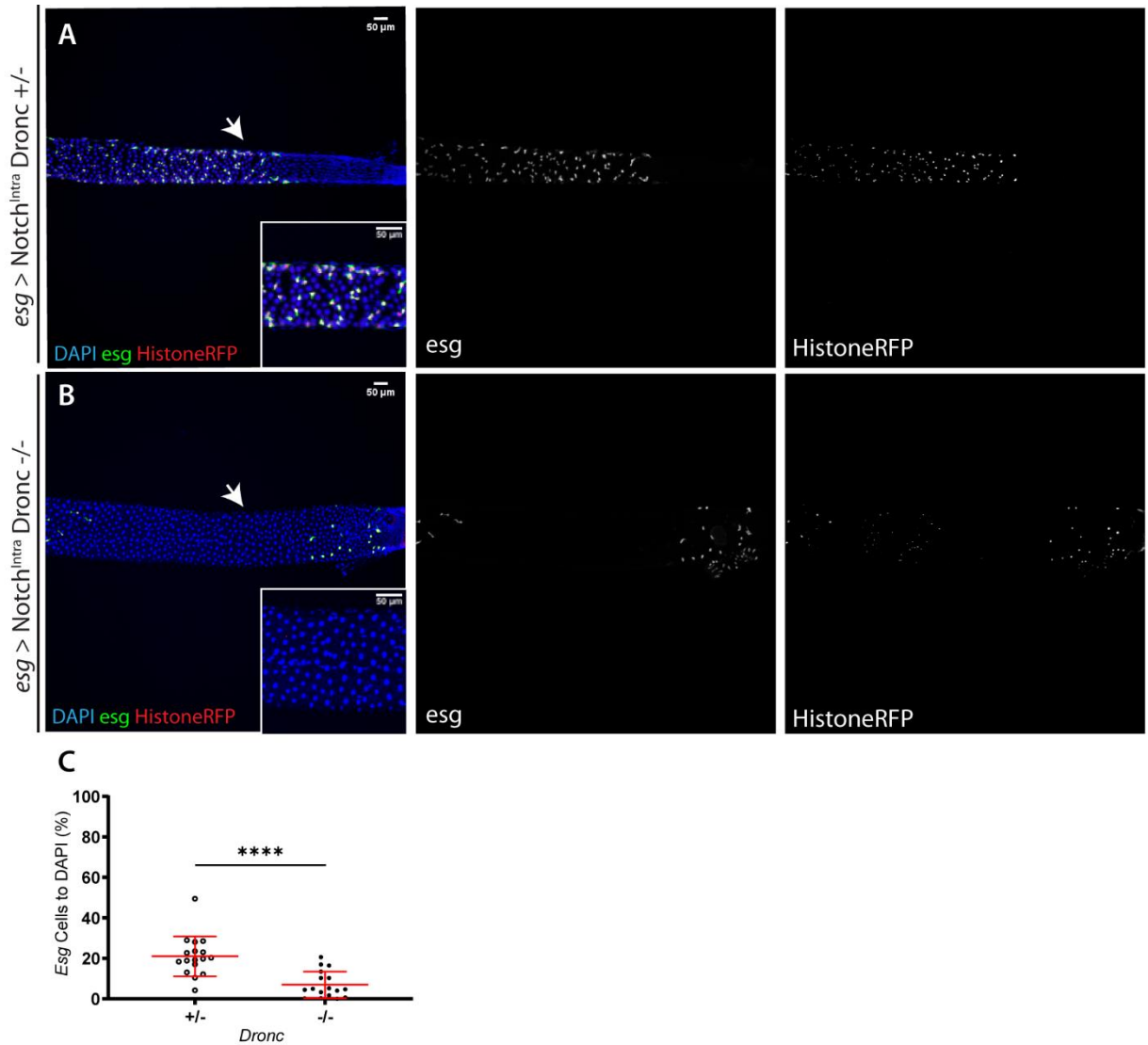


Figure 5-9: Activated Notch signalling exacerbates the rapid differentiation observed in *Dronc* KO intestines

(A) Representative ReDDM labelling of a *Dronc* heterozygous intestine overexpressing the Notch pathway activator, Notch^{Intra}, for 7d. *esg*-expressing progenitor cells (green) and HistoneRFP (red) are depleted in the posterior region of the intestine. (B) *esg*-induced *Dronc* KO intestine overexpressing the Notch intracellular domain for 7d. Note that loss of *Dronc* in the progenitor cells accelerates the elimination of these cells from the epithelium (compare (A) & (B)). The white arrows indicate the position of the insets 500 μ m from the posterior region. Note the complete loss of progenitor cells in this region. (C) Quantification of the number of *esg*-positive cells relative

to DAPI in either *Dronc* heterozygous or *esg*-induced *Dronc* KO intestines, overexpressing *Notch*^{Intra} for 7d. There is a significant reduction in *esg*-expressing cells following *Dronc* KO ($P = < 0.0001$, Mann-Whitney test, +/- N = 17, -/- N = 17). Error bars represent Standard deviation of the Mean in all panels. All of the experiments described in the figure were performed in Oxford medium with an experimental regime which protects epithelial integrity. Scale Bars represent 50 μ m. The genotype of the *esg* > *Notch*^{Intra} *Dronc* +/- *Drosophila* is *w*¹¹¹⁸; *esg-Gal4 UAS-CD8-GFP* / *UAS-Notch Intra*; *TubG80ts UAS-Histone-RFP DroncKO* / +. The genotype of the *esg* > *Notch*^{Intra} *Dronc* -/- *Drosophila* is *w*¹¹¹⁸; *esg-Gal4 UAS-CD8-GFP* / *UAS-Notch Intra*; *TubG80ts UAS-Histone-RFP DroncKO* / *UAS-Flp FRT Dronc-GFP-APEX FRT QF*.

Following the observation that *Dronc* LOF results in the increased differentiation of intestinal precursors following the activation of the Notch pathway, I sought to establish whether *Dronc* was directly modulating the activity of the pathway. To explore this hypothesis, I performed qPCR on progenitor *Dronc* LOF intestines, using primers against the *enhancer of split complex*. In *Drosophila*, the majority of known Notch Response Genes are located within this complex, and therefore constitute a reliable readout of Notch pathway activity (292). In progenitor *Dronc* LOF intestines, I observed around a 2-fold upregulation in the transcription of *Enhancer of split α* (Alpha), *Enhancer of split β* (Beta) *Enhancer of split M3* (M3) when compared to heterozygous *Dronc* controls [Figure 5-10], suggesting that *Dronc* was indeed regulating the activity of the Notch pathway.

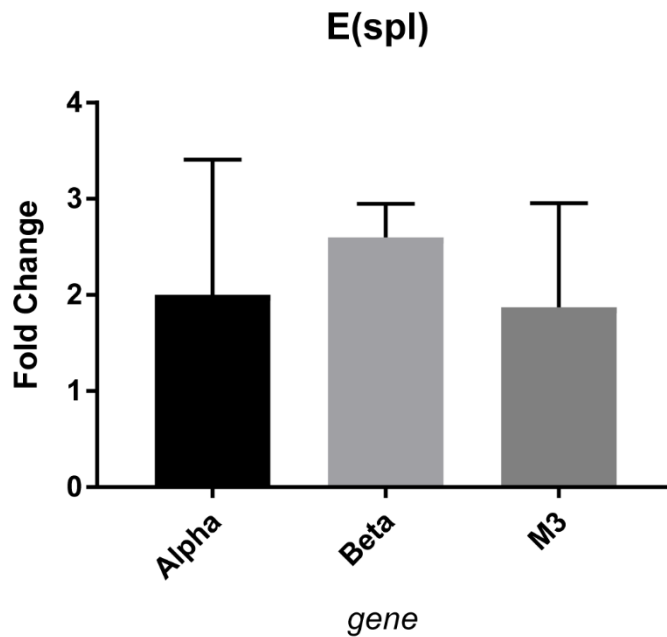


Figure 5-10: Dronc upregulates the Notch Response Genes.

qPCR data of the fold change of *Enhancer of split α (Alpha)*, *Enhancer of split β (Beta)* and *Enhancer of split m3 (M3)* between *Dronc* heterozygous and *Dronc* KO intestinal progenitors. There is around a 2-fold upregulation in the transcription of these genes in response to *Dronc* LOF. Error bars represent Standard deviation of the Mean. All of the experiments described in the figure were performed in Oxford medium with an experimental regime which protects epithelial integrity. The genotype of the +/- *Drosophila* is $w^{1118}; esg-Gal4 \text{ UAS-}mCD8-GFP; \text{ UAS-HistoneRFP TubG80ts } Dronc^{KO} / SM6A-TM6B$. The genotype of the -/- *Drosophila* is $w^{1118}; esg-Gal4 \text{ UAS-}mCD8-GFP / +; \text{ UAS-HistoneRFP TubG80ts } Dronc^{KO} / \text{ UAS-Flp FRT } Dronc-GFP-APEX \text{ FRT } Suntag-HA-Cherry$. Dronc modulation of Notch pathway activity is likely upstream of the Insulin/TOR pathway.

Following the observation that Dronc modulates the activity of the Notch pathway, I next explored whether the Insulin-TOR pathway was required downstream of the Notch pathway. Previous studies have demonstrated that the Notch-mediated repression of

the Tuberous Sclerosis Complex 2 (TSC2), mediated by Su(H) [Figure 5-11], was required for the differentiation of Enteroblasts into Enterocytes (196).

The insulin/TOR pathway is activated through the binding of a *Drosophila* insulin-like peptide (Dilp) to the insulin receptor. Subsequently, the activated insulin receptor then phosphorylates its substrate Chico. The downstream phosphorylation cascade leads to the activation of the phosphatidylinositol 3-kinase (PI3K) complex, before phosphorylating phosphatidylinositol-4, 5-bisphosphate (PIP2) to phosphatidylinositol-3, 4, 5-triphosphate (PIP3) (293). The activity of Phosphatase and tensin homolog on chromosome 10 (PTEN), acts as a negative regulator of this process (294). Activated PIP3 results in the activation of Phosphoinositide-dependent kinase-1 (PDK1) and AKT, leading to the nuclear localisation of forkhead box sub-group O (dFOXO), and the suppression of growth and translation; in addition to the activation of the stress response. In addition to regulating dFOXO, AKT also inhibits the activity of the Tuberous Sclerosis (TSC1/TSC2) complex, activating the downstream factors, Ras homolog enriched in brain (Rheb) and the target of rapamycin (dTOR). The modulation of these factors initiates transcription, translation, autophagy and endocytosis.

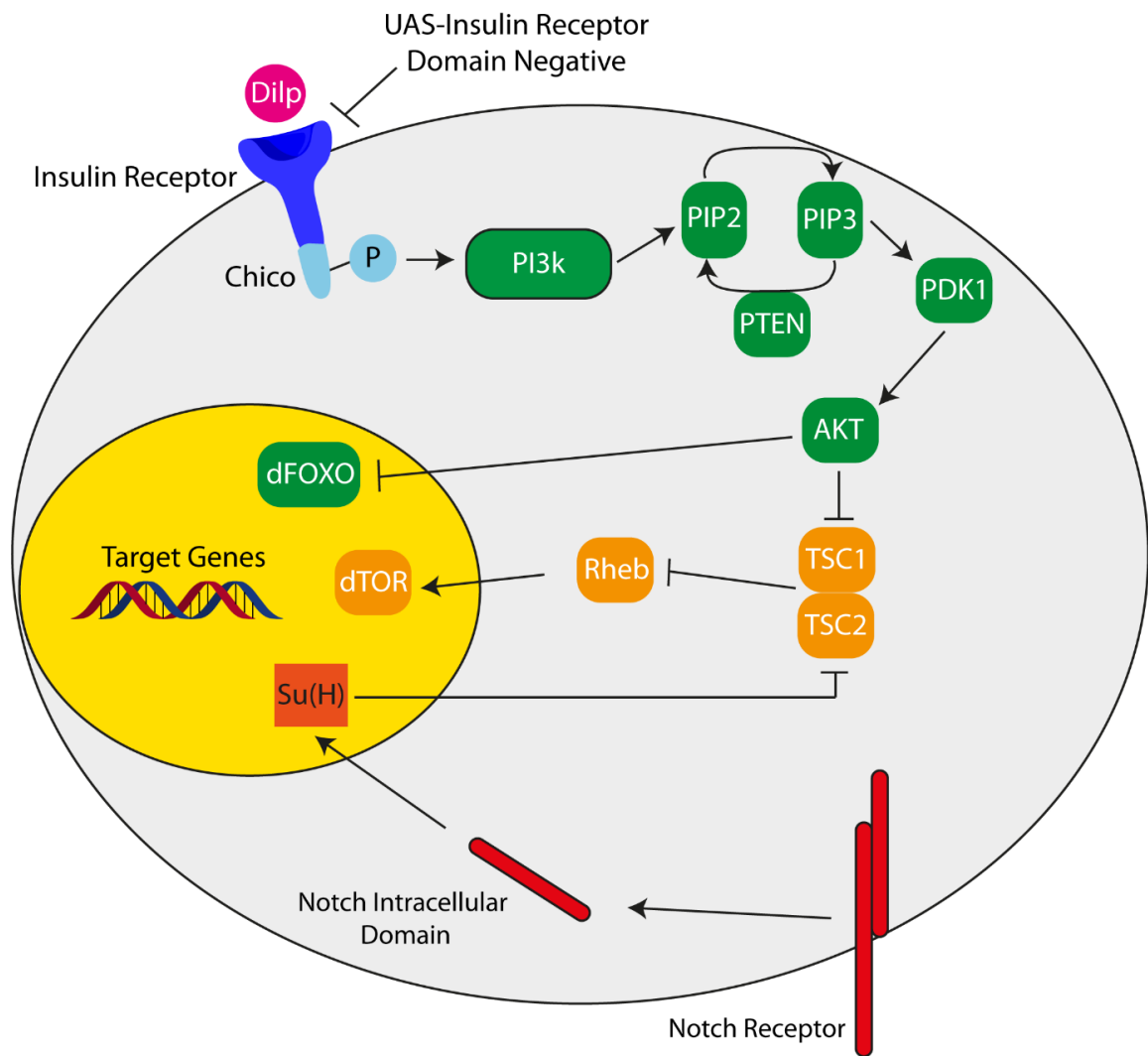


Figure 5-11: Schematic of the Interaction between the Insulin and Notch Pathway.

The insulin/TOR pathway is first activated through the binding of one of the eight *Drosophila* Insulin-like peptides (Dilps; insulin or Insulin-like growth factor in mammals) to the Insulin receptor (Insulin receptor or Insulin-like growth factor receptor in mammals). Binding of Dilps leads to the phosphorylation of the Insulin receptor substrate, Chico (IRS1-4 in mammals). The phosphorylation of Chico activates the phosphatidylinositol 3-kinase (PI3K) complex. This complex phosphorylates Phosphatidylinositol 4,5-bisphosphate (PIP2) into phosphatidylinositol 3,4,5-trisphosphate (PIP3). The phosphatase and tensin homologue on chromosome 10 (PTEN), acts as a repressor of the insulin pathway, utilising PIP3 as a substrate (295, 296). Elevated PIP3 levels lead to the activation of Phosphoinositide-dependent kinase-1 (PDK1) and the subsequent

activation of AKT. The activation of AKT then leads to the inhibition of the transcription factor, forkhead box sub-group O (dFoxo), and the inhibition of the Tuberous Sclerosis (TSC1/2) complex. This complex represses the activation of Ras homolog enriched in brain (Rheb), and the activator of the target of rapamycin (TOR). Downregulation of this pathway is achievable through overexpression of a dominant-negative form of the Insulin receptor, impeding its activation. The Notch signalling pathway is also able to modulate the activity of the Insulin pathway. Following cleavage of the Notch intracellular domain, upon activation of the Notch receptor, this fragment translocates to the nucleus. Here the Notch intracellular domain interacts with Su(H), transcriptionally downregulating TSC2. This culminates in the activation of the pathway, through the downregulation of the inhibitor complex. Figure redrawn and adapted from (196, 288, 293).

To downregulate the activity of the Insulin/TOR pathway to determine whether there was a genetic hierarchy between the two signalling pathways in this context, I overexpressed a dominant-negative form of the Insulin receptor under the regulation of the *esg*-GAL4 driver [Figure 5-11], while simultaneously excising the *Dronc* rescue-cassette. As expected, the loss of Insulin/TOR signalling in *esg*-positive cells rescued the premature differentiation phenotypes observed through the loss of *Dronc* [Figure 5-12]. Collectively, these results confirmed the requirement for Insulin signalling in the process of differentiation, and corroborated with previously published studies. demonstrating a genetic hierarchy between the Insulin/TOR and Notch signalling pathways, during the terminal differentiation of Enteroblasts into Enterocytes.

