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Mucin 55B and its effect on healthy ageing

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Mucin 55B and its effect on healthy ageing

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Abstract:

Chronic inflammation is a major symptom of many age-related diseases such as, Alzheimer's and dementia. This inflammation has been linked to the movement of bacteria from the gut to outside of the lumen into lymphoid tissues and the bloodstream. Understanding the importance of the gut barrier and its integrity is therefore of the upmost importance. Here we use the model organism, *Drosophila melanogaster*, to investigate how this barrier is regulated.

Mucin genes have long since been known to be a contributing factor to the maintenance of the gut intestinal barrier. We use a drug-inducible UAS-Gal4 system in order to knockdown one of these mucins, mucin 55B. Unpublished data had previously shown a knockdown of this gene extended lifespan and maintained gut intestinal barrier function later into life.

We used AMPs as readouts for the Toll and JNK pathways and found that the mucin 55B knockdown has no effect on this immune response. Further markers were used to find the same result in the insulin pathway. Using antibiotic lifespans we were not able to confirm that the presence of the gut microbiota extended lifespan. We sought to find how mucin 55B was regulated as this could aid in treating late life inflammation in both *Drosophila* and humans. Here we show that the JNK pathway is likely to regulate the expression of mucin 55B.

This research has broadened the understanding of the impact mucins have on lifespan as well as health span and provide new insight into how approaches can be used to maintain the gut barrier and reduce morbidity.

Contents

Table of figures

Introduction

The ageing population and increased inflammation

As each year passes there is significant growth in the percentage of the population over 65 years old, and with this growth there is a proportional increase in age-related diseases. For example, by the year 2040 the percentage of the US population over 65 is set to rise to 21% (US Department of Health and Human Service, 2020). Structural and functional changes associated with ageing have been linked to precursors of many age-related health problems, including increased fibrosis and inflammation (Selman et al., 2016; Guarner, 2015). There are large economic impacts to unhealthy ageing, with dementia alone costing the NHS £26.3 billion in 2014 (Azheimer's UK, 2014). This issue is only becoming more prominent as estimations in the US expect this cost to quadruple by 2030 (Alzheimer's Disease International, 2022). The slowing of age-related diseases is therefore of the utmost importance in order to make sure the NHS does not become overstretched, which is becoming more likely due to tighter financial restrictions.

Many diseases associated with mortality, such as Parkinson's disease, are linked to chronic inflammation (Pajares et al., 2020). This inflammation can be the result of several stimuli, including bacteria, which is more pronounced when gut permeability is high. Excessive induction of the immune system can begin to cause tissue damage and promote ageing. Unfortunately, there is no single universal healthy microbiome configuration, so it is important to understand the dynamic relationship between the gut microbiome, host and age-related disease.

The effect of gut permeability on ageing

In a recent review, Sommer and Backhed (2013) predicted the microbial cell content per gram to be 10^3 in the duodenum, 10^4 in the jejunum, 10^7 in the ileum, 10^{12} in the colon. Maintaining the homeostasis of the gut microbiome, within any animal, is key to longevity and healthy ageing. The gut microbiota has been subject to a number of studies, with the ability to maintain its stability shown to have links to reduced obesity and type 2 diabetes cases (Riedel et al., 2021).

A defining factor in whether the gut can maintain homeostasis is the integrity of the intestinal barrier. Several diseases are believed to be associated with its failure. Patients with inflammatory bowel disease develop an abnormal immune response

which is elicited by intestinal microbiota (de Souza and Fiocchi, 2016). Evidence was also found in inflammatory bowel disease patients as well as patients displaying altered mucus layers to show that permeability is increased compared to controls (Salim and Soderholm, 2011; Johansson et al., 2014). Although these results offer good evidence that permeability increases with age and disease, the results often differ depending on the methodology of the study and type of patients. Most *in vivo* studies do show increased permeability (40-50%) in patients with an active disease (Michielan and D'Inca, 2015).

There are differing findings on whether gut permeability increases with age. A study measuring lactulose and mannitol absorption in the small intestine concluded that the permeability of the ageing human gut does not increase compared to younger controls (Saltzman et al., 1995). But a separate study suggested that there is reduced transepithelial electrical resistance in younger controls, a marker for permeability in the monolayer (Man et al., 2015). The same study investigated possible biomarkers associated with barrier dysfunction and found that increased claudin-2 expression was linked to reduced transepithelial electrical resistance. It is likely that intestinal barrier function or loss thereof is secondary to other ailments or disruptions. A recent study in mice showed that middle cerebral artery occlusion increased gut permeability in both younger and older mice (Crapser et al., 2016). Interestingly, and relevant to this study, this gut permeability caused bacterial translocation was resolved quickly in younger mice but not in the older.

A main hypothesis connecting the disruption of barrier function with ageing is the 'leaky gut' hypothesis, which states that damage causes the barrier to become permeable. This permeation is then predicted to result in bacteria and other pro-inflammatory toxins being secreted into the blood stream, activating an immune response and initiating inflammation (Maynard and Weinkove, 2018). This highlights the importance of investigating the gut barrier and why it may become weakened with age.

Drosophila as a model organism in research

Drosophila melanogaster has a long history of being used as a model organism, first being studied experimentally in 1906 (Castle, 1906). Their low generation time (10-14 days) and high reproduction rate allow for a large number of flies to be produced

quickly, allowing a large quantity of experiments to be completed over a short period of time.

Made up of four chromosome pairs, the *Drosophila* genome is 180Mb in length and is 60% homologous to humans. Although 20-fold smaller than the human genome, the *Drosophila* genome encodes a similar number of gene families (Taormina et al., 2019). This allows the study of gene function in humans to be simplified. Possibly the most important feature of *Drosophila* is that it is a convenient genetic system with many available mutants and transgenic tools. For example, the UAS/GAL4 system allows tissue specific transgene expression and temporal control to be activated either by temperature or drug exposure (Osterwalder et al., 2001). One of the many functions the UAS/GAL4 system uses is Ribosomomal Nucleic Acid (RNA) in order to create a sequence-specific suppression of gene expression, called RNA interference (RNAi). These RNAi studies can be conducted easily, with the availability of RNAi lines which target most transcripts (Perkins et al., 2015).

Drosophilae have a long history of usage in ageing experimentation (Loeb and Northrop, 1916). As complex organisms, *Drosophila* offer a wide range of features which can be monitored with age which are lacking in other model organisms (yeast or *C. elegans*). This is proven as *Drosophila* have been shown to have a reduced resting metabolic rate, decreased protein and fat synthesis and reduced flying with age (Piper and Partridge, 2018). Utilising *Drosophila* in the context of intestinal barrier studies provides another advantage in the form of sexual dimorphism. The virgin female midgut is significantly larger than that of the male with this difference growing after mating. As well as this, adult female intestinal stem cells (ISCs) divide more readily than in males. For these reasons, female *Drosophilae* tend to be used for easier experimentation (Miguel-Aliaga et al., 2018).

Drosophila's use in gut microbiota studies

Fly and human intestines share similar tissue, anatomy and physiological function (Rubin, 2007) (Fig. 1). For example, both mammalian and fly guts are made up of a monolayer of epithelial cells, enterocytes. A major difference between human and fly guts is folding; human guts are highly folded with the formation of crypts and villi which increase surface area, whereas fly guts are not. Both mammalian and fly intestines do

utilise microvilli which protrude into the lumen, creating a brush border on which a layer of mucus separates the host from intestinal microbes. Insects contain a semi-permeable, non-cellular structure known as the peritrophic matrix which protects the epithelium from physical damages as well as regulating the transfer of particles between lumen and enterocytes. In *Drosophila,* a peritrophic matrix is present to protect the midgut and hindgut (Apidianakis and Rahme, 2011). It has been shown that with age this mucus layer depletes along with the peritrophic matrix causing increased likelihood of damage to the intestinal barrier (van Beek et al, 2016). *Drosophila* also show key responses to infection of microbes which can be measured, allowing insights into systemic and local responses with age. Overall, conserved cellular processes, shared morphology, practicality, and ease of genetic intervention make *Drosophila* an important model organism of the human intestine (Apidianakis and Rahme, 2011).
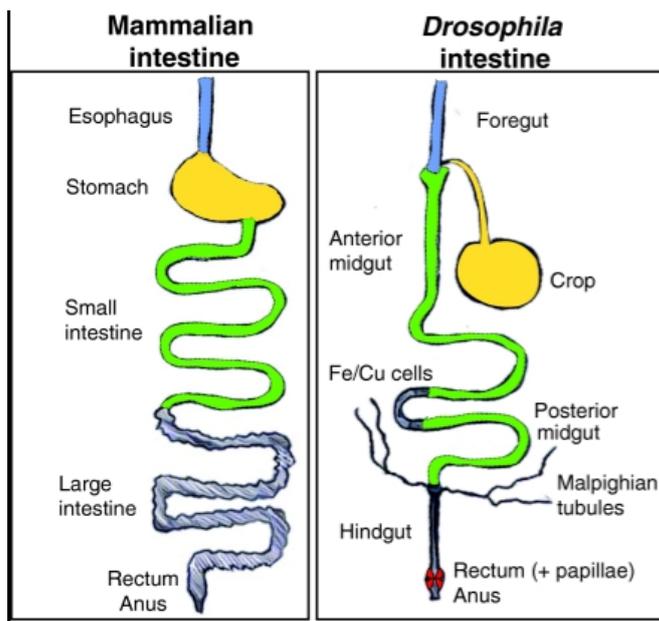
Figure 1: Schematics of mammalian and Drosophila intestines (Apidianakis and Rahme, 2011). Similarities in the oesophagus and foregut (blue), stomach and crop (yellow), small intestine and midgut (green), and large intestine/rectum/anus and hindgut/rectum/anus (grey). Differences include the presence of Fe/Cu cells and Malpighian tubules found in Drosophila.

Both humans and *Drosophila* contain no true core microbiota (Miguel-Aliage et al., 2018; Turnbaugh et al., 2007). A core microbiota broadly refers to any set of microbial taxa, or the genomic and functional attributes associated with those taxa, that are characteristic of a host or environment of interest (Neu, Allen and Roy, 2021). With age, the microbiota of *Drosophila* has been shown to increase and become more diverse (Kim and Jazwinski, 2019; Bartosch et al., 2004). In response to this, aged flies produce a higher level of reactive oxygen species (Sanz et al., 2010) and antimicrobial peptides (Kounatidis et al, 2017) to aid in controlling the growing microbiota. Clark and colleagues found that changes to the ecology of the gut microbiota (for example, an increased proportion of Proteobacteria and decreased proportion of Firmicutes) occurred before cellular markers of intestinal barrier failure (Clark et al., 2015). These changes have been hypothesised to cause inflammation, creating a feed forward loop where inflammation causes further microbiota dysbiosis (Clark and Walker, 2017). This systemic inflammation throughout the *Drosophila* has been shown to inactivate key metabolic pathways, such as the insulin signalling pathways, and drain metabolites causing muscle wastage in chronically infected flies (Dionne et al., 2006). These ideas were then applied using smurf flies in another publication which showed that gut dysbiosis did limit lifespan (Lee et al., 2022).Smurf assays are a common experimental design in *Drosophila* studies that assess food intake by the co-ingestion of a blue dye. This allows researchers to view food not absorbed by the digestive tract. This property can therefore assess gut permeability *in vivo* (Rera et al., 2012). Strengthening the intestinal barrier could therefore reduce gut dysbiosis and prevent inflammation, delaying the onset of age-related diseases (Maynard and Weinkove, 2018).

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Mucins effect on ageing

Mucins have been studied extensively within mammalian models and are a family of large, complex, glycosylated proteins with a highly conserved 'mucin domain' (Johansson and Hansson, 2016; Johansson et al., 2013). Within the protein core of mucins are so-called PTS regions (involving proline, threonine and serine) which are often repeated in tandem (Paone and Cani, 2020). Serine and threonine pairs are targeted for O-glycosylation in which several different sugars can be added to the amino acids affecting protein stability and regulating protein activity. Due to the

multitude of function changes O-glycosylation can achieve it is the focus of ageing, cancer and neurogenerative research (Franzka et al., 2021; Burchell et al., 2018; Akasaka-Manya and Manya, 2020). Separately, proline enables this process, ensuring the mucin remains unfolded in the Golgi apparatus allowing glycosylation to occur (Fig. 2). O-glycosylation also provides protection to itself; the glycan coat shelters the protein core of mucins preventing endogenous protease degradation as well as enabling mucins to bind water and form a gel (Hansson, 2020; Etienne-Mesmin et al., 2019; Johansson et al., 2011). Glycosylation can differ along the digestive tract depending on each individual's diet and overall health. This has been suggested to be partly influenced by microbial colonisation (Hansson, 2020; Pelaseyed and Hansson, 2020; Etienne-Mesmin et al., 2019). The formation of this structure has been found to be important to human health, with healthy humans displaying a O-glycosylation profile of MUC2 which is qualitatively and quantitatively more uniform compared to patients with active colitis (Johansson et al., 2011).
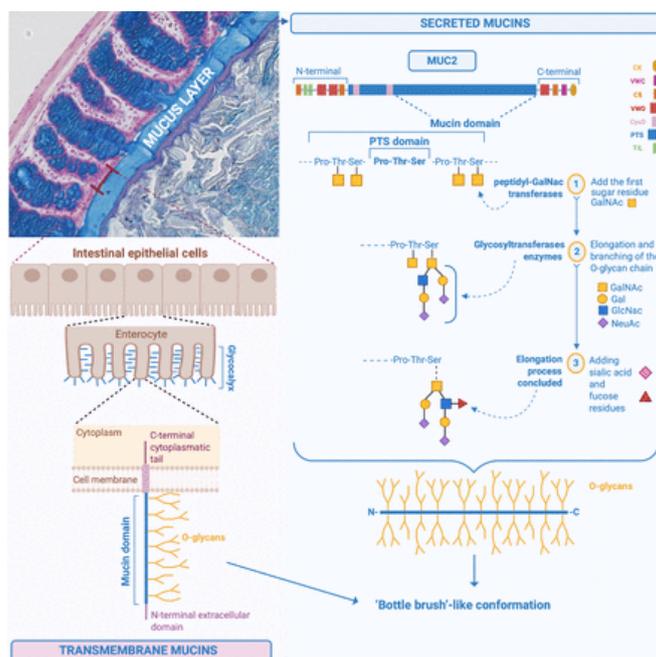
Figure 2: Stages of synthesis of mucus in the gut (Paone and Cani, 2020). Left side of figure: Shows an illustration of the transmembrane mucins on the surface of an enterocyte. Right side of figure: Specific steps in the synthesis of MUC2 in the

intestine. Including the steps involved in adding the first glycosylation made by the peptidyl-GalNAc transferases which allows the first sugar to the serine and threonine of the PTS sequence. Ser, serine; Pro, proline; Thr. Threonine.

Within *Drosophila*, mucin-like proteins have been poorly characterised compared to their human counterparts as the PTS repeats are unable to be detected due to poor sequence conservation (Syed et al, 2008). This is because heavy glycosylation's obstruct biochemical mucin isolation, making proteins large and difficult to extract. Lang and colleagues, 2007 attempted to avoid this practical issue by using a bioinformatic search for mucin-associated domains combined with PTS repeats and serine/threonine rich content. This study was initially hampered due to low representation of PTS repeats in cDNA; however, gel-forming mucins were eventually returned in several divergent species. This makes mucins a very interesting subject for ageing research as it suggests mucins have a wider function in different species and organs than previously thought (Kumar et al, 2017).

Specifically, little research is readily available on the mucin gene, *Muc55B* (Fig. 3A&B). Currently, the gene has been found in *Drosophila melanogaster* and is thought to be an extracellular matrix structural constituent. Mucins can generally be separated into two categories: transmembrane mucins and gel-forming mucins. *Muc55B* is a transmembrane mucin which are synthesised in the rough endoplasmic reticulum and attached to the cell membrane of the enterocytes until they cover the apical surface (Johansson, Sjovall and Hansson, 2013). The estimated function of these proteins is not to form gels as one may picture when imagining a mucin but to provide cell protection and have been predicted to be involved in host-microbe interactions (Pelaseyed and Hansson, 2020; Johansson, Larsson and Hansson, 2011). Although a predictive model has been generated using AlphaFold2 (Fig.3A), annotations are sparse and the inner workings of the protein are less understood. Figure 3B shows the primary sequence of the protein and highlighted in red is a predicted signal peptide. The first 17 amino acids in the sequence was predicted to be a putative singal peptide by the SignalP programme (Nielsen et al., 1997; Chandra et al., 2004). The light blue highlighted section of primary sequence matches strongly with a protein of unknown function (DUF725). Although it is a protein of unknown function, there is reason to suggest it is a transmembrane region due to its alpha-helix prediction. The PTS region is predicted by AlphaFold to be towards the end of the primary sequence and is

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highlighted in purple in Figure 3B. This matches with Syed and colleagues, 2008 due to it containing at least four repeats and high ST content. Overall, the structure given from the AlphaFold model is useful as it allows us to view a potential location for a transmembrane region, however, the PTS region has poor confidence. This is representative of other research groups struggles as Syed et al., 2008 found low conservation of PTS regions.
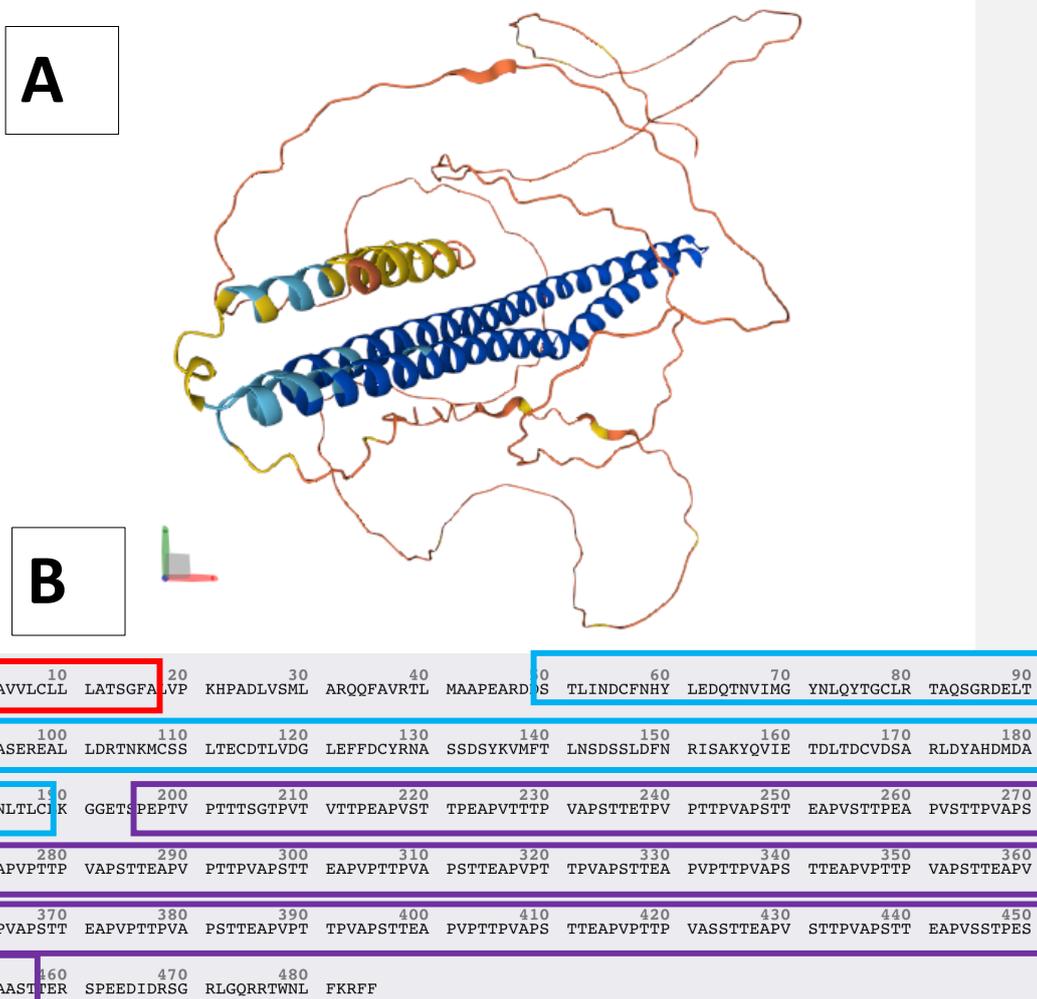