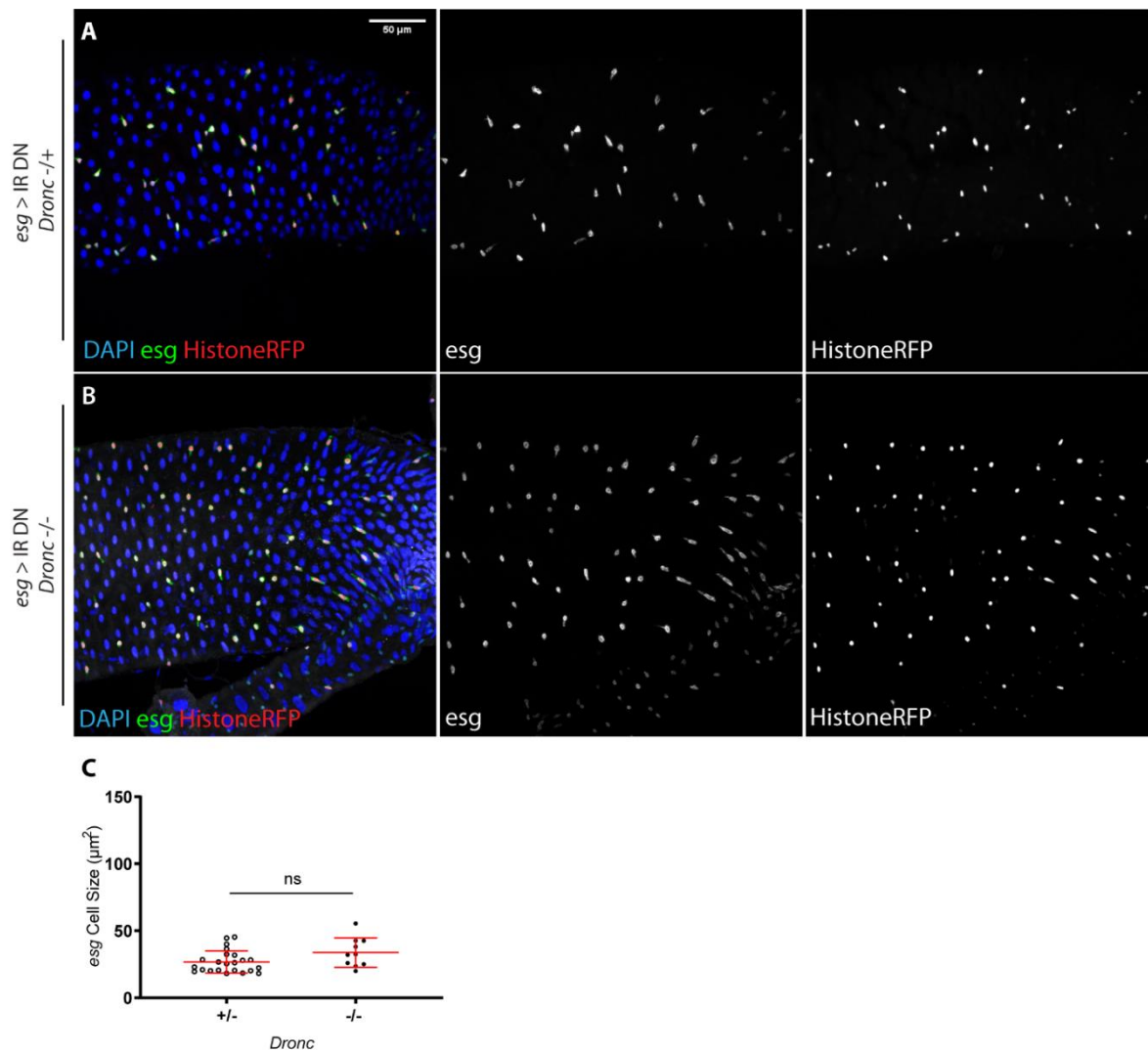


Figure 5-12: Loss of Insulin signalling rescues the *Dronc* KO hyperplastic phenotypes.

(A) Representative image of ReDDM labelling in a *Dronc* heterozygous intestine, overexpressing the dominant-negative form of the Insulin receptor under the regulation of the *esg* promoter for 3d. *Esg* (green, GFP) labels Intestinal Stem Cells and Enteroblasts, HistoneRFP (red) is a semi-permanent marker retained in differentiated cells. (B) Representative image of ReDDM labelling in a *Drosophila* intestine in which the progenitor cells are deficient in *Dronc*, and have been overexpressing the dominant-negative form of the Insulin Receptor under the regulation of the *esg* promoter for 3 days. Note that (A) and (B) demonstrate similar morphological features, i.e. lack of fully differentiated HistoneRFP (red) cells without *esg* expression (green, GFP), collectively indicating a rescue from the *Dronc* knockout phenotypes. (C) Quantification of the average cell

size (μm^2) of *esg*-expressing cells corresponding to the genotypes illustrated in panels (A) and (B). There is no statistically significant change in the average cell size between the two conditions. ($P = 0.2484$, unpaired two-tailed t-test, +/- N = 24, -/- N = 10). DAPI (blue) labels the nuclei of all cells in panels (A) and (B). Error bars represent Standard deviation of the Mean in all panels. All of the experiments described in the figure were performed in Oxford medium with an experimental regime which protects epithelial integrity. Scale Bars represent 50 μm . The genotype of the *esg* > IR DN Dronc +/- *Drosophila* is w^{1118} ; *esg-Gal4 UAS-CD8-GFP / UAS-Insulin Receptor DN; TubG80ts UAS-Histone-RFP DroncKO / +*. The genotype of the *esg* > IR DN Dronc -/- *Drosophila* is w^{1118} ; *esg-Gal4 UAS-CD8-GFP / UAS- Insulin Receptor DN; TubG80ts UAS-Histone-RFP DroncKO / UAS-Flp FRT Dronc-GFP-APEX FRT QF*.

5.2 Discussion

In this chapter, I have demonstrated that loss of *Dronc* within the Intestinal Stem Cells does not result in any observable phenotypes (i.e. increased number, size or differentiation into Enteroendocrine cells). Thus, suggesting a specific role for Dronc in the regulation of Enteroblasts. Using a transcriptional reporter of Dronc activity, I was able to observe widespread transcription throughout the whole of the intestine, suggesting this specificity is not transcriptionally related. However, using a newly developed Dronc-protein sensor, I was able to observe Dronc accumulation and stabilisation within the Intestinal Stem Cells and the Enteroblasts. Moreover, the accumulation of the protein appeared to be higher in Enteroblasts than in the Intestinal Stem Cells, suggesting a possible explanation for the phenotypic data. Importantly, this accumulation in the Intestinal Stem Cells and Enteroblasts only appeared to occur in

conditions without damage, as upon Paraquat treatment this accumulation disappeared. Next, I demonstrated that loss of Notch signalling in intestines KO for *Dronc*, rescued the hyperplastic phenotypes. Whereas, the overactivation of the pathway lead to an exacerbation of the differentiation phenotypes, with a reduction of *esg* expressing cells observed upon simultaneous overactivation of the pathway and loss of *Dronc*. Furthermore, qPCR experiments using downstream readouts of the Notch pathway demonstrated a 2-fold upregulation of the pathway in *Dronc* LOF intestines. Finally, by overexpressing the dominant-negative form of the Insulin receptor while simultaneously removing *Dronc*, I was able to rescue the loss of *Dronc* phenotypes. This confirmed previously published data and suggested that *Dronc* regulation of Enteroblasts is likely to be situated above the Insulin/TOR pathway.

Loss of *Dronc* proliferation phenotypes could be achieved through several distinct mechanisms.

In the *esg*-Gal4 induced knockout for *Dronc*, I observe a statistically significant increase in Intestinal Stem Cell and Enteroblast cell number at 3 days. However, I only observe increases in differentiated cell number at 7 days. Jointly, this would suggest that initially an excess in proliferation occurs first, which subsequently triggers the differentiation of the excess pool of *esg*-positive cells. The principle proliferative cells in the intestinal system are the Intestinal Stem Cells (165, 166) with the Enteroblasts dividing rarely, if ever (183); however when using the *Delta*-Gal4 driver I failed to observe an increase in proliferation. This, therefore, suggests three likely possibilities to account for the

proliferation. Firstly, loss of *Dronc* within the Enteroblasts non-autonomously signals to the Intestinal Stem Cells to divide. Secondly, the premature and aberrant differentiation of Enteroblasts following loss of *Dronc* induces proliferation of the Intestinal Stem Cells; or thirdly, loss of *Dronc* causes the Enteroblasts to proliferate.

Numerous studies have implicated apoptotic caspase activation with the induction of proliferation in neighbouring tissues (297). The phenomenon of Apoptosis-induced proliferation (AiP), occurs as a result of irreparable damage to the cell triggering the activation of apical-caspases, leading to the release of mitogens inducing proliferation in neighbouring cells (297). However this is unlikely to be the cause of the proliferation in my experimental conditions, as the caspase activation observed is non-apoptotic and occurs as a consequence of loss of *Dronc* rather than its activation.

Several studies have demonstrated that impeding the differentiation of Enteroblasts can promote hyperproliferation of the Intestinal Stem Cells. For example, Enteroblasts mutant for the group B Sox protein, Sox21a, cannot differentiate or proliferate; however, the expression of mitogens is increased. These subsequently act as paracrine signals to simulate proliferation of Intestinal Stem Cells. While *Dronc* knockout Enteroblasts can complete the differentiation step into Enterocytes, their differentiation process is abnormal, i.e. retention of the stem cell marker *esg*, and enlargement of cells with the expression of Pdm1. In this prolonged and/or premature differentiation process, *Dronc* knockout Enteroblasts may stimulate the excessive proliferation of Intestinal Stem Cells, through loss of negative feedback and excessive

secretion of mitogens (298). Importantly, this could explain the initial increase in proliferative cells, followed by the excessive differentiation and hyperplastic phenotypes observed following the loss of *Dronc*. While from this experimental data, it is impossible to rule out Enteroblast proliferation as a cause of increased *esg* cell number, proliferation in this cell type is not widely documented. Thus, a significant contribution of Enteroblast proliferation to the phenotypes described previously is unlikely.

Dronc is sub-lethally accumulated in Enteroblasts, but is unlikely to be regulated by the previously described mechanisms.

A comprehensive body of literature has demonstrated an ability for the caspases to stimulate the proliferation and differentiation of Intestinal Stem Cells into Enteroblasts, through the apoptotic activation of signalling pathways in damaging conditions (104, 291, 299). Conversely however, the signalling pathways orchestrating the differentiation of Enteroblasts into the terminally differentiated Enterocytes remains less well understood. Through utilising an experimental condition in which the intestines remain free from apoptosis, and basal cell turnover for at least 7 days post activation of the ReDDM tool, we have been able to demonstrate that *Dronc* is accumulated in the Intestinal Stem Cells and Enteroblasts.

Whilst the observation of Dronc protein retention within specific cells of the intestinal system is novel, the subcellular localisation of Dronc in other tissues is widely accepted. In the wing imaginal discs, Myo1D has been shown to initiate the translocation of Dronc to the plasma membrane, activating Duox and causing the release of ROS to promote haemocyte attraction (268). Similarly, in the salivary gland, tango7 regulates the activity of Dronc at the cortex, with Dark regulating its cytoplasmic activity. Importantly a sub-lethal dose, activated by reaper, is required to initiate this cortical activity (285).

In this thesis, my ability to observe activation of the DBS-S-QF reporter, but not the specific subcellular activation of Dronc using the modified GFP, would correlate with the sub-lethal activation of Dronc in this context. While sub-lethal activation of Dronc through reaper regulates the salivary gland activity, this cannot be the case within the intestinal system, as overexpression of the micro-RNA against Hid, Grim and Reaper did not recapitulate the *Dronc* KO phenotypes. Collectively however, the accumulation or stabilisation of Dronc preferentially within the Enteroblasts, and to a lesser extent the Intestinal Stem Cells, could suggest a possible explanation for the phenotypes associated with the loss of *Dronc* in Enteroblasts but not Intestinal Stem cells.

Dronc function requires the Notch and Insulin-TOR pathways

Previous studies have demonstrated a role for Dronc in the regulation of neuroblast proliferation and self-renewal, through a protein-protein interactions with the Notch

regulator, Numb (89). This in combination with the crucial requirement for the Notch pathway in the regulation of intestinal progenitors, lead me to explore this pathway as a potential mediator of Dronc activity. I discovered that the activity of Notch is indispensable for the non-apoptotic function of Dronc in Enteroblast differentiation. Furthermore, the loss of *Dronc* phenotypes are exacerbated through the overactivation of this pathway. Additionally, the Notch Response Genes appear transcriptionally 2-fold upregulated in *Dronc* loss of function intestines. Along the same vein, the Insulin/TOR pathway appears to act downstream of the Notch pathway in this context (196, 197). Experimental evidence provided in this thesis suggests that the catalytic activity of Dronc is required for the regulation of Enteroblasts, thus ruling out the likelihood of an interaction with Numb (89). The interaction partner orchestrating this regulatory role by Dronc remains elusive. The Notch pathway, whilst initially appearing a simple process, can be modulated by a numerous and complex network of interactors. The Notch-intracellular domain itself plays host to many phosphorylation, ubiquitination, hydroxylation and acetylation sites, all modulating the fine-tuning of the signalling cascade (290). This collectively means that the elucidation of the interaction partner is unlikely to be achievable through classic genetic epistasis, without a substantial screen of known Notch interaction partners; favouring the utilisation of more complex biochemical approaches such as Mass-Spectrometry techniques.

6. Single Cell Sequencing of *Dronc* knockout intestines reveals aberrant expression of metabolic genes and increased viral load.

6.1. Results – *Dronc* knockout progenitors have deregulated gene expression

In the previous chapter, I demonstrated that *Dronc* activity appeared to be restricted to the Enteroblast cells. Furthermore, using a new *Dronc* protein sensor, I was able to observe its accumulation within the progenitor cell populations. However, this specificity was lost in tissue-damaging conditions, where it appeared uniformly spread across the intestine. Molecularly, I was able to demonstrate that *Dronc* appears to be located upstream of the Notch Pathway, as loss of Notch signalling inhibits Enteroblast differentiation, whereas its overactivation exacerbates *Dronc* loss of function phenotypes. Finally, I was able to demonstrate that the Insulin/TOR pathway is likely to reside below the Notch pathway in this context.

Drop-sequencing t-SNE plots cannot be clustered using canonical methods

Following the demonstration that *esg-gal4* induced loss of *Dronc* resulted in the premature and aberrant differentiation of Enteroblasts into Enterocytes; I sought to further characterise the transcriptional profile of these cells, while attempting to understand the molecular events orchestrating the differentiation process of *Dronc* mutant progenitor cells. To achieve this, I collaborated with Vincent Croset and

Christoph Treiber from the Scott Waddell Laboratory, to use the recently established RNA single sequencing technique, termed Drop-Seq (225).

Drop-seq allows the simultaneous analysis of many thousands of individual cellular transcriptomes, while retaining the ability to reassign these transcriptomes to individual cells. Thus, allowing the transcriptional profiling of complex tissues consisting of multiple cell types. Initially the complex tissue (i.e. the *Drosophila* intestinal system), is dissociated into single isolated cells [Figure 6-1 A]. Following dissociation, the cells are filtered and passed through a multifluidic device. The first flow contains the microparticles and lysis buffer, the second contains the dissociated cells in suspension, and the final flow contains oil; this process leads to the formation of oil droplets containing, one microparticle and one cell [Figure 6-1 B]. The encapsulation of the microparticle and lysis buffer, within the oil droplet, leads to the instantaneous lysis of the cell. This lysis releases the mRNA contents of the cell into the droplet. The mRNA is then hybridised to primers on the surface of the microparticle. These primers have PCR handles, a unique sequence (acting as a barcode for the cell), and a unique molecular identifier for each transcript [Figure 6-1 C]. The droplets are then broken, and a reverse transcription step converts the mRNA to cDNA. The cDNA is subsequently amplified through a PCR reaction and sent for sequencing. Each sequence is then mapped to the *Drosophila* genome. Using the cell barcode, each sequence can be traced back to its source cell, allowing analysis of individual cell transcriptomes (225, 226).

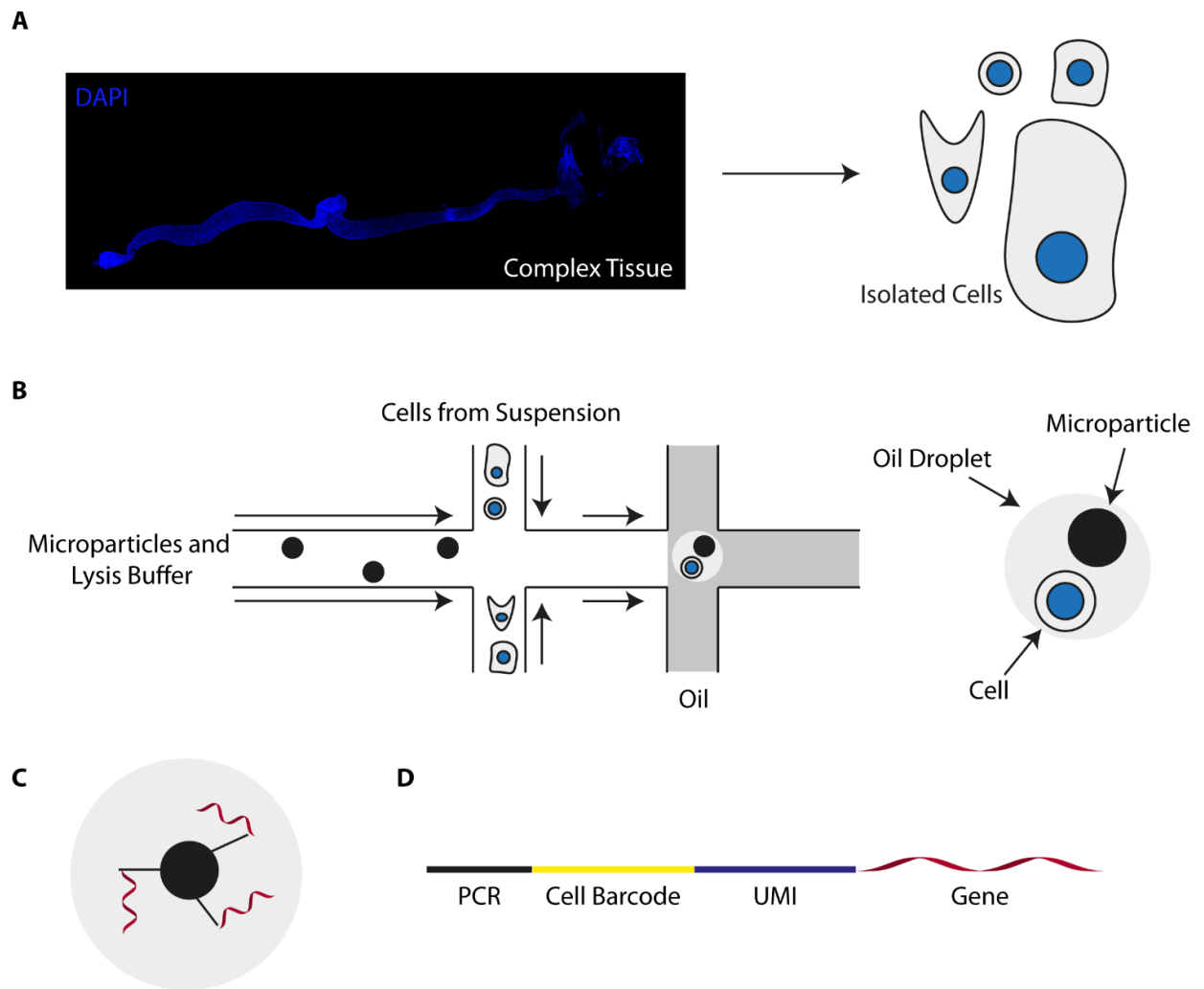


Figure 6-1: Drop-seq methodology.

(A) Representative image of a female *Drosophila* intestine (left), DAPI (blue) labels the nuclei of all cells within the gut. Initially, the *Drosophila* intestinal system (left) is dissociated through biochemical and mechanical methods into isolated single cells. (B) A graphical representation of a multifluidic device. Pumps force microparticles in lysis buffer through one chamber, another pump forces the dissociated cells through another, and finally, another pump forces oil through another chamber. This results in the formation of droplets containing a microparticle and cell. The speed of the pumps is adjusted in order to form droplets. Droplets containing more than one cell, or no cells, are excluded later during analysis of the data. (C) Graphical representation of an oil droplet when the microparticle and lysis buffer have dissociated the cell, within this droplet the cellular mRNA is hybridised to the microparticle primer. (D) Graphical representation of the

primer sequence hybridised to the microparticle primer. The primer contains a PCR sequence, which following reverse transcription to cDNA, allows amplification of the genetic material. A cell barcode sequence allows the association of each transcript to the cell of origin. A Unique Molecular Identifier (UMI) allows the identification of each gene transcript and the identification of PCR duplications (225, 226).

In collaboration with the Scott Waddell laboratory, I dissected and dissociated the cells from 30 *Dronc* heterozygous intestines, and 30 progenitor cell *Dronc* KO intestines. To detect the presence of single cells and the absence of clumps, the cell isolates were checked using a microscope. These cells were then processed using the aforementioned process before being sent for sequencing. The sequencing data was subsequently processed using the Drop-seq tools software, produced by the McCarroll Laboratory (225, 226), before processing using the Seurat Drop-sequencing toolkit designed by the Satija Lab (See materials and methods) (300).

Following initial interrogation of this data, using the STAR: ultrafast universal RNA-seq aligner, I obtained a low mapping rate, with around only 10% of our sequencing reads mapping to the *Drosophila* genome. Additionally, I also obtained a low number of cells in each condition (226). Using the Seurat Drop-Sequencing toolkit I obtained similar data, observing a low number of Unique molecular identifiers (UMI) per cell, indicative of a low number of sequencing reads per cell [Figure 6-2 A]. Additionally, of the number of UMIs detected per cell, the number of genes assigned to those identifying sequences were also low [Figure 6-2 B]. Collectively, this suggests that the data obtained from this

experiment is likely to be of low quality, and thus it may be difficult to obtain meaningful results.

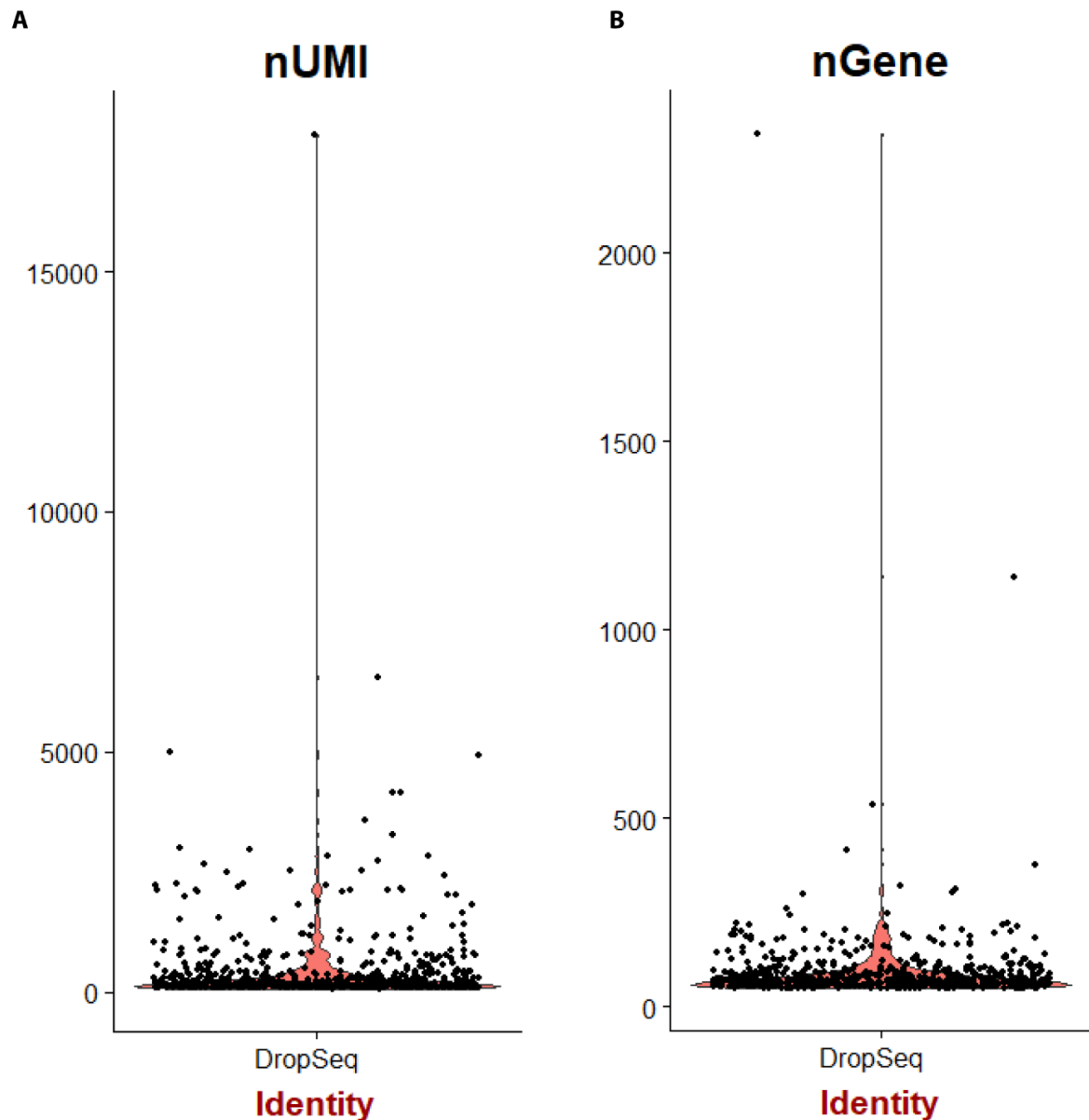


Figure 6-2: A low number of reads and assigned genes were obtained from the Drop-Seq data.

(A) A violin-plot denoting the number of Unique Molecular Identifiers (UMI, y-axis) per cell (black dot). The number of UMIs detected per cell appears low, and most appear between 0 and 5000.

(B) A violin-plot denoting the number of mRNA sequences assigned to UMIs, that also correspond to a gene. There are a very low number of genes attributed to the UMIs (between around 0 and 500), suggestive of a low read depth per cell. The genotypes of the *Drosophila* in this figure are

w¹¹¹⁸; esg-Gal4 UAS-mCD8-GFP ; UAS-HistoneRFP TubG80ts Dronc^{KO} / SM6A-TM6B and *w¹¹¹⁸; esg-Gal4 UAS-mCD8-GFP / +; UAS-HistoneRFP TubG80ts Dronc^{KO} / UAS-Flp FRT Dronc-GFP-APEX FRT Suntag-HA-Cherry*.

Despite these initial indications of low-quality Drop-seq data, I decided to proceed with the analysis of the data. As a first step, I discarded any cell which had a gene count of less than 5, as these were likely to be low-quality cells; or above 1000, as these could have been droplets containing more than one cell. Subsequently, following normalisation of the data, I performed Principal Component Analysis on the transcriptomes in order to reduce the dimensionality of these data sets to 50. Then, I performed t-SNE analysis to reduce these 50 dimensions to just two dimensions producing the clustering data observed in [Figure 6-3].

Initially, I compared the spread of the heterozygous and progenitor *Dronc* knockout cells depicted in the t-SNE plot. Reassuringly, there appeared to be a spread of cells from both conditions across the plot [Figure 6-3 A], allowing the intra-cluster comparison of genes between the two conditions in the future. On subsequent interrogation of the data, it appeared that the analysis had detected 13 distinct clusters of cells [Figure 6-3 B] (144, 147). While there are only four subtypes of cells within the *Drosophila* intestinal epithelium, previous studies have also demonstrated region-specific transcriptional variation, possibly accounting for the increased number of clusters (181). Furthermore, nervous and muscular tissue were also likely to be included in these samples.

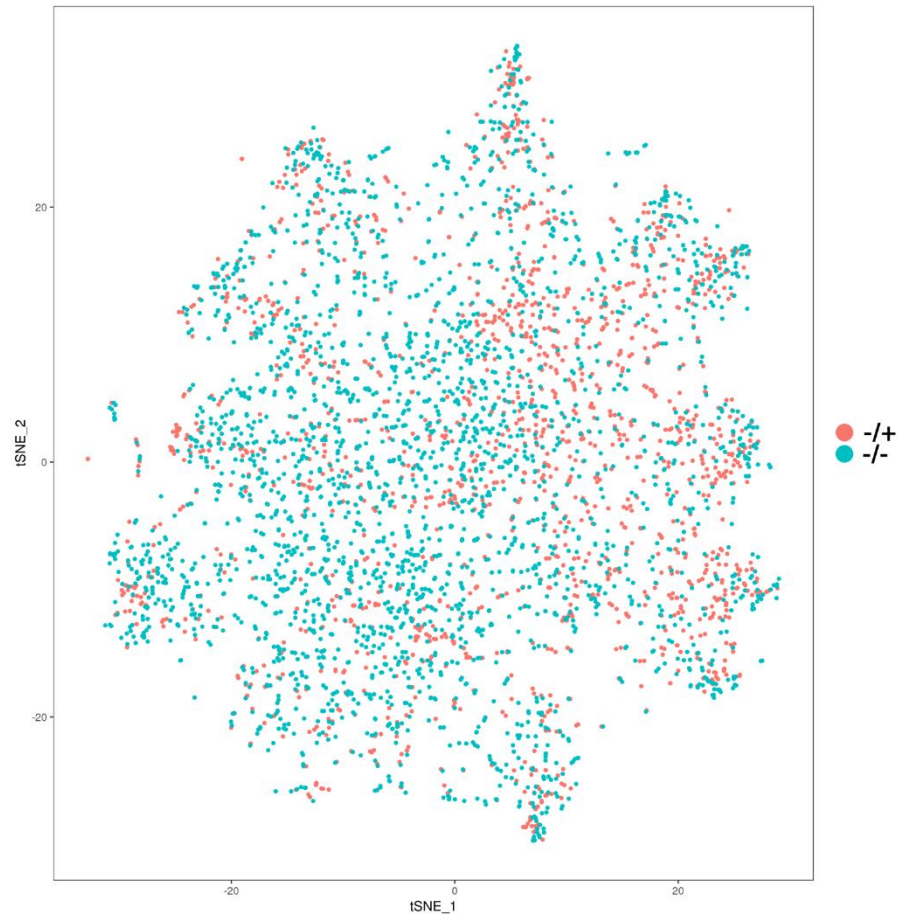
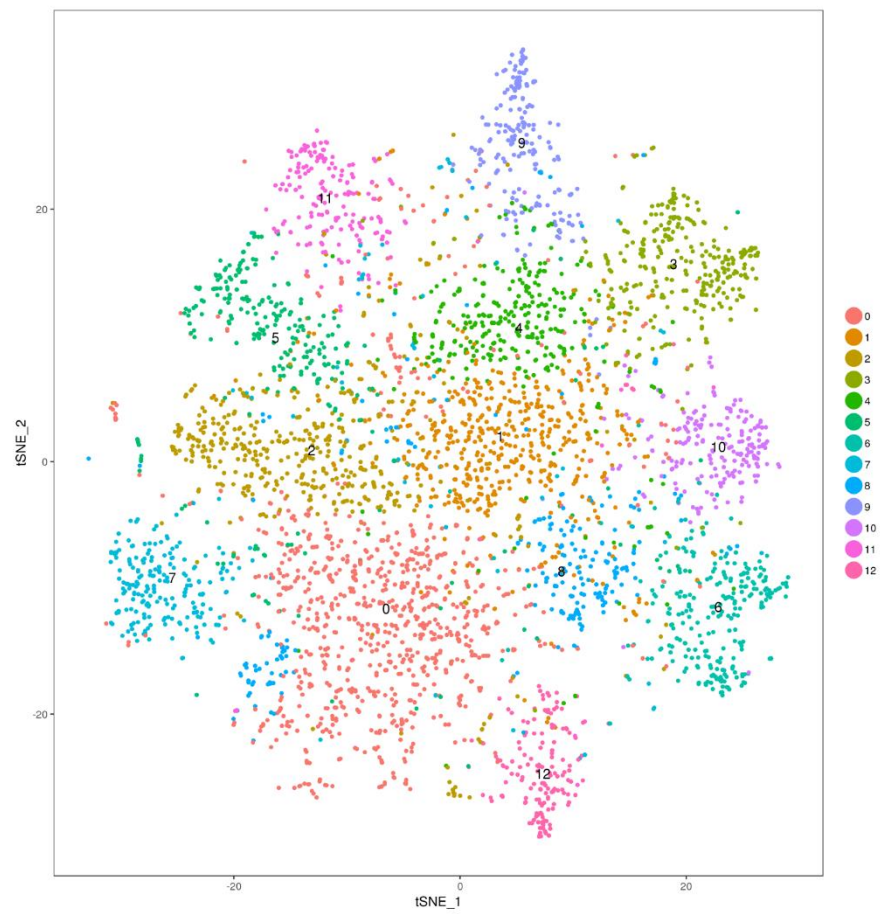
A**B**

Figure 6-3: PCA and t-SNE analysis of the intestinal system reveals 13 distinct clusters of cells.