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          10         20         30         40                 50         60         70         80         90
MKFAVVLCLL LATSGFALVP KHPADLVSML ARQQFAVRTL MAAPEARDDS  TLINDCFNHY LEDQTNVIMG YNLQYTGCLR TAQSGRDELT
          100        110        120        130        140        150        160        170        180
KESASEREAL LDRTNKMCSS LTECDTLVDG LEFFDCYRNA SSDSYKVMFT LNSDSSLDFN RISAKYQVIE TDLTDCVDSA RLDYAHDMDA
          190        200        210        220        230        240        250        260        270
CDENLTLCIK GGETSPEPTV PTTTSGTPVT VTTPEAPVST TPEAPVTTTP VAPSTTETPV PTTPVAPSTT EAPVSTTPEA PVSTTPVAPS
          280        290        300        310        320        330        340        350        360
TTEAPVPTTP VAPSTTEAPV PTTPVAPSTT EAPVPTTPVA PSTTEAPVPT TPVAPSTTEA PVPTTPVAPS TTEAPVPTTP VAPSTTEAPV
          370        380        390        400        410        420        430        440        450
PTTPVAPSTT EAPVPTTPVA PSTTEAPVPT TPVAPSTTEA PVPTTPVAPS TTEAPVPTTP VASSTTEAPV STTPVAPSTT EAPVSSTPES
          460        470        480
TTEAASTTER SPEEDIDRSG RLGQRRTWNL FKRFF
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Supporting preliminary data for this research

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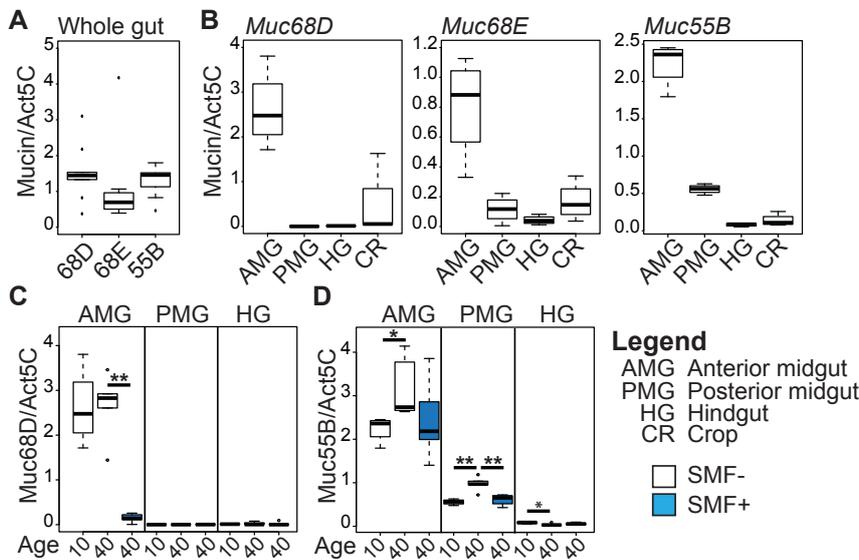
Figure 4: Drosophila intestine mucin expression changes with age and health (Clark, unpublished data). (A-B) Mucin expression in intestines from 10-day old females. (A) Whole guts, n = 10x5 guts. (B) Intestinal regions, n = 5x20 guts. (C-F) Mucin expression in intestinal regions at 10 and 40 days old and in 40-day old smurf flies. (C) Muc68D. (D) Muc55B. All females, n = 5x20 intestines. Boxplots display the first and third quartile, with the horizontal bar at the median. *p<0.05, **p<0.01, ***p<0.001, t-test.

Preliminary data has been very promising as several mucin genes that are expressed in the fly gut and whose expression changes with age have been identified. Muc68D suppression caused intestinal barrier failure, whereas Muc55B suppression caused a strengthening of intestinal barrier function and therefore an hypothesis can be made that mucin genes influence health spans (Fig. 5).

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Data showed that Muc55B and Muc68D had similar levels of expression throughout the whole gut, with Muc68E showing slightly lower levels. Muc68D showed almost no expression in the anterior midgut of the smurf population compared to their healthy

14

counterparts (Fig. 4C). Interestingly, Muc55B showed no expression difference between smurf and healthy flies in the AMG or HG (Fig. 4D).
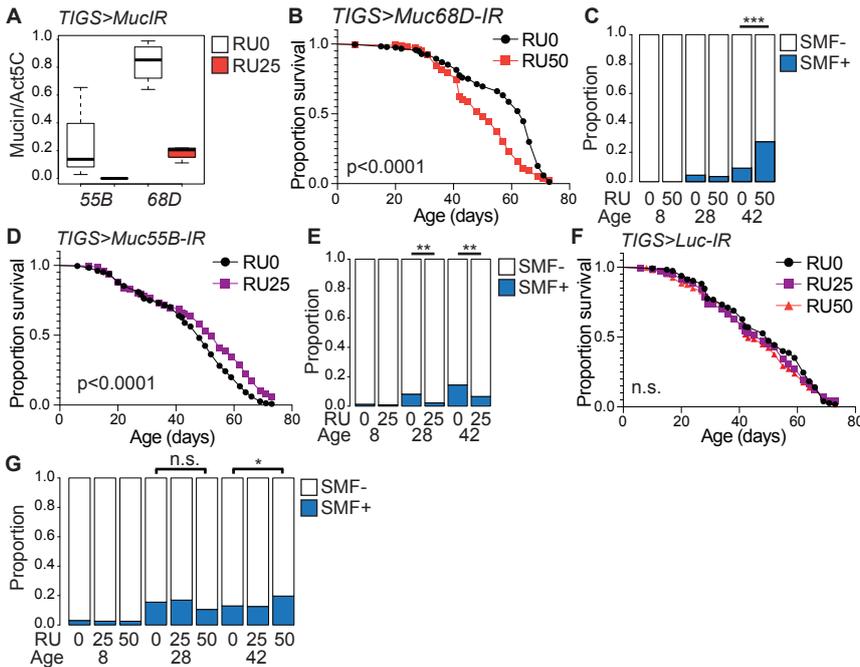
Figure 5: Muc68D and Muc55B shown to regulate intestinal barrier function and lifespan (Clark, unpublished data). (A) Mucin expression level in gut (TIGS>MucIR). (B-C) Lifespan and smurf proportion in TIGS>UAS-Muc68D-IR. (D-E) Lifespan and smurf proportions in TIGS>UAS-Muc55B-IR. (F-G) Lifespan and smurf proportions in TIGS>UAS-Luc-IR. Samples taken from female flies, drug fed from day 5 of adult life. N>200 flies/condition. Data from Muc68E was also collected but was almost identical to Muc68D

It was also shown in preliminary data that Muc55B suppression significantly extends lifespan (Fig. 5D). Arguably more important to human studies, mucin 55B suppression leads to an extended health span as there are significantly fewer smurfs in the knockdown group compared to the control (Fig. 5E).

Aims of the project

Based on a review of the literature and preliminary data, there is a definite gap in knowledge surrounding the mucin 55B gene and its effect on health and lifespan. For

this reason, it is the basis of my MSc research and thesis. In this I will investigate the effects of the gene, *Muc55B*, on lifespan with an emphasis on the smurf population in which we will be able to draw interesting parallels to human lifespan and quality of life.

This project has the overall goal to investigate the effect of mucin expression on the lifespan of *Drosophila melanogaster*. To achieve this, two main aims have been devised:

1. To identify why changes in mucin expression regulate lifespan.

2. To identify the pathways involved in regulating mucin expression.

Methods

Fly culture and Lifespan

This work was carried out with w;; TIGS flies crossed with the relevant UAS line. UAS lines used during this experiment were y^1, v^1;; UAS-Muc55B-RNAi/TM3, Sb^1 (line from the TRiP collection, obtained from the Bloomington Stock Centre. Referred to in this paper as UAS-Muc55B-IR), UAS-Muc55B-IR (Obtained from the Vienna Drosophila Resource Centre. Referred to as UAS-Muc55B.kk-IR in this paper.), w, UAS-PGRP-Lc, w^{1118};; UAS-Hep^{CA}, w^{1118}, UAS-Bsk^{DN} , w;;UAS-Upd3, and w;;UAS-Dome$^{delta-Cyt}$.

Female *TIGS* virgins were then collected and stored in vials of 20 before being crossed with the appropriate genotype line. Crosses were left for 10 days before collections of offspring were taken for experimentation. Flies were cultured in a humidified temperature-controlled incubator with a 12 hours on/off light cycle at 25ºC, in vials containing 4µl of standard cornmeal medium (1% agar, 3% brewer's yeast, 1.9% sucrose, 3.8% dextrose and 9.1% cornmeal). During collection, flies were anaesthetised under light carbon dioxide and were subsequently housed at a density of 27-32 flies per vial. These vials were then flipped into fresh vials and deaths were counted every 2-3 days through adult life.

Geneswitch activity was induced using RU486 (Cayman Chemical Company) within the drug-inducible UAS-Gal4 system (outlined in the section below). This allowed flies

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16

to be induced temporally at day 10 and remain on RU for the remainder of the lifespan as the development of the flies may have been affected if the knockdown had been induced earlier. RU486 was dissolved in ethanol at a concentration of 20mg/ml and mixed into the media during vial preparation. The dose of RU486 used was 25 μg/ml final concentration and control foods were prepared with ethanol alone. The volume of ethanol remained the same throughout. Antibiotic treatment was modified from previous publications, as flies were fed antibiotic food for the first 10 days before being kept in an incubator (Brummel et al., 2004). Antibiotic flies and corresponding controls were flipped in a sterile hood on the same timescales.

The smurf assay, used to confirm barrier integrity, was carried out as described in Rera et al., 2012, with the only amendment being that flies were kept on blue food for a 24-hour period before smurfs were counted. In every experiment, regardless of condition used, control and experimental animals were always transferred to fresh food at the same time-points. This controls bacterial growth in the food throughout these experiments.

Knockdown of mucin 55B

Here we used the UAS-Gal4 geneswitch system in order to knock down mucin 55B (Fig. 6). This allowed us to induce the knockdown temporally after day 10 as we did not want to affect the development of the flies. The geneswitch works by first combining the Gal4 DNA-binding domain with a steroid hormone receptor transcriptional activation domain which requires ligand binding to become active. This is added next to a tissue specific enhancer coding region, for our studies we wanted to target the gut and salivary glands, so we used the TIGS driver. We used the drug RU486 to activate the transcription of the Muc55B inverted repeat (Fig.6). This inverted repeat subsequently bound the Muc55B mRNA, suppressing the creation of the protein. The system was initially used by Osterwalder and colleagues, 2001.

Transgene expression can be detected at around 3-5 hours and maximal expression after 1-2 days (Osterwalder et al.,2001; Baril, Martin-Duque and Vassaux, 2010). A useful mechanism of this system is that ligand-inducible systems also provide the benefit of being able to control the level of transgene expression by varying the dosage of steroid ligand. During preliminary experimentation two knockdowns were

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investigated, 25 μg/ml and 50 μg/ml, and it was found that 25 μg/ml was more beneficial to lifespan, so this dosage was used throughout all experiments in this study.
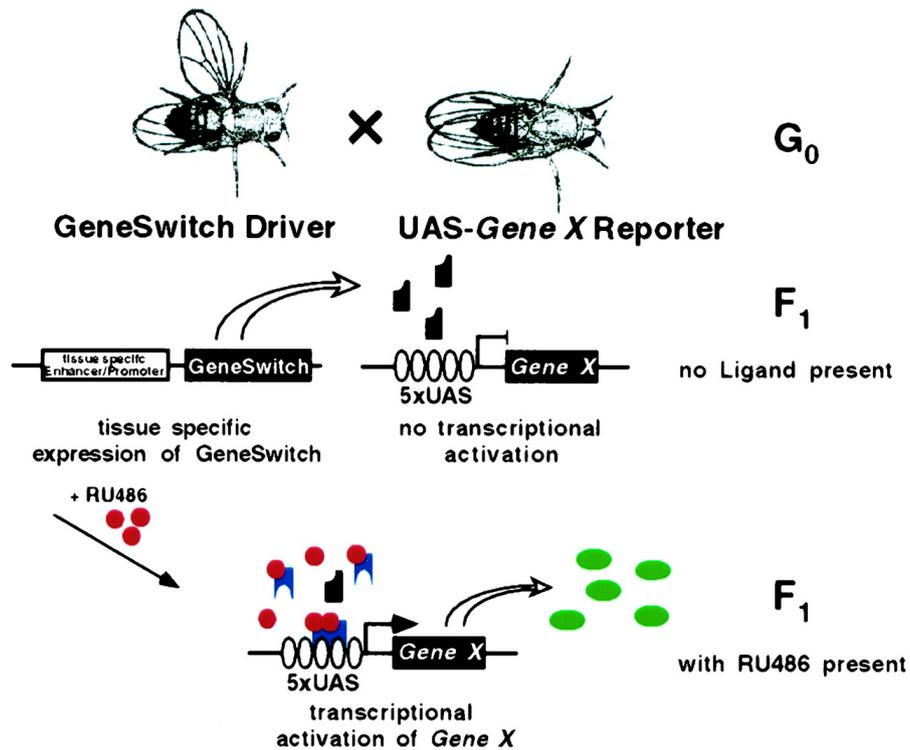
Figure 6: Schematic of the UAS-Gal4 system (Osterwalder et al., 2001). *Black and white image shows the model without RU486 and therefore no expression of the inverted repeat. Coloured image shows RU486 binding to the UAS, therefore activating transcription of the inverted repeat.*

RNA extraction

Flies were initially collected using light CO_2 (as noted above) before dissections took place over ice. During dissections, four different tissue samples for RNA processing were taken and were as follows: Gut; Head; Abdominal carcass, and Thorax carcass. Five flies were used per sample, each body part was then added to a microcentrifuge tube containing 100μl of Trizol before being homogenised with a mechanical pestle.

18

All samples were kept on ice for the entirety of this process. Once all samples were collected and homogenised, they were left to incubate at room temperature for 10 minutes before being stored at -80ºC.

In order to separate phases, samples were first thawed for 10 mins before 20µl of chloroform was added. This was shaken vigorously for 10 seconds by hand before incubating at room temperature for 3 minutes. Samples were then spun in a pre-cooled (4ºC) centrifuge at 12,000g for 15 minutes. After this, 45µl of the aqueous phase was transferred to and stored in a clean microcentrifuge tube.

4.5µl of 3M sodium acetate (RNA grade) was then added to the aqueous phase and pipette mixed before 2µl 20mg/ml glycogen (RNA grade) was added. 51.5µl of isopropanol (RNA grade) was then added and then the mixture was vortexed briefly (1-2 seconds). Samples were then incubated at room temperature for 10 minutes, then spun at 12,000g for 10 minutes in a pre-cooled centrifuge. The supernatant was then removed to leave a pure RNA pellet, and 200µl 75% ethanol (RNA grade) was added to wash the pellet, removing any residual supernatant before storage. One last spin for 5 minutes at 12,000g in a pre-cooled centrifuge took place before samples were stored at -80ºC prior to cDNA synthesis until cDNA synthesis was ready to take place.

cDNA synthesis

Following storage described above, samples were spun for 5 minutes in a pre-cooled centrifuge. The ethanol supernatant was removed, and the RNA pellets were air dried for 5 minutes. Pellets were resuspended in 20µl ddH$_2$O before being placed on a 55ºC heat block for 10 minutes.

DNase treatment was as follows: 8.5µl sample RNA, 1µl DNase buffer and 0.5µl DNase 1. This reaction was incubated at 37ºC for 30 minutes. Once the incubation was complete, 0.5µl 50mM EDTA was added, and pipette mixed. This mixture was then incubated at 65ºC for 10 minutes.

The 10µl Dnase treated RNA was briefly spun down before 1µl random hexamers (0.2mg/ml) were added and pipette mixed. The samples were then incubated at 70ºC for 5 minutes. Samples were cooled on ice before a master mix was added. Master mix was made up as follows: 4µl 5X First Strand Buffer, 0.5µl Ribolock RNase Inhibitor,

2µl 10mM dNTPs, 0.2µl Reverse Transcriptase and 2.3µl ddH$_2$O per sample with 9µl being added to each. Samples were then pipette mixed briefly and incubated at room temperature for 10 minutes. Followed by a 37ºC incubation for one hour then a 70ºC incubation for 10 minutes. 1/5 dilutions were then made with 5µl of the treated RNA and 20µl ddH$_2$O.

Quantitative PCR

A standard for qPCR was then made using 5µl from each undiluted sampled and mixed in a new tube. A series of seven 1/5 serial dilutions were then made to give the following relative concentrations: 1, 0.2, 0.04, 0.008, 0.0016, 0.00032, 0.000064). All samples (undiluted, 1/5 diluted and standards) were then all stored at -20ºC to await qPCR.