(A) A merged t-SNE plot containing cells from *Dronc* heterozygous (red) and *Dronc* knockout progenitor cell intestines (teal). To reduce dimensionality to facilitate clustering, the top 50 variable genes common to both conditions are utilised to form computational clusters of cells. Subsequently, t-SNE analysis reduces the 50 dimensions into a 2D plot as depicted in (A). The *Dronc* heterozygous and *Dronc* knockout progenitor intestinal cells appear spread throughout the plot. (B) A merged t-SNE plot of cells from the *Dronc* heterozygous and progenitor cell *Dronc* knockout intestines, where 13 distinct clusters are depicted. Note that some clusters appear larger with an increased cell density. All of the experiments described in the figures were performed in Oxford medium under an experimental regime which protects epithelial integrity. The genotype of the +/- *Drosophila* is $w^{1118}; esg-Gal4 \text{ UAS-}mCD8-GFP ; \text{ UAS-HistoneRFP TubG80ts } Dronc^{KO} / SM6A-TM6B$. The genotype of the -/- *Drosophila* is $w^{1118}; esg-Gal4 \text{ UAS-}mCD8-GFP / +; \text{ UAS-HistoneRFP TubG80ts } Dronc^{KO} / \text{ UAS-Flp FRT } Dronc-GFP-APEX \text{ FRT Suntag-HA-Cherry}$.

Next, I attempted to assign an identity to these clusters, using markers known to be expressed within the subtypes of cells in the *Drosophila* intestinal system. As a first step, I sought to use the transcriptional profile of the stem cell progenitor marker, *esg* (177). However, the expression of *esg* did not appear restricted or highly expressed in any of the clusters [Figure 6-4 A]. Subsequently, I attempted to identify whether any of the clusters expressed the Enteroblast identity marker, *Su(H)*; however, it was equally as widespread as the *esg* marker (128) [Figure 6-4 B]. Similar results were obtained using the Intestinal Stem Cell marker, *Delta* (*DI*, [Figure 6-4 C]); the Enterocyte marker, *Pdm1* (*nub*, Figure 6-4 D); the Enteroendocrine maker, *Prospero* (*Pros*, [Figure 6-4 E]); the muscle marker, *Mef2* [Figure 6-4 F] and the neuronal marker, *elav* [Figure 6-4 G].

Subsequently, I investigated the transcript levels of *Dronc* to see if it aligned with the t-SNE plot produced in Figure 6-4 A. Nevertheless, the expression pattern of *Dronc* did not correlate strongly with the *Dronc* heterozygous or progenitor *Dronc* KO expression patterns [Figure 6-4 H], nor could I recapitulate this through detection of the expression of flippase-recombinase (Flp) [Figure 6-4 I]. Conversely, I investigated whether I could observe the transcription of the Gal80 transgene, expressed in all cells in both experimental conditions; however, again its transcription did not appear to correlate as expected [Figure 6-4 J]. Finally, I attempted to identify the clusters using the expression patterns obtained from the ReDDM reporter. As with the previous data, I was unable to detect the transcription of the *Gal4* and mCD8-GFP [Figure 6-4 K]. The HistoneRFP (*H2B-RFP*) appeared to be barely detectable and non-specific [Figure 6-4 L]. Collectively, the data correlated with the previously described low number of reads per cell, and strongly suggested that assigning identity to these clusters using canonical markers of intestinal cell identity would not be possible.

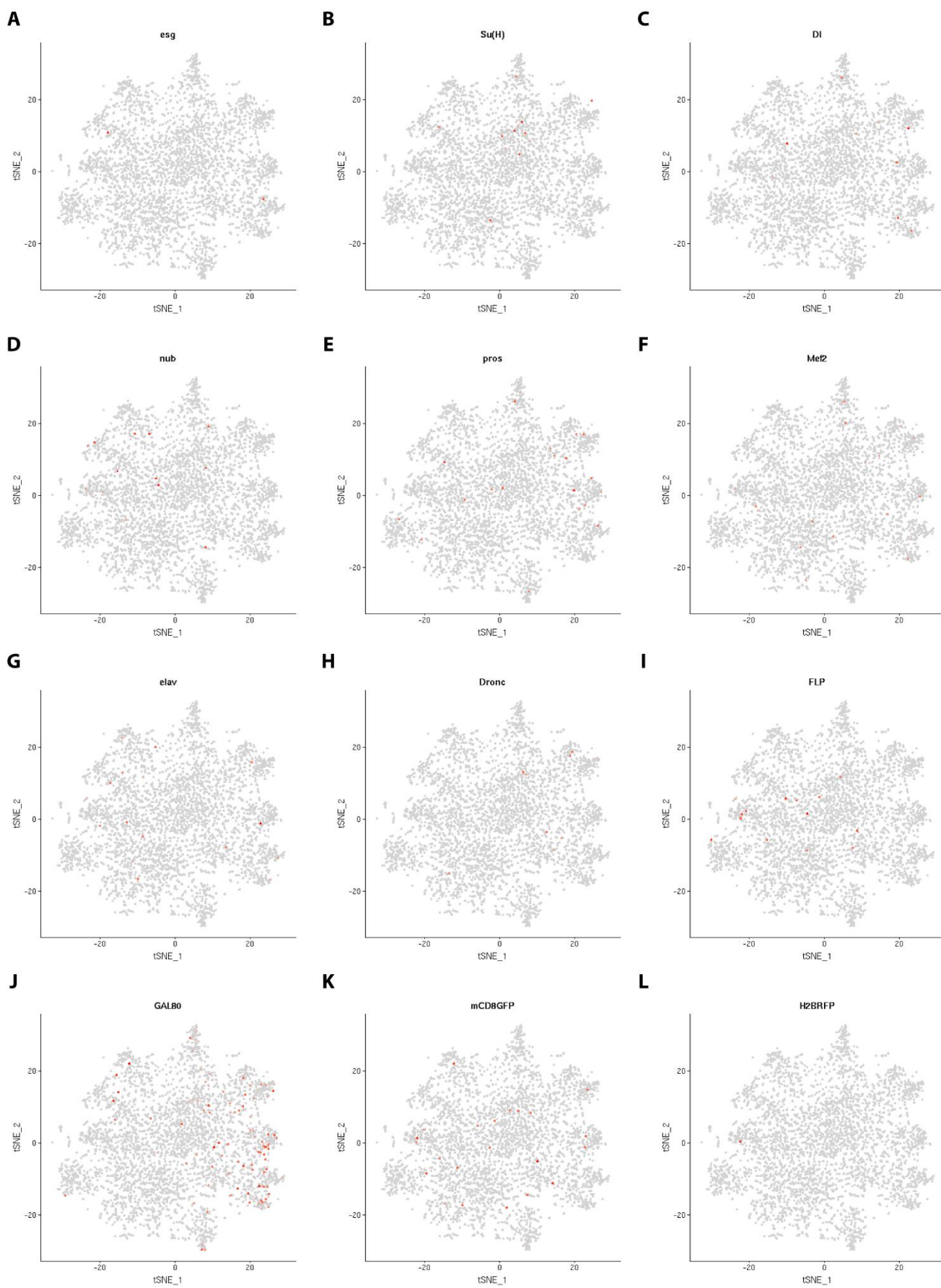


Figure 6-4: Cluster identity cannot be assigned from cell markers or transgenes.

(A) t-SNE plot depicting the expression of the Intestinal Stem Cell and Enteroblast marker, *esg*. Each dot represents one cell, grey represents low or no expression; red represents high transcript expression. Only two cells (top-left and bottom-right) appear to have high transcript expression of *esg*. (B) t-SNE plot depicting the expression of the Enteroblast marker, *Su(H)*. Despite a notable increase in the number of cells expressing this marker, similar to (A) the expression of the *Su(H)* marker is relatively low. (C) t-SNE plot depicting the expression of the Intestinal Stem Cell marker, *Delta (Dl)*. The expression of this marker appears equally as non-cluster specific as the previous examples in (A) and (B). (D) t-SNE plot depicting the expression of the Enterocyte marker, *Pdm1 (nub)*. The expression of this marker although shifted towards the top left clusters is still relatively lowly expressed. (E) t-SNE plot depicting the expression of the Enteroendocrine marker, *Prospero (pros)*. The expression of this marker appears non-cluster specific and has a relatively low expression pattern. (F) t-SNE plot depicting the expression of the muscle marker *Mef2*. The expression of this marker appears non-cluster specific and has a relatively low expression pattern. (G) t-SNE plot depicting the expression of the neuronal marker *elav*. The expression of this marker appears non-cluster specific and has a relatively low expression pattern. (H) t-SNE plot depicting the expression of the apical-caspase *Dronc*. Expression patterns of this transcript should mimic a similar pattern to the $-/-$ condition t-SNE plot depicted in [Figure 6-3 A], as the efficiency of *Dronc* KO using the *esg*-Gal4 driver is around 90%. However, the expression of *Dronc* mRNA appears non-cluster specific and demonstrates a low expression pattern similar to the markers previously described in A-G. (I) t-SNE plot depicting the expression of the *flippase-recombinase protein (FLP)* transgene. The expression of this transgene owing to the genetics used in this experiment, should only occur in the *Dronc* KO condition and thus should also mimic a similar pattern to the $-/-$ condition depicted in [Figure 6-3 A]. Similar to (H), I am unable to recapitulate a similar t-SNE pattern using *FLP* expression. (J) t-SNE plot depicting the expression of the Gal4 repressor transgene, *GAL80*. This transgene is expressed under the regulation of the *Tubulin* promoter in

all of cells in both conditions, and therefore all cells should depict high expression levels of this gene. While considerably more cells show high expression of the transcript when compared to the other genes described in A-I, the number of cells remain considerably lower than expected. (K) t-SNE plot depicting the expression of the ReDDM membrane localised green fluorescent protein, *mCD8GFP*. As previously described, the expression of this transgene is under the regulation of the *esg* promoter and thus labels Intestinal Stem Cells and Enteroblasts. Expression of this transgene appears non-cluster specific and furthermore, does not correlate with the cells depicted in the t-SNE plot for *esg* expression depicted in (A). (L) t-SNE plot depicting the expression of the ReDDM HistoneRFP (*H2BRFP*). The expression of this transgene should mimic the expression patterns denoted in (A) and (K) due to its expression under the *esg* promoter. As previously described with (L) the expression pattern of this transgene does not co-localise with the other genes. The genotypes of the *Drosophila* in this figure are *w¹¹¹⁸; esg-Gal4 UAS-mCD8-GFP ; UAS-HistoneRFP TubG80ts Dronc^{KO} / SM6A-TM6B* and *w¹¹¹⁸; esg-Gal4 UAS-mCD8-GFP / +; UAS-HistoneRFP TubG80ts Dronc^{KO} / UAS-Flp FRT Dronc-GFP-APEX FRT Suntag-HA-Cherry*.

Due to the non-specific and low expression of canonical cell markers, a different approach was required to assign an identity to the clusters. A previous study by the Lemaitre laboratory (150) was able to comprehensively describe varying gene expression in different regions of the intestine, and furthermore through FACS sorting, were able to measure the transcript abundance of genes corresponding to each cell type within the *Drosophila* intestinal system (150). I hypothesised that by comparing the cluster identity markers obtained from the Seurat software, associated with each of the 13 clusters, to the cell-specific gene expression data from the Lemaitre laboratory (150), I could assign an identity to the clusters.

Following the acquisition of cluster identity marker genes, using the Seurat Drop-sequencing toolkit (300), I entered these into the FlyGut-seq tool (150) (<http://flygutseq.buchonlab.com/>). The genes and the expression levels were then imported into Excel [Figure 6-5 A]. The numerical expression data obtained for each gene is described as read per kilobase per million (RPKM) [Figure 6-6 A]. As these RPKM values varied widely between genes, and thus very highly expressed genes, or genes expressed at very low levels could potentially skew the data, I averaged the RPKM values between all of the cell types. Subsequently for each of the cell types, I calculated the percentage RPKM of this average [Figure 6-5 B]. As the identity markers of a cluster could be up or down-regulated when compared to the other clusters, I multiplied the log-fold change value obtained from the Seurat software with the percentage of the average RPKM [Figure 6-5 C]. Finally, in order to determine the intestinal cell most likely to correspond to the cluster, I took the average across all of the marker genes with the highest value determining the most likely identity [Figure 6-5 D].

A

	A	B	C	D	E	F	G	H	I
1	Search Gene	Found FBGN	Found Gene	gut,7-10day female,ISC	gut,7-10day female,EB	gut,7-10day female,EC	gut,7-10day female,EE	gut,7-10day female,VM	
2	Amy-d	FBgn000007	Amy-d	3.73	1.85	4.79	25.12	60.81	
3	Amy-p	FBgn000007	Amy-p	5.56	4.49	806.44	95.03	364.23	

B

J	K	L	M	N	O
Average RPKM	ISC (%) of Average	EB (%) of Average	EC (%) of Average	EE (%) of Average	VM (%) of Average
19.26	19.36656	9.6054	24.8702	130.4258	315.7321
255.15	2.17911	1.759749	316.0651	37.24476	142.7513

C

Q	R	S	T	U	V
Drop-Seq (log-fold change)	ISC	EB	EC	EE	VM
2.036462089	39.43927	19.56103	50.64721	265.6071	642.9764
1.849360212	4.02996	3.25441	584.5181	68.87897	263.9986

**VM (%) of Average
x
Drop-seq (log-fold change)**

←

D

ISC	EB	EC	EE	VM
-19.2776	-17.1179	21.39438	14.26397	86.72975

Average (% of Average x Drop-seq-log fold change)

↑

Figure 6-5: Method of assigning cluster identity using Seurat and FlyGut-seq tools

(A) Genes which were used to cluster the cells in [Figure 6-3 B], were extracted using the Seurat tool and entered into FlyGut-seq tool, produced by the Lamaitre laboratory (150). The resulting data was exported to Excel. The gene name is indicated in the left panel under column A. The read per kilobase per million (RPKM) can be seen under each of the cell types of the *Drosophila* intestinal system in Column D-H. Note the varying values between each cell type and between

each gene displayed. (B) The RPKM values are averaged (Column J) and the percentage of the average RPKM is calculated for each cell type (Column K-O), using the raw RPKM values obtained from FlyGut-seq in (A). (C) Cluster identity marker genes can be up or downregulated when compared to other clusters. To account for this, the values calculated in (B) were multiplied by the log-fold change value obtained from Seurat (Column Q), resulting in the multiplication values denoted in columns R-V. (D) To take into consideration the expression of all identity marker genes, the average of the columns were calculated for each of the individual cell types. The highest value was then selected as the most likely identity of the cluster (See yellow highlighting, denoting visceral muscle (VM)).

Following the application of the aforementioned method, I was able to identify the cellular identity of the 13 clusters [Figure 6-6]. Five clusters corresponded to cell types of the *Drosophila* epithelia, i.e. the Intestinal Stem Cells, Enteroblasts, Enterocytes and Enteroendocrine Cells, and 8 of the clusters appeared to correspond to visceral muscle [Figure 6-6].