PCR was performed with *Power* SYBR Green master mix (Applied Biosystems) on a CFX Connect Real Time PCR System. Cycling conditions were as follows: 95ºC for 10 minutes; 95ºC for 15 seconds then 60ºC for 60 seconds, cycled 40 times; 65ºc for 5 minutes; 95ºC for 5 mins. All calculated gene expression values were normalised to the value of the loading control gene, rp49. The primer sequences used to assess gene expression in this study were as follows:

Act5c_L   TTGTCTGGGCAAGAGGATCAG

Act5c_R   ACCACTCGCACTTGCACTTTC

Dpt_L   ACCGCAGTACCCACTCAATC

Dpt_R   CCCAAGTGCTGTCCATATCC

Drs_L   GTACTTGTTCGCCCTCTTCG

Drs_R   CTTGCACACACGACGACAG

InR_F   AAGCGTGGGAAAATTAAGATGGA

InR_R   GGCTGTCAACTGCTTCTACTG

Muc55B_L   GTCTGACTGAGTGCGATACCC

Muc55B_R   CCTTGTAGCTGTCGGAGGAG

RP49_F   TACAGGCCCAAGATCGTGAA

RP49_R   TCTCCTTGCGCTTCTTGGA

Thor_F   CAGATGCCCGAGGTGTACTC

Thor_R   CATGAAAGCCCGCTCGTAGA

Universal primers for the 16S ribosomal RNA gene were against variable regions AGAGTTTGATCCTGGCTCAG and CTGCTGCCTYCCGTA, as previously published (Clark et al., 2015).

Cell proliferation counts
Dissections

Dissections took place using two 9-well pyrex spot plates in 150µl of chilled phosphate buffered saline (PBS). Ten whole midguts were collected for each condition. One plate was used for dissections after being chilled for 30 minutes on ice, another plate remained on ice with 150µl of sterile PBS in each well to allow the transfer of individual dissected midguts to reduce degradation as much as possible. These dissections took place between 10am and 2pm for a maximum time slot of 90 minutes to account for the circadian rhythm of *Drosophila*. *Drosophilae* have an interesting circadian rhythm meaning there is a small peak in ISC mitosis at 6am and again during to the evening (Karpowicz et al., 2013). Performing dissections around midday meant that these peaks had reduced effects Malpighian tubes were removed during dissections to prevent interference with imaging and cell counts.

Staining

After each 90-minute dissections session, each well of dissected guts was fixed at room temperature. In a fume cupboard, 50µl of 16% paraformaldehyde (pfa, final concentration 4%) was added to the 150µl PBS and then placed on a rocker (60 rpm) for 30 minutes. The 200µl solution was then removed and three 150µl PBS 10-minute washes were carried out. After the final wash, 150µl blocking buffer was added and left on a rocker (60 rpm) for 30 minutes. Blocking buffer was made up of 250 mg BSA

+ 250µl Triton X100 in 50 ml PBS with a 5% addition of NGS into aliquot before adding to plate. Blocking buffer was then removed and 150µl primary antibody mixture was added in PBS + 0.5% triton (250µl Triton X100 in 50ml PBS) to each well and incubated in a cold laboratory (4ºC) overnight. Phosphohistone H3 (PH3) was used as the primary antibody (1:1000 dilution) biomarker of proliferation as it has increased specificity over other commonly used markers such as Ki67. PH3 stains condensed chromatin just before chromosomal segregation therefore cells in the late G2 and mitosis stages of the cell cycle are stained. Goat anti-rabbit 555 was then used as the secondary antibody (1:500 dilution) biomarker. The next morning, the primary antibody mixture was removed and three 10-minute 150µl PBS washes were carried out. Secondary antibody mixture, 150µl, was then added to each well and placed on a rocket at room temperature for 2 hours. This mixture was then removed and a nuclei stain, DAPI (1mg/ml stock), counterstain was added in 140µl PBS + 0.5% triton and left to incubate on a room temperature rocker for 10 minutes. The counterstain mixture was removed, allowing for three 10-minute 150µl PBS washes. Plates were then stored for a maximum of an hour before being mounted. Parafilm was used during all incubations to prevent evaporation of the reagents and a tin foil cover was used when applying light sensitive reagents or antibodies.

Mounting

Dissected and stained guts were removed from their wells and mounted on glass microscope slides with pre-spread Vectashield (Antifade mounting medium Vector Labs H-1000). A coverslip was then placed on top and subsequently sealed with nail varnish. Slides were stored in a light-blocking box in a fridge until imaging.

Confocal microscopy

Stained samples were imaged using a Leica TCS SP5 II microscope. An oil emersion 60x objective lens with no zoom was used for all images. Images were taken at a resolution of 1024x1024 pixels, at 200Hz using a bidirectional X scan with a line average of 2. To image the fluorophores used in proliferation staining we used the following lasers and wavelengths: 405 Diode Laser: 415-460nm wavelength. Images were taken at the posterior midgut located next to the midgut-hindgut junction to maintain consistency and repeatability.

Images were subsequently analysed and proliferating cells were counted. Composite images were generated on the Fiji Image J platform, brightness and contrast were adjusted to enhance clarity of images, but this was consistent across all images.

Statistics

The comparison of survival curves was performed using the log-rank test in the GraphPad Prism software. Comparison of smurf proportions per time-point were carried out using a binomial test to calculate the probability of having as many smurfs in population B as in population A, in R $v^{4.2.1}$. Proliferation count data was analysed using an ANOVA, followed by an appropriate post-hoc test as sample sizes were above 25. All other data comparisons were tested for significant differences using Wilcoxon-Mann-Whitney U tests and student t-tests where appropriate. Number of biological replicate samples is highlighted in each figure legend. All statistical tests were conducted using GraphPad Prism.

Results

Mucin 55B was effectively knocked down in Drosophila in the UAS-Muc55B-IR line, not in the UAS-Muc55B.kk-IR line

As *TIGS* flies were used throughout the experiment, it was expected that this would cause a knockdown of mucin 55B expression within the gut and salivary glands. Data was collected to view mucin 55B expression levels in control and treated groups.

Commented [CR16]: You cannot use a T test if you know your data is not normally distributed. My question is, how do you know this? Did you test for normality? If so you need to detail how and you need to figure out an alternative test. If not, I'd remove any reference to the distribution of your data.

Commented [CR17]: Add UAS and IR or to muc55B and Muc55B.kk when ever these lines are referred to, i.e UAS-Muc55B-IR line, UAS-Muc55B.kk-IR line. This is needed to clearly denote that these are RNAi lines, as opposed to overexpression lines. I believe Muc should always have a capital M.
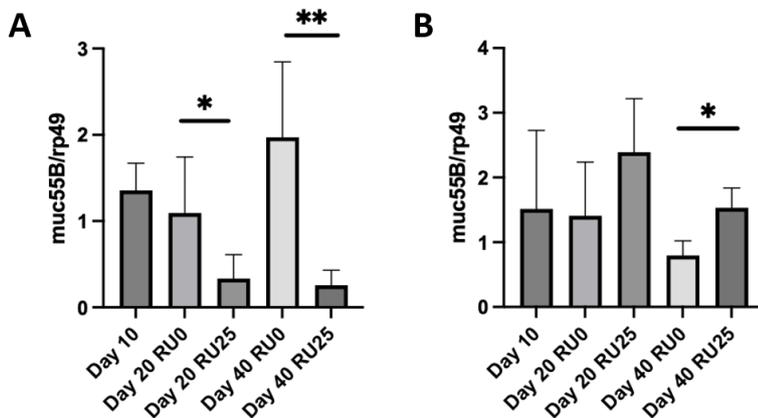
Figure 7: Muc55B expression levels throughout lifespan. (A-B) Mucin 55B expression in the gut from 10-, 20- and 40-day old females. (A) Using the UAS-Muc55B-IR line of flies. N=6x5 guts for all timepoints. (B) Using the UAS-Muc55B.kk-IR line of flies. N=6x5 guts for day 10 and 20 timepoints and N=5x5 guts for day 40. Standard deviation is displayed on both graphs. *p<0.05, **p<0.01, Mann-Whitney U test.

It was found that in the UAS-Muc55B-IR line the knockdown of mucin 55B was successful, showing significant differences at day 20 (p=0.026) and day 40 (p=0.0048) between control and treated groups (Fig. 7A). There was shown to be no significance in the change of mucin 55B expression with age in each control indicating that it is expressed at the same level throughout life. Although this contradicts preliminary data, here we show data for whole gut samples whereas specific gut regions were used in preliminary data. In the UAS-Muc55B.kk-IR line, expression demonstrated a similar level of variance throughout all timepoints indicating there was no issue with the general low expression of mucin 55B. However, there was no significance in the difference between control and treated groups at day 20 and an unusual significant increase at day 40 (p=0.0159) (Fig.7B). Unfortunately, this clearly shows that the knockdown did not work in this line.

Mucin 55B knockdown caused strengthening of gut barrier function in the UAS-Muc55B-IR line

To confirm preliminary data, a repeat of the UAS-Muc55B-IR standard line crossed with *TIGS* flies was performed. This knockdown of mucin 55B had the expected effect

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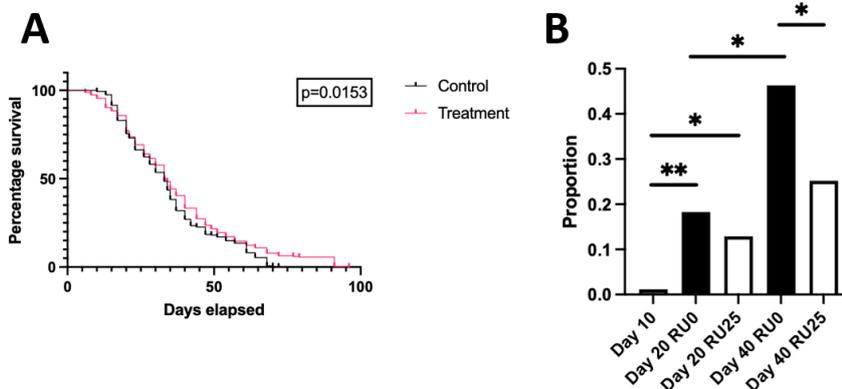
Commented [CR20]: Not appropriate to state a desired outcome. Simply state what the data showed and that this agreed with what was expected from preliminary data (expected rather than desired).

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as the knockdown was shown to significantly extend lifespan (p=0.0153) (Fig. 8A). However, this may have been caused due to a later life extension, as there was no change in the T_{50} values (33 days in both control and treated groups) but there was a longer maximum lifespan in the treated group (96 days compared to 72 days). This is confirmed by a change between maximum lifespan and T_{50} of 218.2% in the control group and 290.1% in the treated group. During this lifespan, we also conducted a smurf assay to assess the integrity of the gut barrier. The smurf assay showed a clear progression; the older the population, the larger the smurf proportion becomes as there was significantly more smurf flies at the day 20 control compared to day 10 (p=0.0022) and at day 40 control compared with the day 20 control (p=0.0112). Thus, confirming previous publications results that gut barrier function depletes with age (Rera et al., 2012) (Fig.8B). This line also showed that the knockdown of mucin 55B was linked to a reduction in smurf proportion. This is highlighted as at day 40 there was a significant reduction (p=0.0202) in the number of smurf flies in the treated group compared to the control, which is likely the cause of the extended lifespan seen in figure 8A.

***Figure 8**: **UAS-Muc55B-IR knockdown lifespan curve and smurf data.** (A) Survival curve of UAS-Muc55B-IR female flies maintained in normal conditions. Muc55B knockdown significantly extended lifespan (Mantel-Cox Test, p=0.0153). RU0 control flies shown in black, RU25 treated flies shown in red. (n=279, n=267 respectively). T50 for both lifespans were 33 days. (B) Plots display the proportion of smurf flies within the population at day 10, 20 and 40. *p<0.05, **p<0.01, binomial test.*

The T_{50} and maximum lifespan were much closer together in the UAS-Muc55B.kk-IR lifespan compared with the UAS-Muc55B-IR curve (Control: 129.4%; Treated:

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Commented [CR22]: Was there a change in the T50 or only in the maximum lifespan? The difference you can see between groups around about day 40 probably also contributed to the significance, so I wouldn't dismiss this data in this way. I would acknowledge that the difference in lifespan is subtle. Especially as the Smurf data is in line with a lifespan extension.

**Commented [CR23]:** But only in the 4 flies you've blamed this extension on? Do you see what I'm saying?

Commented [CR24]: Remove, it's methods detail and not needed.

Commented [CR25]: 33 what?

Commented [CR26]: Maximum lifespan

134.6%) (Fig. 9A). The two curves were significantly different with the control group living longer than the treated group (p=0.0008). Expression level data showed an increase in Muc55B levels at day 40 in RU25 flies so this could have been a contributing factor as we would expect an overexpression of mucin 55B to significantly reduce lifespan. This result is in line with our hypothesis that mucin 55B suppression extends lifespan
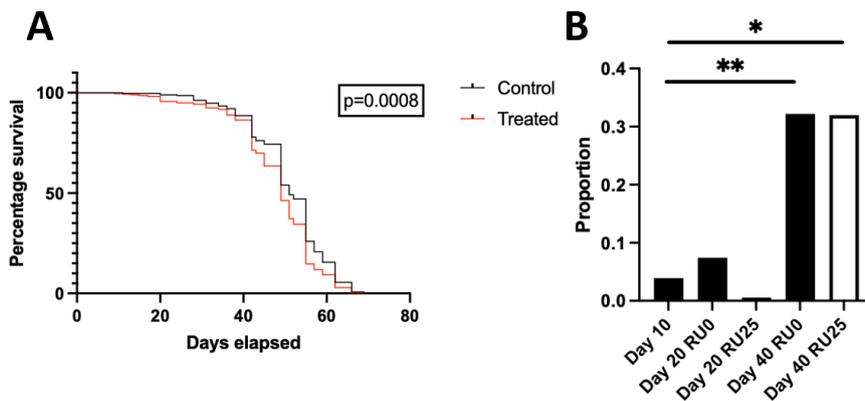
Figure 9: UAS-Muc55B.kk-IR knockdown lifespan curve and smurf data. *(A) Survival curve of UAS-Muc55B.kk-IR female flies maintained in normal conditions. Muc55B knockdown significantly shortened lifespan (Mantel-Cox Test, p=0.0008). RU0 control flies shown in black, RU25 treated flies shown in red. Median survival for each curve was: Control = 51, Treated = 48. (N=289, N=279, respectively). (B) Plots display the proportion of smurf flies within the population at day 10, 20 and 40. N=6x5 female flies, *p<0.05, **p<0.01, binomial test.*

At day 20, the UAS-Muc55B.kk-IR line showed a trend towards a lower proportion of smurf flies in the treated group (Fig. 9B). The proportion then spiked at day 40 and both control and treated flies have very similar smurf proportions. This is slightly unusual given gene expression data showed an increase in the treated group so we may have expected this to have been reflected by a greater proportion of smurfs in this population. These results again confirm that gut barrier integrity is reduced with age as smurf proportions increased from day 10 to day 40 in both control and treated groups.

Commented [CR27]: A good point but needs to be more clearly stated. You're suggesting that the reduced lifespan is in line with the increased level of muc55B at late life. Shorter lifespan is what we would expect from an overexpression assuming we're convinced that knockdown extends lifespan.

Commented [JF28]: This doesn't actually make any sense

Commented [JF29]: Sample size?

AMP expression within the gut does not significantly change with mucin 55B knockdown in the UAS-Muc55B-IR line

It has widely been reported that antimicrobial peptides (AMPs) are upregulated with age (Badinloo et al., 2018; Sheffield et al., 2021). This is due to their response to microbial signals which emanate from the gut, which grow stronger with age due to an increase in bacterial load (Arias-Rojas and Iatsenko, 2022). Mucin proteins are believed to aid in the maintenance of the peritrophic matrix which gets weaker with age. Currently, the only mucin to show evidence of this is Muc68D (Kim et al., 2020). Once this layer has been degraded with age or mucin depletion, the epithelium becomes more susceptible to both bacteria and toxic compounds present in the gut lumen. This can cause an increased immune response. We decided to assess the difference in two of these AMPs expression with age and with respect to the muc55B knockdown.

We assessed diptericin (Dpt), which is regulated by the IMD pathway and is active in commensal control within the gut. Dpt targets gram negative bacteria and rises proportionally with the gut bacteria population during ageing (Hanson et al., 2019; Imler and Bulet, 2005). We therefore expected an increase in Dpt levels with age as has previously been shown (Sciambra and Chtarbanova, 2021) and a reduction of Dpt expression in flies showing a mucin 55B knockdown. As well as this we also assessed drosomycin (Drs), another AMP, which is regulated by the Toll pathway. The production of Drs is activated through a completely different pathway (Toll Pathway) to Dpt and targets gram positive bacteria and fungi. Unlike Dpt, Drs is not as active in reducing the bacterial population directly within the gut (Tauszig et al., 2000). However, Drs has been studied as previous data has shown an increase in aged smurf fly guts (Clark et al., 2015)

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Commented [CR31]: They are believed too. Only Muc68D has any evidence available that this is in fact the case.

Commented [CR32]: Citation?

Commented [CR33]: Misleading terminology. Degraded maybe?

Commented [CR34]: Always with a capital D I believe.

Commented [CR35]: As has been previously shown with age, citations please. I'd say increase with age rather than **large** increase

Commented [CR36]: It's not because its activated by a different pathway, the activation of which occurs in quite a different way

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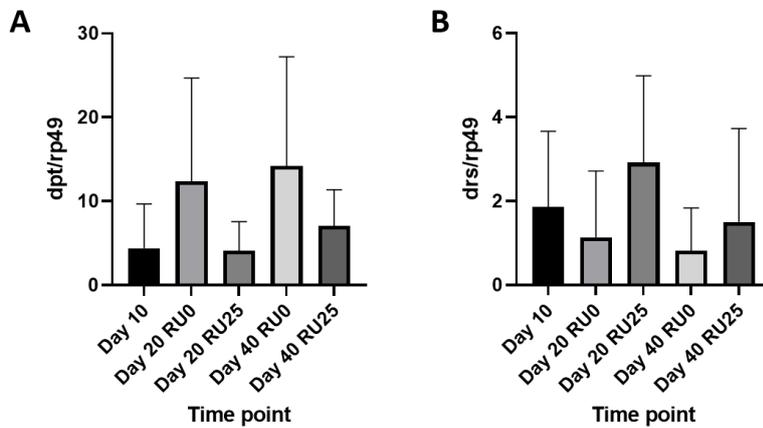
Figure 10: Diptericin and drosomycin expression within the gut in UAS-Muc55B-IR flies. *(A-B) Diptericin and drosomycin expression levels in guts from 10-, 20- and 40-day old females. (A) Diptericin expression. (B) Drosomycin expression. N=6x5 guts. Standard deviations are shown in each graph. There were no significant changes in diptericin or drosomycin expression, Mann-Whitney U test.*

Overall, it was shown in the gut that Dpt expression trended towards an increase with age (Fig. 10A). Although the data showed exactly what we expected, no values were significant. A possible reason for this insignificance is that the sample size of 30 flies means the experiment is underpowered, however, previous studies have used this sample size and found significance (Clark et al., 2015; (Paredes et al., 2011). This, therefore, suggests the insignificant result is due to variable data. Drs expression in the gut can be seen to slightly reduce with age (Fig. 10B), although this deterioration is insignificant through days 20 and 40. It is interesting to see that the knockdown of mucin 55B appears to show a trend toward an increase in Drs expression in the gut. This change is not statistically significant, however, these results do show trends that the knockdown of mucin 55B does reverse the effect of ageing for both Dpt and Drs expression.