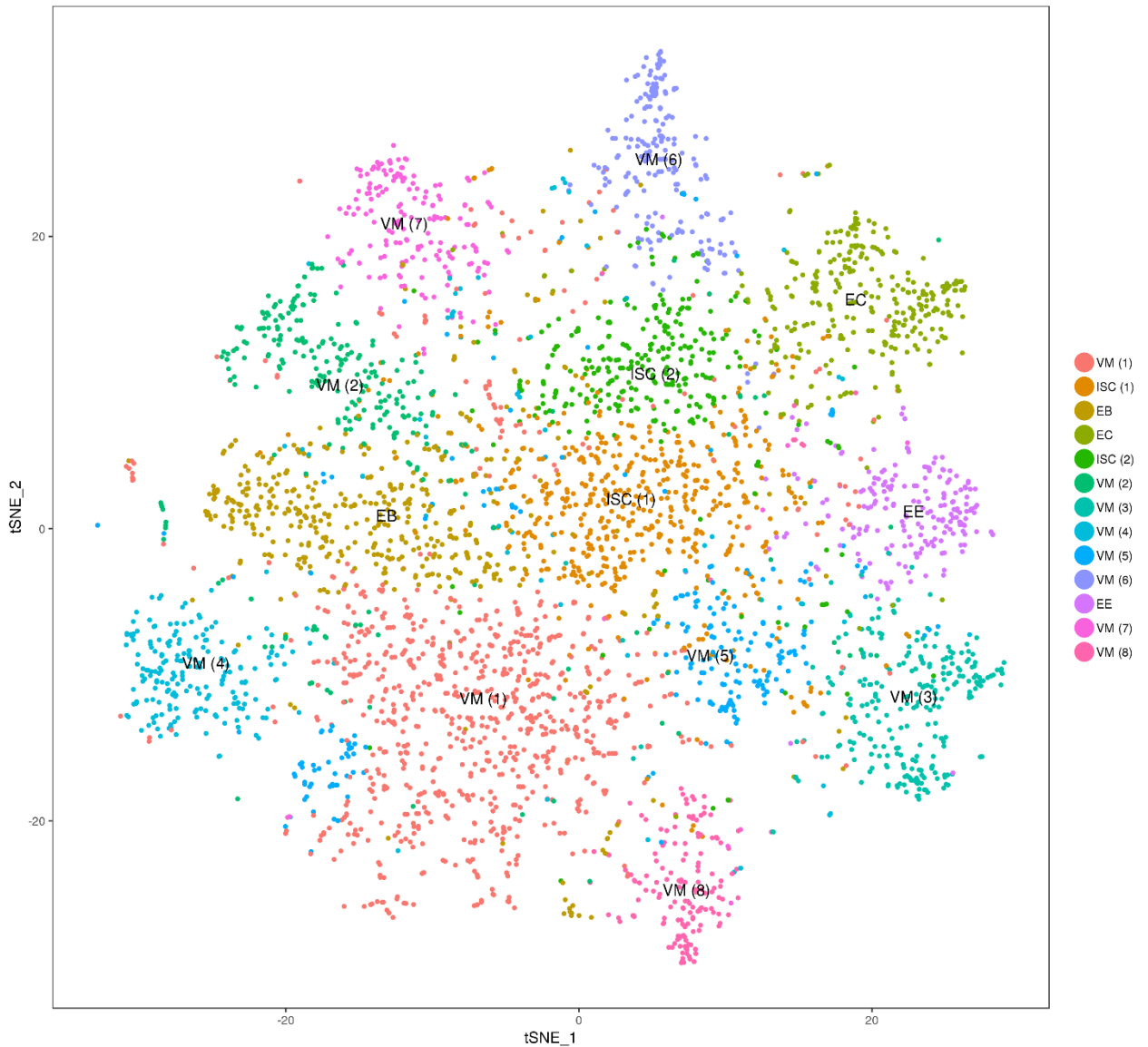


Figure 6-6: Identity can be assigned to the clusters using FlyGut-seq tools.

t-SNE plot depicting cells from adult female *Drosophila* intestines. The two conditions (*Dronc* heterozygous and progenitor cell *Dronc* knockout intestines) are merged into a single plot of 13 clusters. Identity has been assigned to these clusters using the log-fold change of each gene obtained from Seurat, and the previously published expression patterns of these genes in individual intestinal cells obtained from FlyGut-seq (150, 300). This method has identified eight visceral muscle clusters (VM (1); VM (2); VM (3); VM (4); VM (5); VM (6); VM (7) and VM (8)); two Intestinal Stem Cell clusters (ISC (1) and ISC (2)); an Enteroblast (EB) cluster; an Enterocyte (EC) cluster and an Enteroendocrine cell cluster (EE). The genotypes of the *Drosophila* in this figure

are $w^{1118}; esg-Gal4 \text{ UAS-}mCD8-GFP; UAS-HistoneRFP \text{ TubG80ts } Dronc^{KO} / SM6A-TM6B$ and $w^{1118}; esg-Gal4 \text{ UAS-}mCD8-GFP / +; UAS-HistoneRFP \text{ TubG80ts } Dronc^{KO} / UAS-Flp \text{ FRT } Dronc-GFP-APEX \text{ FRT } Suntag-HA-Cherry$.

I next sought to identify any genes which might be differentially transcribed between the *Dronc* heterozygous and progenitor cell *Dronc* KO intestines. As previous experimental evidence demonstrated a functional for *Dronc* in the Enteroblasts, I decided to interrogate the transcriptional changes occurring within this cluster, validating these variances using qPCR. However, as the progenitor cell population in homeostatic conditions only constitutes around 20% of the total cells of the intestinal epithelia, I decided to FACS sort the dissociated intestinal cells in order to obtain a pure population of progenitors.

Following RNA extraction from the sorted cells, reverse transcription and amplification of the cDNA; I attempted to validate candidates by selecting genes which were transcriptionally changed by a log-fold of 1.5 or more. However, I was unable to validate significant changes in the transcription of the desaturase enzyme, *Fad2* [Figure 6-7 A]; the lipid gamma-hydroxylase, *cytochrome P450-4g1* (*Cyp4g1*) [Figure 6-7 B]; the α -*Trypsin* (α Try) enzyme [Figure 6-7 C] and the *Amylase Proximal* (*Amy-P*) enzyme [Figure 6-7 D].

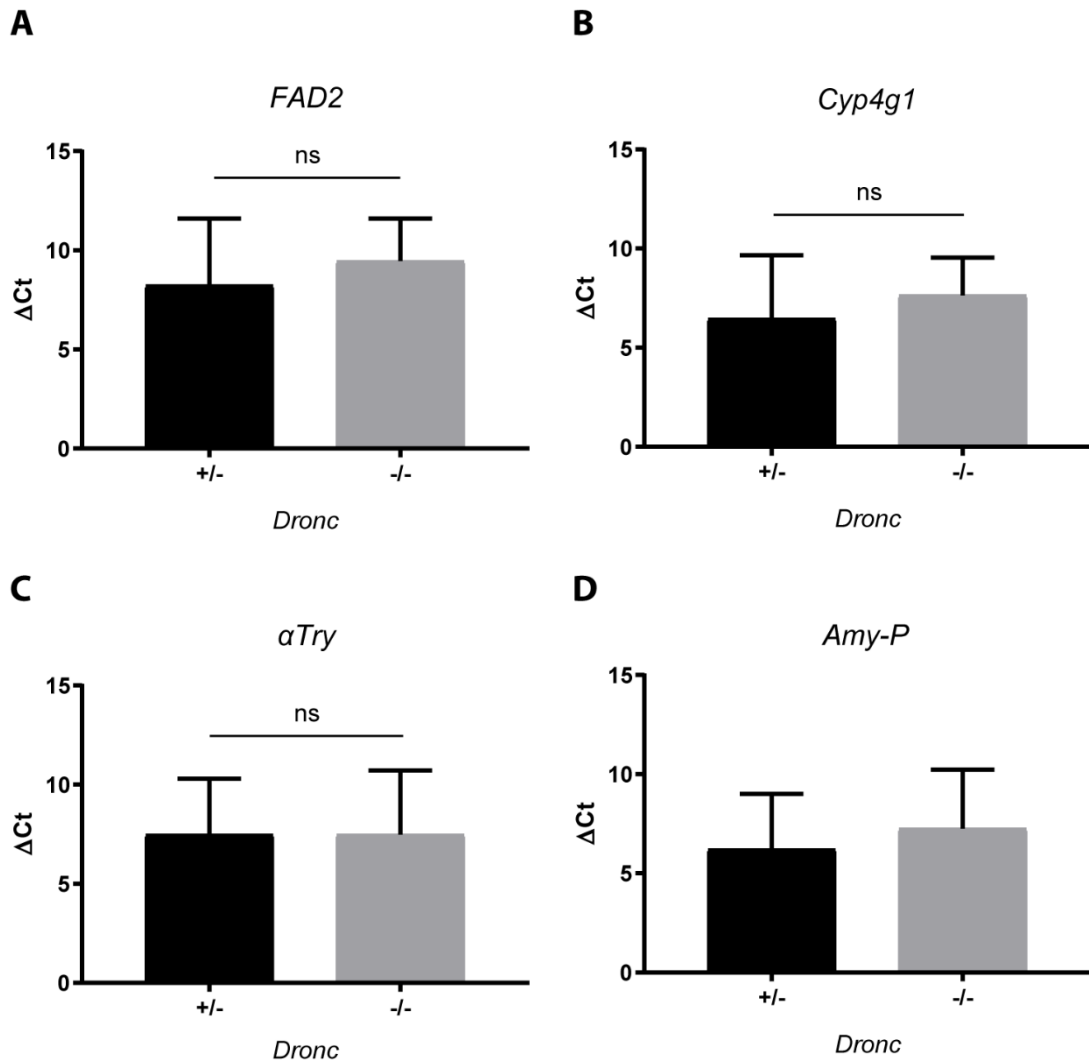


Figure 6-7: Intestinal progenitor FACS sorting and qPCR fails to validate Drop-seq candidates.

(A) qPCR data of *FAD2* expression in *Dronc* heterozygous and *Dronc* KO intestinal progenitors. There is no significant change in the gene expression of *FAD2* between the two conditions ($P = 0.6255$, unpaired two-tailed t-test, +/- N = 3 -/- N = 3). (B) qPCR data of *Cyp4g1* expression in *Dronc* heterozygous and *Dronc* KO intestinal progenitors. There is no significant change in the gene expression of *Cyp4g1* between the two conditions ($P = > 0.9999$, Mann Whitney test, +/- N = 3 -/- N = 3). (C) qPCR data of αTry expression in *Dronc* heterozygous and *Dronc* KO intestinal progenitors. There is no significant change in the gene expression of αTry between the two conditions ($P = 0.9995$, Unpaired two-tailed t-test, +/- N = 3 -/- N = 3). (D) qPCR data of *Amy-P* expression in *Dronc* heterozygous and *Dronc* KO intestinal progenitors. There is no significant

change in the gene expression of *Amy-P* between the two conditions ($P = 0.6849$, Unpaired two-tailed t-test, $+/- N = 3$ $-/- N = 3$). All of the qPCRs described were completed following the dissociation of the intestinal cells and FACS sorting for non-DAPI, *esg* positive cells (*esg-Gal4 UAS-GFP*). The RNA was subsequently reverse transcribed, and the cDNA amplified before qPCR experiments. Error bars represent Standard Deviation of the Mean in all panels. All of the experiments described in the figure were performed in Oxford medium under an experimental regime which protects epithelial integrity. The genotype of the $+/-$ *Drosophila* is $w^{1118}; esg-Gal4 UAS-mCD8-GFP ; UAS-HistoneRFP TubG80ts Dronc^{KO} / SM6A-TM6B$. The genotype of the $-/-$ *Drosophila* is $w^{1118}; esg-Gal4 UAS-mCD8-GFP / +; UAS-HistoneRFP TubG80ts Dronc^{KO} / UAS-Flp FRT Dronc-GFP-APEX FRT Suntag-HA-Cherry$.

Whole intestine Drop-seq analysis reveals the misexpression of genes

As previous efforts to validate Enteroblast specific candidates appeared to be ineffective, I decided to scale back attempts at discovering Enteroblast specific candidates, and search for transcriptional changes across all cells of the intestinal system. Using the Seurat software, I was able to obtain candidates which appeared to be transcribed at a log-fold change of 1.5 above, or below the control intestines. Subsequently, I screened these candidates using the qPCR techniques discussed before. However, I did not dissociate the tissue before RNA extraction or amplify the cDNA. I was unable to observe statistically significant changes in the transcription of *β -Trypsin* (*β Try*) [Figure 6-8 A]; *Cytochrome P450-4g1* (*Cyp4g1*) [Figure 6-8 B]; *FAD2* [Figure 6-8 C]; *Eps15 homology domain containing protein-binding protein 1* (*Ehbp1*) [Figure 6-8 D]; *Drosomycin* (*Drs*) [Figure 6-8 E]; *Maltase A1* (*Mal-A1*) [Figure 6-8 F]; *Maltase A6* (*Mal-*

A6) [Figure 6-8 G]; *Maltase A7 (Mal-A7)* [Figure 6-8 H]; *Yolk protein 1 (Yp1)* [Figure 6-8 I]; *Yolk protein 2 (Yp2)* [Figure 6-8 J] and *Yolk protein 3 (Yp3)* [Figure 6-8 K]. Although, I was able to observe a statistically significant up-regulation in α -Trypsin (α -Try) [Figure 6-8 L]; *Amylase Distal (Amy-D)* [Figure 6-8 M] and *javelin-like (Jvl)* [Figure 6-8 N] expression in progenitor cell *Dronc* KO intestines, and downregulation of *Jonah 65Aiii (Jon65Aiii)* [Figure 6-8 N]. Both α -Trypsin and *Jon65Aiii* are markers of Enterocyte cells (301) and their misexpression could reflect the premature and aberrant differentiation observed in progenitor cell *Dronc* KO intestines. Furthermore, upregulation of the carbohydrate digestive enzyme *Amylase Distal* is also a likely reflection of this aberrant differentiation (144). The role of the misexpression of Javelin-like is less clear; however its activity has been linked to nuclear migration events in the Oocyte (302). In a previous manuscript produced by the Baena-Lopez laboratory of which I contributed experimental data to, using a variant of the DBS-S-QF reporter (DBS-S), we observed nuclear migration events before the apoptosis program, perhaps suggesting a role for *Dronc* and Javelin-like in this process (219).

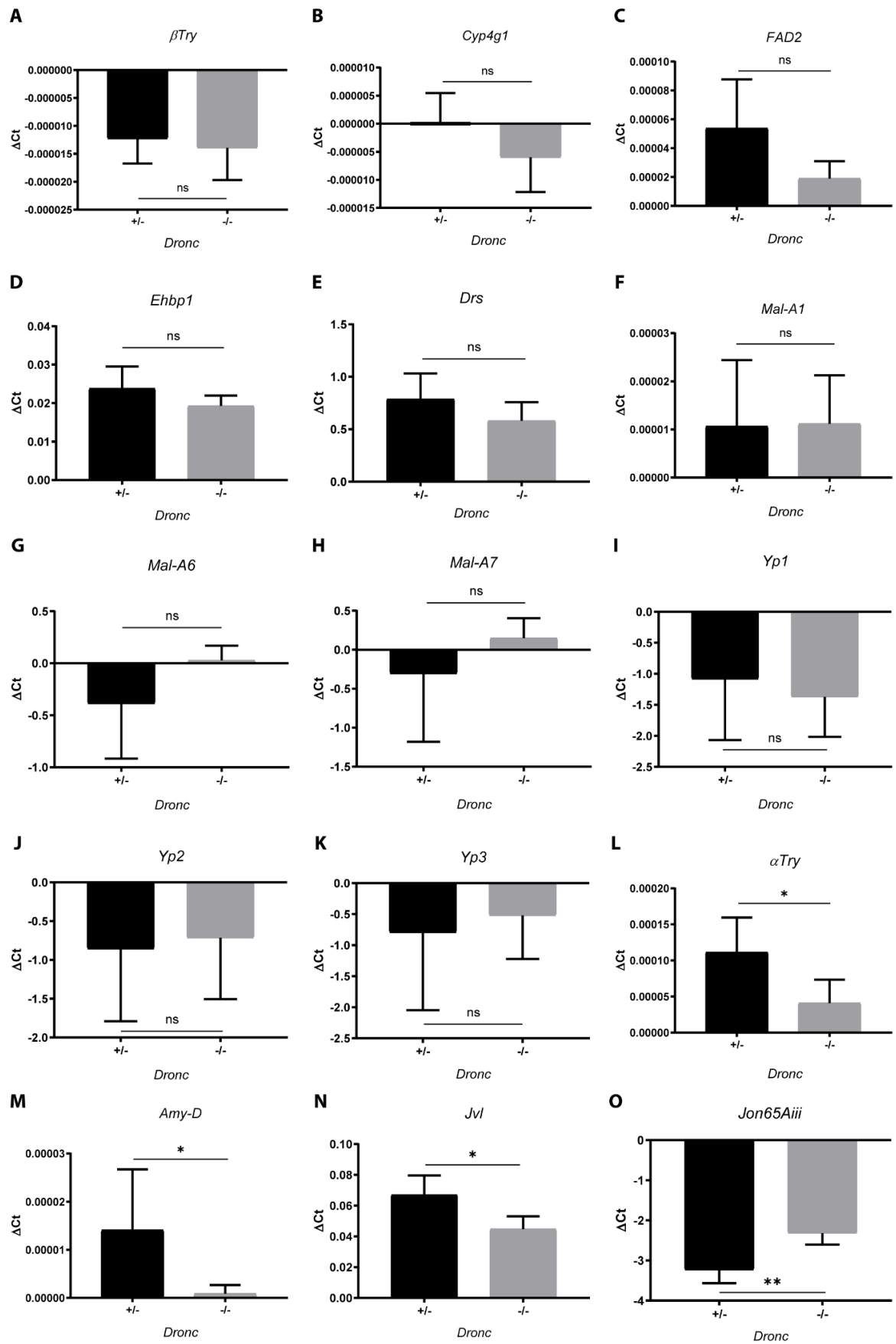


Figure 6-8: *α-Trypsin*, *Amylase Distal*, *Javelin-like* and *Jonah 65Aiii* are misexpressed in *esg*-induced *Dronc* KO intestines.

(A) qPCR data of *βTry* expression in *Dronc* heterozygous and progenitor cell *Dronc* KO intestines. There is no significant change in the gene expression of *βTry* between the two conditions ($P = 0.6287$, unpaired two-tailed t-test, $+/- N = 5$ $-/- N = 5$). (B) qPCR data of *Cyp4g1* expression in *Dronc* heterozygous and progenitor cell homozygous KO intestines. There is no significant change in the gene expression of *Cyp4g1* between the two conditions ($P = 0.1296$, unpaired two-tailed t-test, $+/- N = 5$ $-/- N = 5$). (C) qPCR data of *FAD2* expression in *Dronc* heterozygous and progenitor cell *Dronc* KO intestines. There is no significant change in the gene expression of *FAD2* between the two conditions ($P = 0.0630$, unpaired two-tailed t-test, $+/- N = 5$ $-/- N = 5$). (D) qPCR data of *Ehbp1* expression in *Dronc* heterozygous and progenitor cell *Dronc* KO intestines. There is no significant change in the gene expression of *Ehbp1* between the two conditions ($P = 0.1481$, unpaired two-tailed t-test, $+/- N = 5$ $-/- N = 5$). (E) qPCR data of *Drs* expression in *Dronc* heterozygous and progenitor cell *Dronc* KO intestines. There is no significant change in the gene expression of *Drs* between the two conditions ($P = 0.1664$, unpaired two-tailed t-test, $+/- N = 5$ $-/- N = 5$). (F) qPCR data of *Mal-A1* expression in *Dronc* heterozygous and progenitor cell *Dronc* KO intestines. There is no significant change in the gene expression of *Mal-A1* between the two conditions ($P = 0.8413$, unpaired two-tailed t-test, $+/- N = 5$ $-/- N = 5$). (G) qPCR data of *Mal-A6* expression in *Dronc* heterozygous and progenitor cell *Dronc* KO intestines. There is no significant change in the gene expression of *Mal-A6* between the two conditions ($P = 0.1786$, unpaired two-tailed t-test, $+/- N = 5$ $-/- N = 5$). (H) qPCR data of *Mal-A7* expression in *Dronc* heterozygous and progenitor cell *Dronc* KO intestines. There is no significant change in the gene expression of *Mal-A7* between the two conditions ($P = 0.3532$, unpaired two-tailed t-test, $+/- N = 5$ $-/- N = 5$). (I) qPCR data of *Yp1* expression in *Dronc* heterozygous and progenitor cell *Dronc* KO intestines. There is no significant change in the gene expression of *Yp1* between the two conditions ($P = 0.6409$, unpaired two-tailed t-test, $+/- N = 5$ $-/- N = 5$). (J) qPCR data of *Yp2* expression in *Dronc*

heterozygous and progenitor cell *Dronc* KO intestines. There is no significant change in the gene expression of *Yp2* between the two conditions ($P = 0.8236$, unpaired two-tailed t-test, +/- N = 5 -/- N = 5). (K) qPCR data of *Yp3* expression in *Dronc* heterozygous and progenitor cell *Dronc* KO intestines. There is no significant change in the gene expression of *Yp3* between the two conditions ($P = 0.7185$, unpaired two-tailed t-test, +/- N = 5 -/- N = 5). (L) qPCR data of α *Try* expression in *Dronc* heterozygous and progenitor cell *Dronc* KO intestines. There is a significant upregulation in the gene expression of α *Try* in *esg*-induced *Dronc* loss of function intestines ($P = 0.0264$, unpaired two-tailed t-test, +/- N = 5 -/- N = 5). (M) qPCR data of *Amy-D* expression in *Dronc* heterozygous and progenitor cell *Dronc* KO intestines. There is a significant upregulation in the gene expression of *Amy-D* in *esg*-induced *Dronc* loss of function intestines ($P = 0.0489$, unpaired two-tailed t-test, +/- N = 5 -/- N = 5). (N) qPCR data of *Jvl* expression in *Dronc* heterozygous and progenitor cell *Dronc* KO intestines. There is a significant upregulation in the gene expression of *Jvl* in *esg*-induced *Dronc* loss of function intestines ($P = 0.0107$, unpaired two-tailed t-test, +/- N = 5 -/- N = 5). (O) qPCR data of *Jon65Aiii* expression in *Dronc* heterozygous and progenitor cell *Dronc* KO intestines. There is a significant downregulation in the gene expression of *Jon65Aiii* in *esg*-induced *Dronc* loss of function intestines ($P = 0.0056$, unpaired two-tailed t-test, +/- N = 5 -/- N = 5). Error bars represent Standard Deviation of the Mean in all panels. All of the experiments described in the figure were performed in Oxford medium under an experimental regime which protects epithelial integrity. The genotype of the +/- *Drosophila* is $w^{1118}; esg-Gal4 \text{ UAS-}mCD8-GFP; \text{ UAS-HistoneRFP TubG80ts } Dronc^{KO} / SM6A-TM6B$. The genotype of the -/- *Drosophila* is $w^{1118}; esg-Gal4 \text{ UAS-}mCD8-GFP / +; \text{ UAS-HistoneRFP TubG80ts } Dronc^{KO} / \text{ UAS-Flp FRT } Dronc-GFP-APEX \text{ FRT Suntag-HA-Cherry}$.

6.2. Results – Dronc KO intestines have an increased viral load

Following the demonstration of gene misexpression in progenitor *Dronc* KO intestines, I decided to investigate whether this deregulation and/or the intestinal hyperplasia could alter the composition of the microbiota. As previous studies have documented that changes in gene expression altered the microbiota of *Drosophila* and mice models (303, 304). To establish whether there was a microbiome compositional change, I used the taxonomic classification software, Kraken2 (230).

Kraken2 uses a pre-computed database of known organisms and assigns taxonomic labels to DNA sequences corresponding to these organisms. The Kraken2 software then segments the query sequence, in this case, a sequence obtained from the Drop-seq data, into nucleotide sequences of a certain length (*k*-mers). The sequence is then mapped to the lowest common ancestor of the genomes that contain that particular *k*-mer. The mapping of the sequence *k*-mers forms a pruned subtree of a general taxonomy tree used for the classification process. Following the processing of all of the *k*-mers of a given query sequence, the classification tree is scored by summing all of the weights in the path. The highest number is designated as the taxonomic classification assigned to the query sequence (230) [Figure 7-10]. Importantly, *k*-mers which do not correspond to any taxa in the pre-computed database are discarded, thus reducing the number of false positives. This process occurs for every sequence in the Drop-seq dataset, producing a list of all of the organisms identified, with a tally corresponding to the number of reads for each organism.

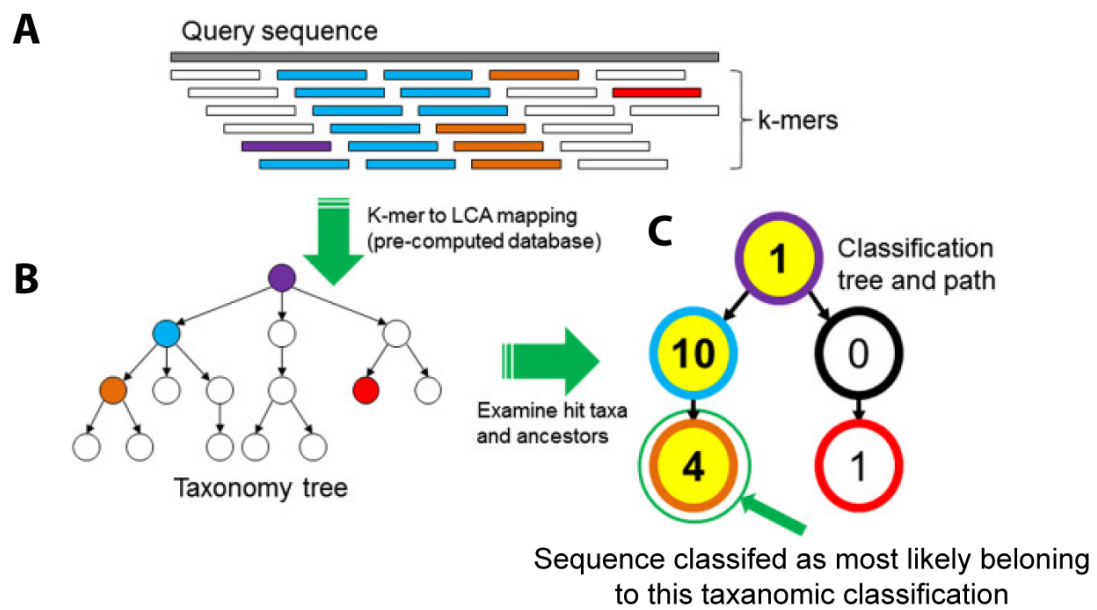


Figure 6-9: The Kraken2 sequence algorithm for metataxonomic analysis of sequencing data

(A) The query sequence obtained from the Drop-seq data is delivered into the Kraken2 algorithm, and split into nucleotide sequences (k -mers) of 31 nucleotides (default). These individual k -mers are then cross-referenced against a pre-built database containing each of the possible k -mer sequences, tagged with the associated taxa. (B) The taxa associated with the sequence k -mers, as well as the taxas ancestors, are then structured into a classification tree which will subsequently be used for classification. (C) In the tree, each of the nodes is weighted against the number of k -mers in the sequence associated with that nodes taxon. Each of the pathways are then scored by adding together all of the weights in the paths, with the highest number denoting the most likely classification path. Note this is the reason why the orange circle is designated the taxa and not the red circle. The figure was obtained and adapted from (230)

In order to determine whether there was a compositional change in the microbiota following loss of *Dronc* in the progenitor cells, I analysed both the *Dronc* heterozygous and the *esg*-induced *Dronc* loss of function datasets, using the Kraken2 software.

Initially, I compared the composition of the microbiota at the domain level, comparing the read counts of the Eukaryota, Bacteria and Archaea, in addition to the number of viral reads. Subsequently, I calculated these reads as a percentage of the total read count. In agreement with previous studies of microbiome composition, the microbiome of *Dronc* heterozygous intestines appeared to consist mainly of reads from the bacteria domain (305, 306) [Figure 6-10 A]. Additionally, a significant proportion of the reads were from the Eukaryota domain [Figure 6-10 A]. On closer inspection of this domain, it appeared that the majority of these reads were obtained from the Fungi, Metazoa and Viridiplantae kingdoms. As with bacteria, previous studies have demonstrated the presence of Fungi and metazoan populations in the *Drosophila* intestinal microbiota (307, 308). The contribution from the Viridiplantae kingdom can presumably be attributed to the plant-based media used to rear the flies, still present in the intestinal system during dissection. The contribution of the viral reads to the composition was low [Figure 6-10 A] and the Archaea domain reads were negligible. Confident in the validity of my data in comparison to the previously described literature pertaining to intestinal microbiota composition, I proceeded to analyse the data obtained from the *esg*-induced *Dronc* knockout intestines.

Strikingly, the composition of the microbiota in *Dronc* KO progenitor intestines varied significantly from the heterozygous controls [Figure 6-10 A-B]. Following loss of *Dronc* in the progenitor cells, I observed a substantial increase in the number of viral reads obtained from these intestines [Figure 6-10 B]. Furthermore, the viral reads appeared

to account for the majority of the reads obtained; with the Bacterial and Eukaryota appearing reduced in this condition [Figure 6-10 A-B].

Next, I sought to investigate whether the increase in viral reads was occurring at the expense of the Bacteria and Eukaryota reads, i.e Dronc was inhibiting the growth of bacteria at the expense of viral; or whether the composition of the Bacteria and Eukaryota remained constant and the increase in viral reads occurred independently. In order to distinguish between these two possibilities, I normalised the reads from the Virus, Bacteria and Eukaryota to the contamination reads associated to the *Homo sapien* taxa. I hypothesised that as all of the samples were processed at the same time, the contribution of the contaminants to the total reads should be independent of experimental changes, and thus should increase linearly with the number of reads in the sample .i.e more sample equates to more reads. Through comparing the fold change in *H. Sapien* reads compared the Virus, Bacteria and Eukaryota; I was able to observe a 116-fold increase in the read count of Viruses and a 1.89 and 1.86-fold decrease in the read count from the Bacteria and Eukaryota respectively [Figure 6-10 C]. Thus, suggesting that while there is a decrease in the number of Bacteria and Eukaryota present in the sample, the increased viral read count appears to increase, in part, independently of their decrease.

Subsequently, I sought to examine which of the viral species were present in the increased viral load following the loss of *Dronc* function. Strikingly, the picorna-like, Nora Virus, appeared to be overwhelmingly represented, with *Drosophila subobscura* Nora Virus variant appearing to constitute a further minor contribution [Figure 6-10 D]. *Macaca mulatta* polyomavirus 1 and *Drosophila immigrans* Nora Virus were present at almost negligible amounts by comparison [Figure 6-10 D].

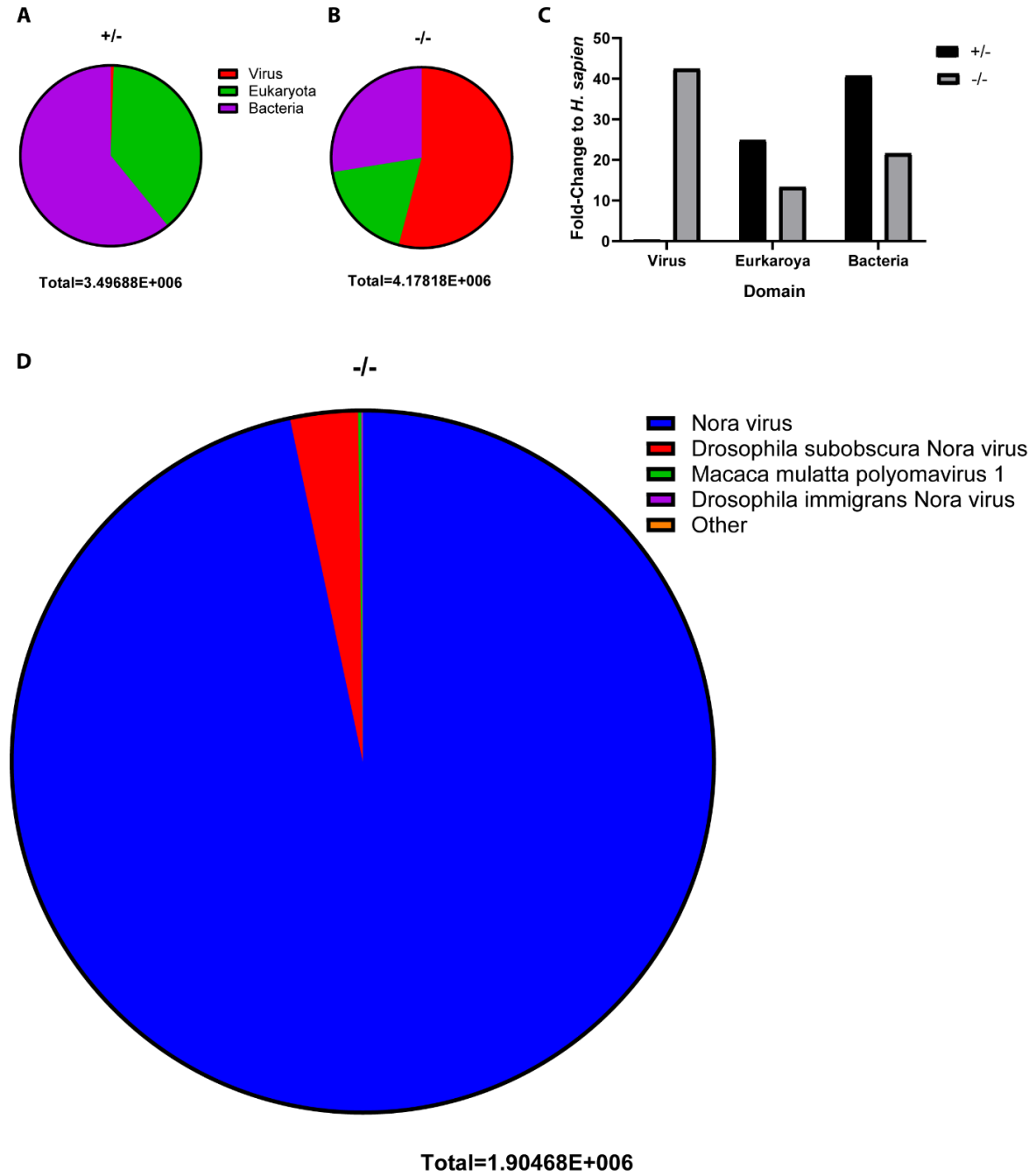


Figure 6-10: *esg*-induced *Dronc* KO intestines have an increased viral load.

(A) Pie-chart depicting the reads associated with the Bacteria and Eukaryota domains, and Viruses. Note that Archaea were included in the analysis; however, their contribution to the chart was negligible and thus not displayed. In *Dronc* heterozygous intestines, Bacterial reads constitute the majority of the total assigned reads from the Drop-Seq data, with Eukaryota the next largest population. Note that the number of viral reads in comparison to the total remains small. (B) Pie-chart depicting the reads associated with the Bacteria and Eukaryota domains, and Viruses. Note that Archaea were included in the analysis however the contribution to the chart was negligible and thus not displayed. In *esg*-induced *Dronc* KO intestines there is a substantial increase in the number of Viral reads compared to (A); with the contribution of the Bacteria and Eukaryota domains constituting a reduced proportion of the total reads. (C) Bar graph depicting the reads associated with Bacteria, Eukaryota or Virus from the *Dronc* heterozygous and the *esg*-induced *Dronc* knockout intestines, normalised to the contaminant reads of *Homo sapiens*. Viral reads appear to increase by 116-fold in progenitor *Dronc* Knockout intestines compared to control. Bacterial and Eukaryota reads decrease by 1.89 and 1.86-fold respectively. (D) Pie Chart depicting the reads of the viral species contributing to the overall increase observed in progenitor *Dronc* KO intestines. Nora virus appears to be overwhelmingly implicated with the increase, with *Drosophila subobscura* Nora Virus also contributing to a lesser extent. Increases in *Macaca mulatta* polyomavirus 1 and *Drosophila immigrans* Nora Virus also appeared to play a negligible contribution. All of the experiments described in the figure were performed in Oxford medium under an experimental regime which protects epithelial integrity. The genotype of the +/- *Drosophila* is $w^{1118}; esg-Gal4 \text{ UAS-}mCD8-GFP; \text{ UAS-HistoneRFP TubG80ts } Dronc^{KO} / SM6A-TM6B$. The genotype of the -/- *Drosophila* is $w^{1118}; esg-Gal4 \text{ UAS-}mCD8-GFP / +; \text{ UAS-HistoneRFP TubG80ts } Dronc^{KO} / \text{ UAS-Flp FRT } Dronc-GFP-APEX \text{ FRT Suntag-HA-Cherry}$.