Drosomycin expression in the gut is significantly increased with age in the UAS-Muc55B.kk-IR line

The same AMPs were investigated in the same way within the UAS-Muc55B.kk-IR line (Fig. 11A&B). Unlike the UAS-Muc55B-IR line, the UAS-Muc55B.kk-IR line showed a significant increase in Dtp between both day 10 and 20 controls (p=0.0152) as well as

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Commented [JF38]: Experimental set-up?? Why big differences are often not significant?? Is this normal in other assays done by other researchers??

Commented [JF39]: Reference

Commented [CR40]: A trend toward an increase again

day 10 and 40 controls (p=0.0238) which is in accordance with previous publications (Clark et al, 2015) (Fig. 11A). There was no significant change in Dpt expression between control and treated groups, which is to be expected given the knockdown did not reduce the levels of mucin 55B.
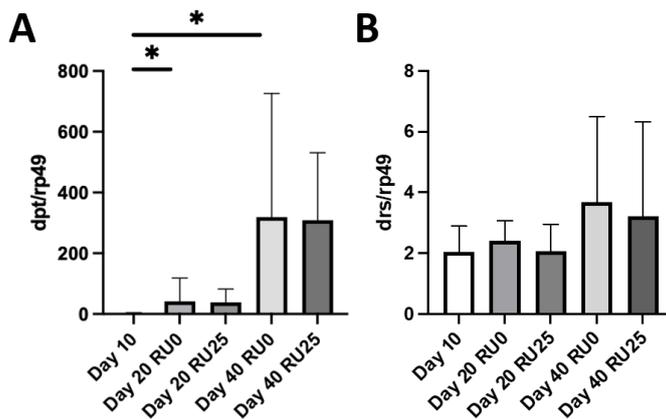
Figure 11: Diptericin and drosomycin expression within the gut in the UAS-Muc55B.kk-IR line. *(A-B) Diptericin and drosomycin expression levels in guts from 10-, 20- and 40-day old females. (A) Diptericin expression, N=6x5 guts. (B) Drosomycin expression. N=6x5 guts. Standard deviations are shown in each graph. *p<0.05, **p<0.05, Mann-Whitney U test.*

Drosomycin levels show no change with age (Fig. 11B). Although a slight increase with age can be seen, the large variance meant this was not significant. This may indicate that a small number of gram-positive bacteria or fungi accumulated within the gut with age and therefore drosomycin levels were not able to rise. There was no change between control and treated groups which was expected as the knockdown was not successful.

Drosomycin and diptericin showed a trend towards reduced expression with age in the abdominal carcass of UAS-Muc55B-IR flies

Abdominal RNA was then investigated as when the gut barrier becomes weakened with age, it was hypothesised that bacteria would move from the gut into the body cavity possibly triggering systemic immune responses in the fat body or haemocytes (macrophage cells). For example, the activation of Toll receptors leads to the mobilisation of NF-κB factors, which have been shown to regulate genes involved in

immune response (Verma et al., 1995; Baeuerle and Baltimore, 1996). A study found that all *Tolls*, with the exception of *Toll-3*, were expressed within the fat body and lymph glands (Kambris et al., 2002). This suggests that bacteria being leaked from the gut could bind these receptors and thus cause an AMP response in the abdomen. Diptericin and drosomycin were again used as biomarkers for this response.
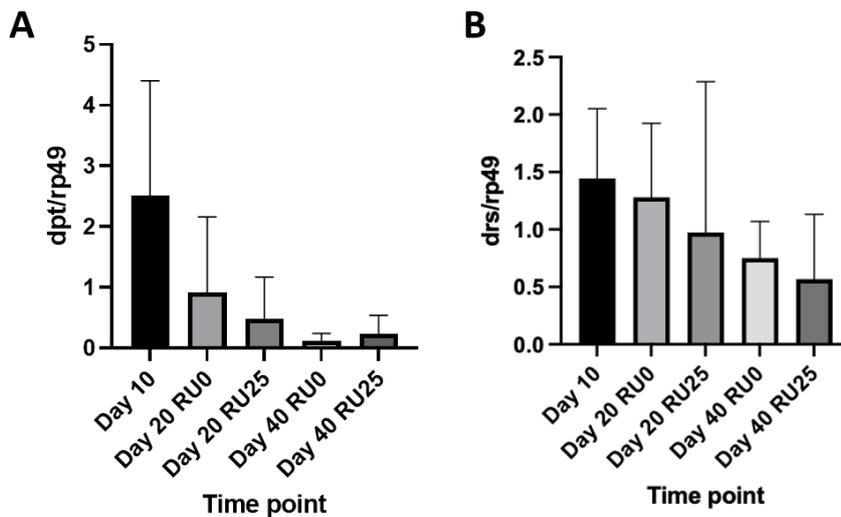
Figure 12: Diptericin and drosomycin expression within the abdomen in UAS-Muc55B-IR flies. *(A-B) Diptericin and drosomycin expression levels in the abdomen from 10-, 20- and 40-day old females. (A) Diptericin expression, N=6x5 abdomen. (B) Drosomycin expression. N=6x5 abdomen. Standard deviations are shown in each graph. There were no significant changes in diptericin or drosomycin expression, Mann-Whitney U test.*

Unexpectedly, the results showed that both Dpt and Drs expression showed a trend towards a reduction over age (Fig. 12&B). Diptericin levels were not significantly changed with age, however there was a trend towards a depletion of Dpt expression with respect to Muc55B knockdown at day 20 (Fig. 12A). Drs also showed a trend towards a reduction of expression after the Muc55B knockdown; this time at day's 20 and 40 (Fig.12B). This suggests that Muc55B suppression may have an effect on Drs and Dpt expression although this experiment was unable to significantly demonstrate it. This is in line with the smurf data above which suggests more bacteria leaks from the gut and therefore causes an immune response in the abdomen.

Commented [CR45]: But these samples were of non-Smurf flies – remember to add that information to all your figure legends where relevant too. It's really important.

30

Insulin receptor expression is significantly reduced in midlife in the UAS-Muc55B-IR line within the abdomen

After studying AMPs, we shifted focus onto metabolic pathways which are markers for the fly's immune response. A reduction in the level of insulin receptor expression has been shown to significantly extend lifespan in *Drosophila* (Tatar et al., 2001; Ikeya et al., 2009). This association between the insulin signalling pathway and ageing is strengthened as the overexpression of FOXO has also found to extend lifespan (Giannakou et al., 2004). It has also been shown in past publications that smurf flies lose metabolic stores with age (Catterson et al., 2018), which has been found to be due to the immune activation pathway becoming so overstimulated that the fly is unable to use metabolic pathways such as the insulin signalling pathway (Dionne et al., 2006).
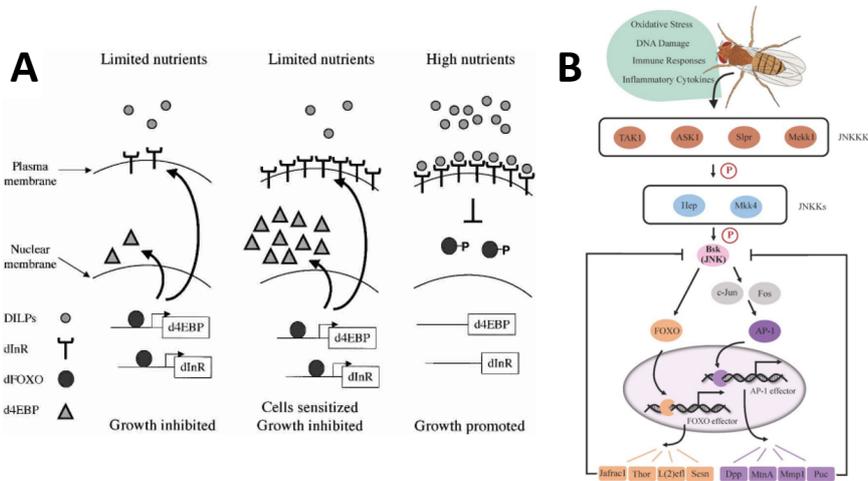
Figure 13: Regulation of insulin receptor and THOR by FOXO (Gan et al., 2021; Puig et al., 2003). *(A) Model to show regulation of growth by a feedback mechanism involving InR and FOXO. (B) Shows how THOR is regulated by FOXO. Unphosphorylated FOXO can bind DNA and promote the expression of THOR.*

Two biomarkers were subsequently selected, insulin receptor (InR) and THOR. InR was selected as a readout as its expression is regulated by FOXO and so reflects