As the aforementioned microbiota changes in the *Dronc* KO progenitor intestines could have resulted from any number of environmental factors, such as intestinal infection, I sought to confirm these observations using qPCR. Importantly, using independent replicates reared in separate vials. On this occasion, I was able to observe a decrease in viral load, observing a statistically significant decrease in the mRNA of the Nora Virus gene, *Gp1* [Figure 6-11 A]. Subsequently, I investigated whether I could observe a change in the abundance of bacterial organisms in *esg*-induced *Dronc* KO intestines; using primers specific for bacteria which have previously been demonstrated to be highly prevalent in the composition of the intestinal microbiota (309). I was unable to observe a statistically significant change in the levels of *L. fructivorans*, *L. brevis*, *L. plantarum*, *A. tropicalis* and *A. pomorum* [Figure 6-11 B-F]; however, with the exception of *L. plantarum*, all appeared to be present at higher levels in the *esg*-induced *Dronc* knockout than in the *Dronc* heterozygous intestines [Figure 6-11 B-F].

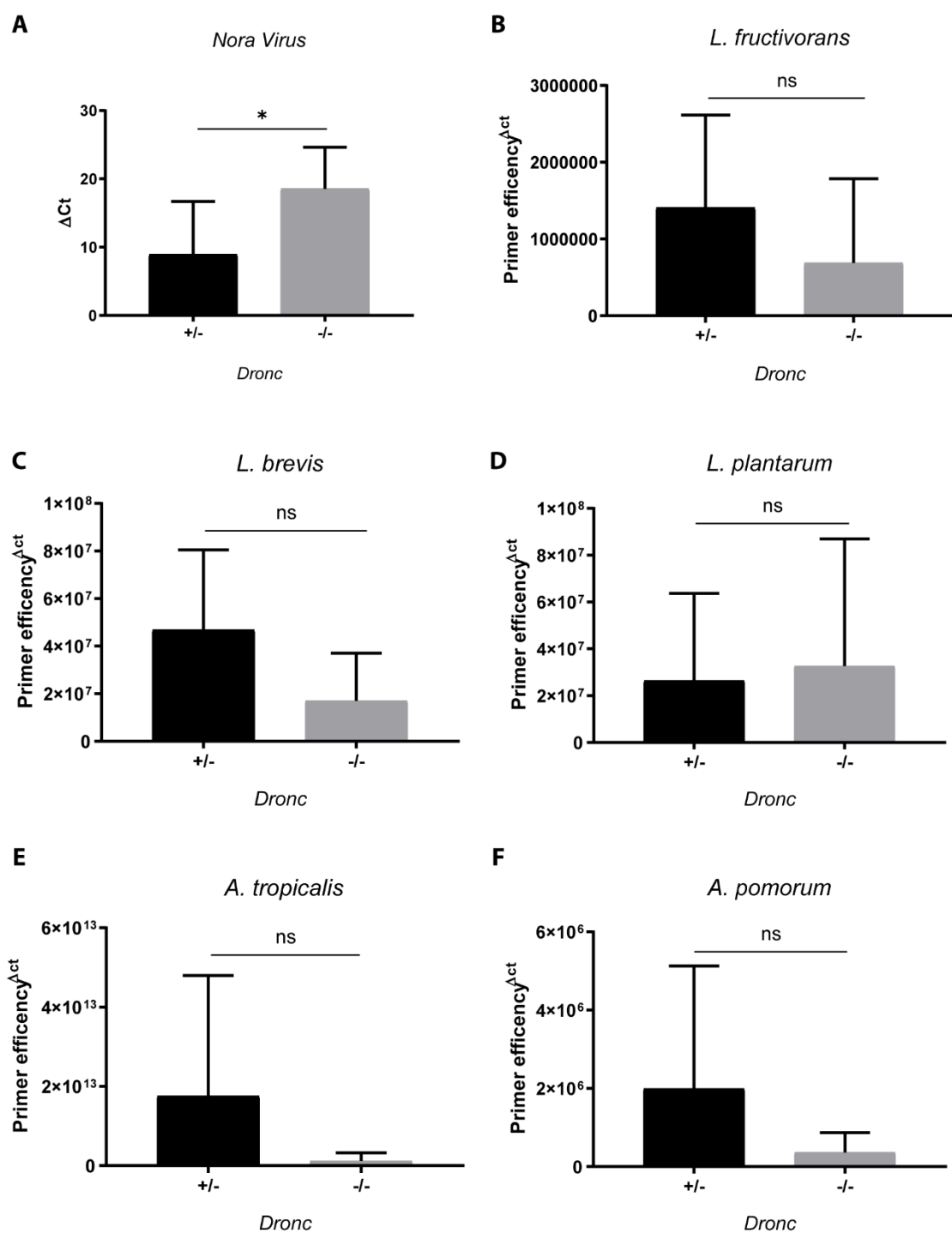


Figure 6-11: A reduction in Nora Virus colonisation and an increase in bacterial colonisation is observed using qPCR.

(A) qPCR data of Nora virus, Gp1, transcription in *Dronc* heterozygous and progenitor cell *Dronc* KO intestines generated using *esg*-Gal4 driver. There is a significant downregulation of *Nora virus* mRNA observed in the *esg*-induced *Dronc* KO intestines ($P = 0.0392$, unpaired two-tailed t-test,

+/- N = 4 -/- N = 4). (B) qPCR data of *L. fructivorans* transcription in *Dronc* heterozygous and progenitor cell *Dronc* KO intestines generated using *esg*-Gal4 driver. There is no significant change in the prevalence of *L. fructivorans* between the two conditions (P = 0.7000, Mann Whitney, +/- N = 3 -/- N = 3). (C) qPCR data of *L. brevis* transcription in *Dronc* heterozygous and progenitor cell *Dronc* KO intestines generated using *esg*-Gal4 driver. There is no significant change in the prevalence of *L. brevis* between the two conditions (P = 0.2570, unpaired two-tailed t-test, +/- N = 3 -/- N = 3). (D) qPCR data of *L. plantarum* transcription in *Dronc* heterozygous and progenitor cell *Dronc* KO intestines generated using *esg*-Gal4 driver. There is no significant change in the prevalence of *L. plantarum* between the two conditions (P = > 0.9999, Mann Whitney, +/- N = 3 -/- N = 3). (E) qPCR data of *A. tropicalis* transcription in *Dronc* heterozygous and progenitor cell *Dronc* KO intestines generated using *esg*-Gal4 driver. There is no significant change in the prevalence of *A. tropicalis* between the two conditions (P = 0.7000, Mann Whitney, +/- N = 3 -/- N = 3). (F) qPCR data of *A. pomorum* transcription in *Dronc* heterozygous and progenitor cell *Dronc* KO intestines generated using *esg*-Gal4 driver. There is no significant change in the prevalence of *A. pomorum* between the two conditions (P = 0.4237, unpaired two-tailed t-test, +/- N = 3 -/- N = 3). Error bars represent Standard Deviation of the Mean in all panels. All of the experiments described in the figure were performed in Oxford medium under an experimental regime which protects epithelial integrity. The genotype of the +/- *Drosophila* is *w*¹¹¹⁸; *esg*-Gal4 UAS-*mCD8*-GFP; UAS-*HistoneRFP TubG80ts Dronc*^{KO} / *SM6A-TM6B*. The genotype of the -/- *Drosophila* is *w*¹¹¹⁸; *esg*-Gal4 UAS-*mCD8*-GFP / +; UAS-*HistoneRFP TubG80ts Dronc*^{KO} / UAS-*Flp FRT Dronc*-GFP-APEX FRT *Suntag*-HA-Cherry.

6.3. Discussion

In this chapter, I have demonstrated that the Drop-seq data was of low quality, owing to a low number of cells obtained, in addition to a low sequencing depth. These flaws ensured that it could not be processed using canonical methods, i.e. with cell identity markers or through using published bioinformatical tools such as FlyGut-seq. I was, however, able to obtain candidates which appeared transcriptionally deregulated in *Dronc* knockout progenitor cells, through observing changes across the whole intestine rather than individual clusters. In doing so, I was able to validate the deregulation of α -*Trypsin* (α -Try); *Amylase Distal* (*Amy-D*); *javelin-like* (*Jvl*) and *Jonah 65Aiii* (*Jon65Aiii*) transcription following loss of *Dronc* in intestinal progenitor cells.

Using metataxonomic approaches through the use of Kraken2, I was able to observe an increase in the viral load of intestines where the progenitor cells were deficient in *Dronc*. Furthermore, while this increased viral load appeared to come at the expense of bacteria to some extent, this could not fully explain the overall total increase observed. Upon closer inspection of the viral species prevalent in this upregulation, Nora Virus and its variants were highly upregulated, in addition to *Macaca mulatta* polyomavirus 1. Finally, I was unable to verify this upregulation in Nora virus transcripts through qPCR.

The low-quality sequencing data could be improved through refinement or other techniques

The quality of the data obtained from the Drop-seq method appeared to be insufficient to accurately determine clusters, the variances in gene expression between these clusters, and the variances between the experimental conditions. The reason for this remains unclear, and the real explanation is unlikely to be apparent without further investigation outside the scope of this thesis. However, there are several methods which would likely improve the quality of the data should extra time and monetary expense be spared. Firstly, I could increase the length of time the samples are run through the multifluidic chamber, increasing the number of droplets obtained and thus notionally, the number of reads. Whilst doing this however, the likelihood of collecting cells undergoing apoptosis is likely to increase. Along these lines, increasing the number of dissected *Drosophila* and thus available cells, could also be a method of increasing the number of reads obtained. Furthermore, increasing the number of repeats of the experiment and combining the datasets would also increase the quantity of the data, at the expense of inter-run variation. Nonetheless, while at a more considerable expense, perhaps the most substantial method of increasing the quality of the data would be through more advanced single-cell sequencing techniques, such as 10x genomics, which have been demonstrated to be more sensitive than the Drop-seq or inDrop methods (310).

Loss of *Dronc* in progenitor cells causes deregulation of gene expression

Whilst further improvements to the Drop-seq experiment would yield improved results, the initial aim was to characterise more fully the defects associated with *Dronc* KO progenitor cells at a transcriptional level. Comparing across all cells of the intestinal system, I was able to determine that several metabolic genes were deregulated following the loss of *Dronc* in the progenitor cells. Although, the directness of the causality between loss of *Dronc* and the deregulation of these genes is unclear.

The trypsinases are considered markers of Enterocyte identity (301); however in my data, I observe an upregulation in the transcription of the α -*Trypsin* gene. This could reflect the increased differentiation within the intestine. Another possible explanation for the deregulation of the digestive enzymes could relate to a loss of regionalisation and compartmentalisation of the intestine. Amylase distal, Jonah 65Aiii and α Trypsin are all mostly expressed in the R1, R2 and R4 respectively. The misexpression of these genes could represent a change in the identity of these cells. The consequence of this and the contribution of *Dronc* in this process again remains unclear (150).

I also observed an upregulation in javelin-like protein. This protein has been implicated in oocyte nucleus migration. A previous study in the Baena-Lopez laboratory demonstrated that nuclear migration events precede apoptosis in the wing imaginal disc

(219), perhaps suggesting that *Dronc* plays a more causal relationship in this process rather than as an indirect consequence of apoptosis.

The implication of loss of *Dronc* on viral immunity remains unclear

Following the loss of *Dronc* in the progenitor cells of the intestinal system, I observed a considerable increase in the viral load of these affected intestines in the Drop-seq data. Mainly from Nora Virus and its derivatives, but also from Macaca mulatta polyomavirus 1 or more commonly known as Simian Virus 40 (SV40). This virus is a known pathogen of the rhesus monkeys and can infect humans. Thus, it was surprising to encounter this in the microbiome of the *Drosophila* intestinal system (311, 312). The reasoning for this, however, can be explained through the design of the constructs used to induce the excision of the *Dronc* cassette. To disengage the polymerase from the *Dronc* locus following transcription of the Suntag-HA-Cherry, the superseding sequence contains the PolyA terminator sequence from the SV40 virus. In this construct, it would appear that, in part, this terminator sequence is expressed and recognised by the Kraken2 software.

The striking increase in the colonisation of Nora virus in the *Drosophila* intestine following loss of *Dronc* in the progenitor cells, is a particularly interesting curiosity; although this increase in viral load was not recapitulated in the qPCR experiments. Nora virus is an enterically transmitted virus, which upon infection has a very mild effect on the fitness of the organism i.e. longevity, eggs laid or the percentage of eggs developing

into adult flies (234, 313). Furthermore, the titres can vary enormously between 10(4) and 10(10) viral genomes per fly, depending on genotype. The non-pathogenic nature and variable titre of Nora virus could suggest a reason for its high presence in the Drop-seq data but downregulation in the qPCR data.

Another curiosity of *Nora Virus* is the current unexplained method of defence against this virus, if any for that matter exists. Previously published literature suggests that this virus is not affected by traditional RNAi machinery, however upon infection, antimicrobial peptides such as the *Drosomycin* genes, the *Nimrod* genes, and the *Attacin* genes are upregulated. The increased viral load in *Dronc* KO progenitor intestines may represent a novel role for *Dronc*, in the regulation of the immune response, or more specifically in the viral response of *Drosophila*. This is particularly interesting as numerous studies have demonstrated a regulatory role for the caspases in the mammalian innate immune response (314). Further research into this area is required to fully determine the role of *Dronc* in anti-viral immunity in *Drosophila*.

My inability to recapitulate the results of the Drop-seq experiments using qPCR could occur for several reasons. One explanation could stem from the differing methods used to process the samples between the two techniques. Prior to conducting the Drop-Seq experiments, the intestines were dissociated through treatment with elastase. Although a gentler method than other biochemical approaches, previous experimental evidence has shown that treatment can result in the death of cells (224). Thus, it is possible that viral material not present inside an infected cell is destroyed in this process (i.e. capsids

within the intestinal lumen). In the instance of the preparation of the qPCR samples, there is no dissociation step and thus, it is likely that all of the material present within the lumen is preserved. If this is true, while cells are still more susceptible to viral infection following *Dronc* KO, the increase in viral load could be masked by the capsid contained RNA still present in the lumen. To distinguish between these two possibilities, dissociation of the qPCR samples could be conducted before RNA extraction. Another possibility for the differences between the Drop-seq and qPCR data is that the loss of *Dronc* in progenitor cells creates a general deficiency in viral immunity, rather than a specific susceptibility to Nora virus infection. Thus despite Nora virus showing a decrease in titre in the qPCR experiment, in actual terms, another virus species titre could be increased (for example, *Drosophila submucosa* Nora virus). Due to the specificity of the qPCR primer to Nora Virus, any increase in other viruses would not be detected using this technique.

7. Discussion

7.1 Thesis Summary

Through the course of this thesis, I have demonstrated that the catalytic activity of Dronc is required to prevent the excessive proliferation and differentiation of progenitor cells. This activity, however, appears completely independent of the canonical apoptosis pathway. On further investigation, the activity of Dronc was shown to be restricted to the Enteroblasts, and this could be linked to its stabilisation within this cell. Subsequently, I demonstrated that Dronc function required the activity of the Notch and Insulin/TOR pathways. Finally, loss of *Dronc* within the intestine, lead to the misexpression of a number of genes and a potential deregulation of the microbiota.

7.2 Caspase activity is unlikely to relate to the anastasis phenomenon.

Anastasis refers to the phenomenon observed when a cell recovers from the process of dying following activation of the apoptosis pathway (70). Its purpose has been hypothesised to occur as a response to limit the permanent damage to a tissue, caused by exposure to a severe but transient injury, chemical stress or radiation exposure (70). Examples of anastasis have been observed in human, *Drosophila* and *C. elegans* models. For example, in mouse models, ischemia-induced apoptosis can be reversed by annexin-A5 binding to phosphatidylserine, leading to their internalisation and the reversal of

apoptosis (315). In *Drosophila*, sublethal activation of the caspases has been observed in response to irradiation, with caspase activity required to surpass a threshold in order to initiate the apoptosis programme (316).

The data obtained in this thesis would suggest that the caspase activation observed within the *Drosophila* intestinal system is not related to the anastasis phenomena. Most examples in the literature suggest that this phenomenon occurs as a response to a damaging insult, for example irradiation. However in chapter 3, I demonstrate that apoptosis and epithelium regeneration does not occur in my experimental conditions. This, therefore, rules out the likelihood of the apical-caspase activation observed with the DBS-S-QF reporter relating to the anastasis phenomena. Furthermore, it suggests that the function of Dronc within the Enteroblasts relates to a novel non-apoptotic function of the caspases, independent of the cell death process.

7.3. The accumulation and activation of Dronc within Enteroblasts prevents hyperplasia

Over the last 20 years, multiple studies utilising the *Drosophila* model organism have employed various tissue-damaging agents, such as environmental or genetically induced stresses, to uncover the molecular factors orchestrating the proliferation and differentiation of Intestinal Stem Cells into Enteroblasts (104, 168, 291). In spite of this, the differentiation step of Enteroblasts into terminally differentiated Enterocytes

remains less well studied. In this thesis, I utilised experimental conditions which ensure that the *Drosophila* intestinal system remains devoid of apoptosis and basal turnover for at least 7 days following activation of the ReDDM reporter; thus allowing for the study of the non-apoptotic role of the caspases within this system. Interestingly, despite the absence of apoptosis, I observed a regulated and reproducible pattern of caspase activation, which appeared to occur to a large extent in Enteroblasts. Furthermore, in this thesis I demonstrated that the specific accumulation and activation of Dronc within this cell type is indispensable for preventing gut hyperplasia, in addition to cell fate commitment into Enterocytes. Collectively, these findings suggest the presence of an intricate genetic network, regulating the differentiation of Enteroblasts into Enterocytes. Additionally, the data also suggests that the regulation of the epithelium homeostasis is not solely reliant on the Intestinal Stem Cells and its regulatory system. Thus collectively, this evidence proposes that the fine-tuning of the processes relating to Enteroblast differentiation could be more important than first considered in regulation of intestinal homeostasis. Importantly, deficiency in the mammalian homologue of Dronc, *caspase-9*, in intestinal precursors triggers excessive proliferation and poorly differentiated tumours (109). More importantly clinically, is that this deficiency is linked with a poor prognosis for patients with colon cancers (109).

Independently, these results support the previously documented observations of non-apoptotic caspase regulation of stem cell properties, particularly in the instances of proliferation and differentiation, separately from their roles in the apoptosis programme (104, 168, 291). Taking a wider view of caspase biology, supported by the

plethora of examples pertaining to their function in a non-apoptotic context (5, 39, 69, 269, 299, 317, 318), the process of apoptosis may be the most apparent phenotypic consequence of their activation, but may not be their most important role in maintaining organism homeostasis. Further studies into the role of metacaspases, caspase-like enzymes present in primitive and unicellular organisms (319), may clarify these more primary functions.

7.4. The Dronc-dependent role in maintaining Enteroblast quiescence requires its enzymatic activity in addition to Notch signalling, but is independent of the apoptosis pathway.

The data presented in this thesis demonstrates that the sole presence of Dronc is not sufficient to successfully regulate Enteroblast quiescence and prevent hyperplasia, with its catalytic activity required for those tasks. This suggests that Dronc does not solely act as a scaffold protein, or regulator through protein-protein interactions, as described with Numb (89), but as a proteolytic enzyme. Further experiments also suggest that the effector caspases are not required to ensure Enteroblast quiescence, and instead an unknown factor “X” must exist for this regulation to occur. Furthermore, similar experiments knocking down the activity of the pro-apoptotic proteins, *Hid*, *Reaper* and *Grim*, suggest that the activation of Dronc is unlikely to originate from these canonical caspase pathway members. The notion that Dronc can act independently of the caspase

pathway in non-apoptotic conditions, may suggest an explanation for the ability of the caspases to be active, but not result in apoptotic cell death.

Furthermore, the genetic epistasis between *Dronc* and the Notch pathway indicates that *Dronc* may mediate the cleavage of factor “X”, to directly or indirectly downregulate Notch signalling [Figure 7-1]. To aid with this hypothesis, I have demonstrated in this thesis that the suppression of either the Notch or Insulin/mTOR pathways can rescue the hyperplasia phenotypes observed in progenitor cells mutant for *Dronc*. Reciprocally, these phenotypes can be exacerbated through upregulation of the Notch pathway in *Dronc* mutant conditions. Collectively, the data strongly suggests a non-apoptotic function for *Dronc* as a regulator of signalling pathway(s) in Enteroblasts.

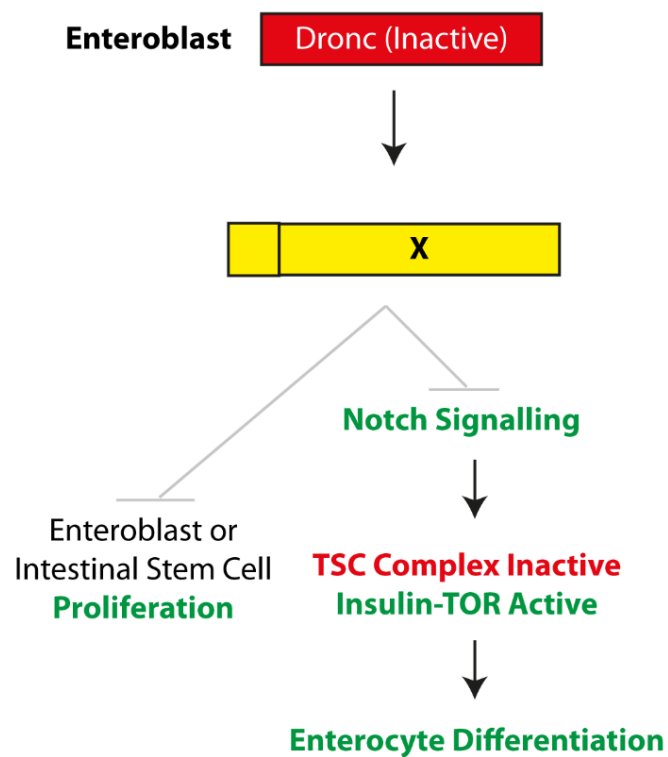
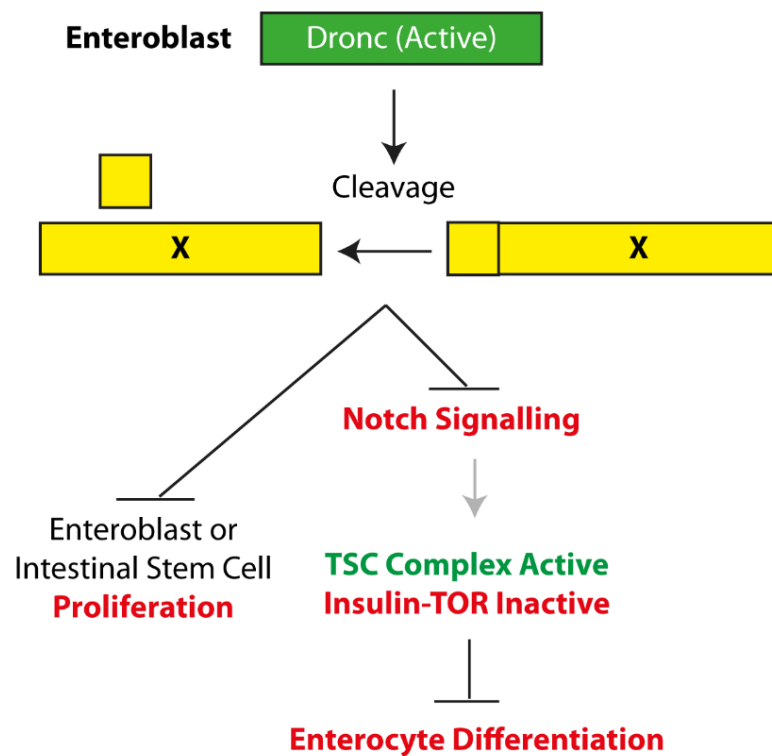


Figure 7-1: Working model describing the interplay between Dronc and the Notch/Insulin-TOR pathway in Enteroblasts.

The specific accumulation of Dronc within the Enteroblasts enables the cleavage of substrate “X”. This cleavage could result in the downregulating of the Notch or Insulin pathways, preventing the premature entry of the Enteroblasts into Enterocytes and subsequent hyperplasia.

7.5. Caspases regulate intestinal cell properties through different cellular mechanisms

An influential body of literature has demonstrated that the caspases can regulate the proliferation and differentiation of intestinal precursors, through the activation of signalling pathways in apoptotic conditions. Indeed, a recent observation by *Jin et al.*, demonstrated that the effector caspases, activated downstream of the Hippo pathway, cleave the chromatin regulator Brahma in order to restrain the proliferation and differentiation of Intestinal Stem Cells following epithelial damage (104) (Transition 1 of [Figure 7-2]). The findings presented in this thesis demonstrate that Dronc is also essential in the regulation of the appropriate timing for the Enteroblasts, to enter into the Enterocyte differentiation programme (Transition 2 of [Figure 7-2]). In contrast to the literature demonstrating effector caspase cleavage of Brahma, this new function of Dronc is unlikely to rely on the same mechanism, as it is effector caspase-independent. Alternatively, the described non-apoptotic Dronc function appears to be strongly associated with the Notch pathway and subsequently, the Insulin/TOR pathway [Figure 7-1]. Collectively, the evidence demonstrated in this thesis, illustrates the ability of the

caspases to regulate the homeostasis of the different intestinal precursor subpopulations, without entry into the apoptosis programme. Additionally, they insinuate the existence of a highly specific, but currently unknown, mechanism of sub-lethal caspase activation which appears to orchestrate this regulation without instigating apoptosis.

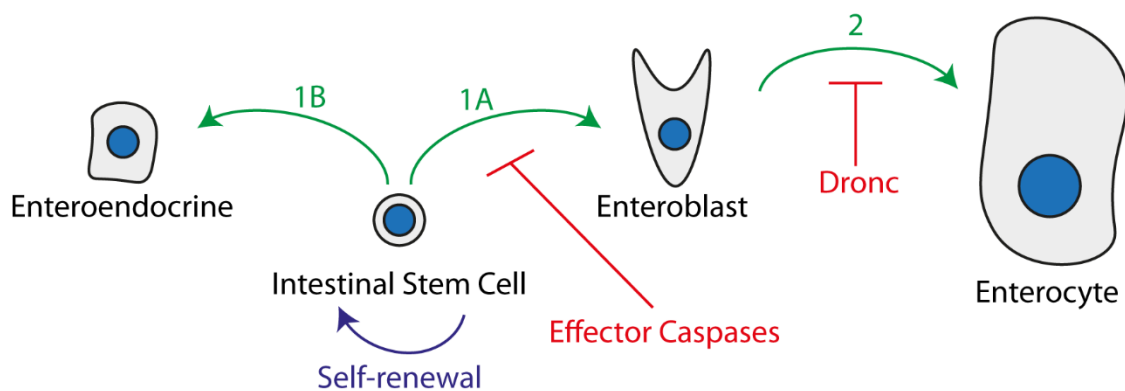


Figure 7-2: The non-apoptotic functional effect of Dronc within the intestinal epithelia

Intestinal Stem Cells are able to self-renew or differentiate into Enteroblasts (1A) or Enteroendocrine cells (1B). The Enteroblast can subsequently differentiate into Enterocytes (2). The data presented in this thesis suggests that Dronc, independently of the effector caspases, is able to prevent this transition, and induce Enteroblast quiescence.

7.6. Caspases as tumour suppressors independently of apoptosis

The deregulation of the caspases has canonically been associated with the evasion of apoptosis and the propagation of tumorigenesis (281, 282). Thus, these enzymes have reasonably been ascribed the title of tumour suppressor genes for this purpose.

However, an emerging body of literature concerning the non-apoptotic functions of these enzymes has described additional roles, relating to the ability of the caspases to regulate the proliferation and differentiation of Intestinal Stem Cells (104, 320). Thus, strongly implying an important regulatory role in the prevention of hyperplasia, in addition to the formation of tumours.

The *Drosophila* findings presented in this thesis suggest a role for the caspases in the regulation of Enteroblast differentiation [Figure 7-1]. These effects thus, depend on the capacity of the caspases to maintain a pool of intestinal progenitors in quiescence (This thesis and (109, 321)). On the other hand, loss of caspase activity can divert the intestinal precursors from proliferative cells to terminally differentiated cells, thus redirecting cells from uncontrolled cancerous growth. Collectively, the data described in this thesis supports the hypothesis that caspases have the capacity to operate as tumour suppressors through various tissue-specific mechanisms, often independently of the apoptosis process.

7.7. Loss of caspase function alters the microbiome/viome

Numerous studies have observed an influence of the microbiota on gene expression, intestinal morphology and behaviour of the host-organism (322). However, the ability of the intestine to modulate through gene expression the composition of the microbiota is less well documented. In this thesis, using Drop-seq techniques, I observed a substantial increase in the viral load in *Dronc* KO progenitor intestines. However,

following qPCR analysis of the data, I failed to recapitulate the results of the Drop-seq experiment. While the definitive cause of this discrepancy remains unknown, it could relate to the difference in the handling of these samples between the two techniques. For example, in contrast to whole gut qPCR, the intestinal cells are dissociated prior to undertaking the Drop-seq protocol. This dissociation using biochemical approaches, could destroy the viral material within the intestinal lumen, not present within the intestinal epithelial cells. Thus, any increase in viral susceptibility using the Drop-seq method, could be masked in the whole gut qPCR experiments by luminal viral material, explaining the discrepancy between the two experiments. Further investigations will surely determine the likelihood of this conclusion. However, despite the contrast between the Drop-seq and qPCR data, relating to the increase or decrease in viral loads respectively; in *Dronc* LOF conditions, the data overall does suggest deregulation of the microbiota when compared to *Dronc* heterozygous intestines. While it remains unclear what influence *Dronc* has on the microbiome, further studies will likely elucidate the function of *Dronc* in this context.

7.8. Future Directions and Concluding Remarks

This project has yielded a number of important insights which may prove critical to our understanding of caspase biology, both in physiological and pathological scenarios; however, a number of questions still remain unanswered which may further improve our understanding of caspase biology and provide attractive follow-up studies. Principally, determining the conservation of these findings in mammalian systems may

prove insightful to our understanding of cancer biology, particularly colon carcinomas deficient in *caspase-9* signalling. Previous studies have demonstrated that loss of *caspase-9* in colon cancer leads to proliferative and differentiation defects, resulting in a poor prognosis for patients (109). In this study, we observe an epistatic effect of Dronc signalling and the Notch pathway. Interestingly, Notch pathway deregulation is frequently observed in many tumours, including those originating from the intestinal system (323). If similar, such deregulation could be therapeutically ameliorated through therapeutic intervention, succeeding where caspase modulating compounds have previously been unsuccessful (71). However, previous unsuccessful attempts at designing caspase-modulating compounds have arisen from tumour specific effects resulting from altering caspase activity, such as an increase in metastasis in some contexts (324). Thus, future treatments with Notch inhibitors, should the similarities between the *Drosophila* and mammalian system be observed, may only be useful as a personalised medicine.

Within the *Drosophila* intestinal system, the substrate(s) by which the caspases regulate Enteroblast quiescence are still unclear. Previous studies have observed that cleavage of Brahma by effector caspases, regulates the activity of the Intestinal Stem Cells; however, in conditions absent of basal turnover or apoptosis, the effector caspases are not active and thus unlikely to be involved. Additionally, previous studies have observed interactions of Dronc with the Notch regulator, Numb. In the evidence presented in this thesis however, the catalytic activity of Dronc is required, thus likely ruling out a significant role for this member in the regulation of the Notch pathway. Future studies

could aim to decipher the signalling molecule(s) pertaining to the fine-tuned regulation of the differentiation of Enteroblasts, through Dronc in the non-apoptotic context.

Equally unclear and the possibility for further study, remains the signalling pathway(s) or molecule(s) tasked with activating Dronc in this non-apoptotic context to maintain Enteroblast quiescence. In this thesis, I have demonstrated that the main pro-apoptotic proteins in *Drosophila*, Hid, Grim and Reaper, are not involved in this process. Thus, suggesting an insignificant role for the canonical apoptotic pathway in this process. Further genetic and biochemical approaches would rule out other likely activators of Dronc and potentially, establish the true mediators of this process.

Overall, this project has made steps towards improving our understanding of the non-apoptotic function of Dronc within the *Drosophila* intestinal system. Utilising novel tools originating from advanced genome engineering and sequencing techniques. I have demonstrated that Dronc regulates Enteroblasts to prevent premature differentiation and hyperplasia. Independently of *Drosophila melanogaster*, we hope these findings will help elucidate the pathologies associated with caspase deregulation in cancer; in order to improve the prognosis of those patients who sadly are afflicted by the uncontrolled signalling of these enzymes.

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