FOXO activity and thus insulin signalling activity. This will indicate whether metabolic activity is being 'traded-off' in favour of higher immune activity. Figure 13A shows that when the fly undergoes periods of low nutrition InR expression increases as FOXO is unphosphorylated but when nutrition is high, FOXO is bound so InR expression is significantly lowered. This is a similar feedback mechanism by which THOR is regulated by FOXO (Fig. 13B). This feedback mechanism allows us to use InR and THOR as readouts for the insulin signalling pathway as when the expression of InR and THOR increases, the insulin signalling pathway becomes inactivated. Hence, we hypothesise that flies with no knockdown of mucin 55B will have higher levels of InR and THOR expression compared to treated flies as we expect there to be a larger immune response in control flies.
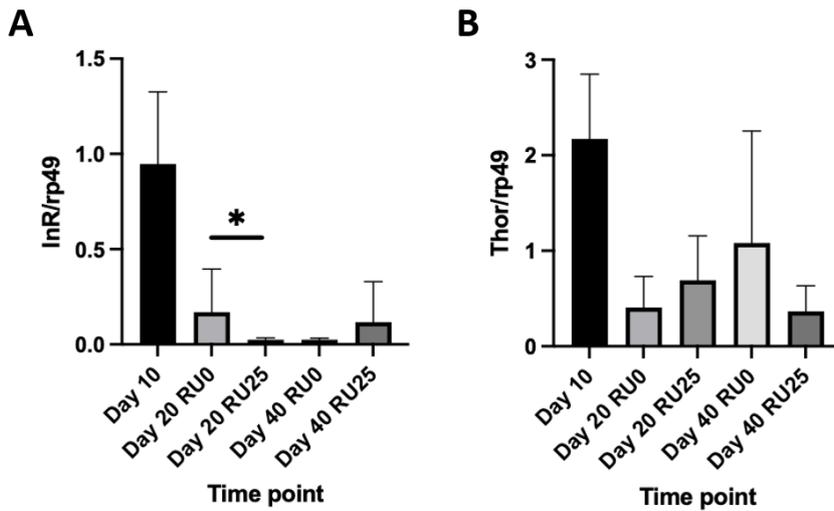
*Figure 14: Insulin receptor and THOR expression within the abdomen from UAS-Muc55B-IR flies. (A-B) Insulin receptor and THOR expression levels in abdomen from 10-, 20- and 40-day old females. (A) Insulin receptor expression, N=6x5 abdomen. (B) THOR expression. N=6x5 abdomens. Standard deviations are shown in each graph. *p<0.05, Mann-Whitney U test.*

The two metabolic biomarkers were tested within the UAS-Muc55B-IR line (Fig. 14A&B). InR showed an overall trend towards a decrease in expression throughout with the day 40 control flies being visibly, but not statistically significantly, lower than

their day 10 counterparts (Fig. 14A). Similarly to results shown in Figure 10 this is likely due to data variation as other publications have shown InR expression had no change with age with a smaller sample size (Tanabe et al., 2017). A repeat experiment using a larger sample size could be performed to view whether the variation lowers. It was shown that InR was expressed at significantly lower levels in treated flies at day 20 than the control (p=0.0317). This indicates that the suppression of mucin 55B allowed the flies to maintain an active insulin signalling pathway by possibly strengthening the intestinal barrier function and stopping immune activation. At day 40, the InR expression levels of control and treated flies are shown to be insignificantly different, suggesting this to be a midlife change which is contributing to the lifespan change.

Similar to InR, THOR expression showed an insignificant decrease between days 10 and 20 but, in contrast, showed an insignificant increase at day 40 (Fig. 14B). There were no other notable differences in THOR expression suggesting there is no difference in insulin signalling levels across age or between conditions. This is interesting as both InR and THOR are readouts of insulin signalling so it was expected that both would indicate the same result. Given the small and limited significance between conditions in InR data, it is more likely that mucin 55B knockdown has no link to insulin signalling pathway activation.

No change in AMP expression in UAS-Muc55B.kk-IR flies within the abdomen

As with the initial UAS-Muc55B-IR line, analysis of AMPs was then conducted within the abdomen of UAS-Muc55B.kk-IR flies (Fig. 15A&B). The UAS-Muc55B.kk-IR line also showed diptericin expression did not significantly change with age, although there does appear to be a small rise between the day 20 and day 40 controls (Fig. 15A). Drosomycin levels also remained statistically insignificant with age (Fig. 15B) with the only slight fluctuation coming with a rise between day 40's control and treated group. This suggests that the damage to the intestinal barrier was not sufficient to allow small molecules derived from bacterial cells to leak out and provoke an AMP response within the abdomen.

Commented [CR46]: Who's to say that a mid-life different can't contribute to lifespan extension?

Commented [CR47]: Remember Thor and InR are two readouts of the same thing. You need to come to one conclusion from both sets of data. You can't conclude that the InR data shows that muc55B levels can impact insulin signalling and then in the next paragraph say that Thor levels show that 55B levels don't impact insulin signalling. Rethink this.
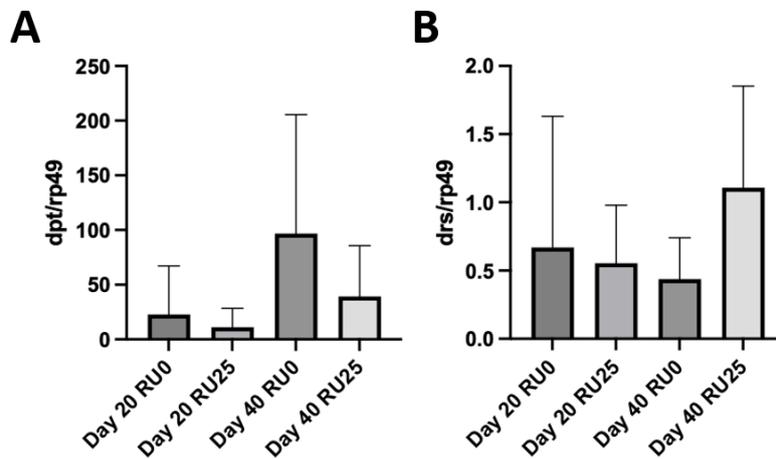
Figure 15: Diptericin and drosomycin expression within the abdomen of UAS-Muc55B.kk-IR flies. (A-B) Diptericin and drosomycin expression levels in abdomen from 10-, 20- and 40-day old females. (A) Diptericin expression, N=6x5 abdomen. (B) Drosomycin expression. N=6x5 abdomen. Standard deviations are shown in each graph. There were no significant changes in diptericin or drosomycin expression, Mann-Whitney U test.

The insulin signalling pathway is inactivated with age in the UAS-Muc55B.kk-IR line within the abdomen

The results initially found in the UAS-Muc55B-IR line were not as expected (Fig. 14). This is mainly because in other publications InR and THOR expression is seen to rise significantly with age, therefore reflecting an inactive insulin pathway (Patridge et al., 2011; Shaposhnikov et al., 2022).
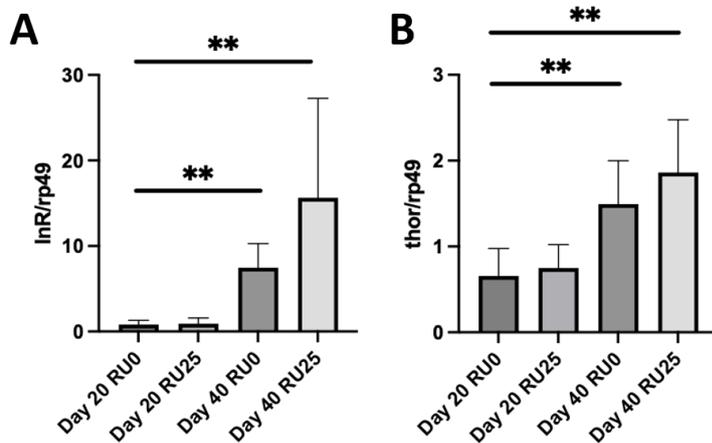
Figure 16: Insulin receptor and THOR expression within the abdomen from UAS-Muc55B.kk-IR flies. *(A-B) Insulin receptor and THOR expression levels in abdomen from 10-, 20- and 40-day old females. (A) Insulin receptor expression, N=6x5 abdomen. (B) THOR expression. N=6x5 abdomen. Standard deviations are shown in each graph. **p<0.01, Mann-Whitney U test.*

This additional data matched initial predictions as it was shown there was a clear increase in both InR and THOR expression with age. InR expression showed increases from day 20 to day 40 control groups (p=0.0043) (Fig. 16A). Interestingly, increases in both control and treated groups had the same p-value despite the treated group looking to increase further, this is due to the standard deviation being higher within this group. The expression data shown in Figure 7 shows it is likely that the UAS-Muc55B.kk-IR flies don't deplete Muc55B with age so this variation is most likely due to noise. THOR expression also showed a significant increase with age (Fig. 16B). Again, the difference between day 20 and day 40 control groups (p=0.0087) was shown to be significant. The graphical display of the control and treated groups looked much closer, which was matched by the insignificant results. InR and THOR increases with age show that immune signalling pathways are overactivated with age, as the high InR and THOR levels show us that important metabolic pathways are inactivated as the flies grow older. Dpt was also increased with age in the UAS-Muc55B.kk-IR line, giving more evidence that systemic immune activation with age causes fly death. There is no difference at day 20 between conditions, which is expected as mucin 55B was not successfully knocked down within the UAS-Muc55B.kk-IR line. The slight

trend towards an increase at day 40 is likely to be due to variation as the knock-down was not successful.

Proliferating cells are more abundant in the ageing fly

Proliferation counts were also taken during the UAS-Muc55B.kk-IR lifespan at three different timepoints (days 10, 20 and 40) in order to determine the number of mitotic divisions in the gut. The hypothesis behind this is that if the gut becomes damaged with age, or through the change in expression of muc55B, the gut will have to be repaired by the fly and thus a higher level of proliferating cells would be visible.
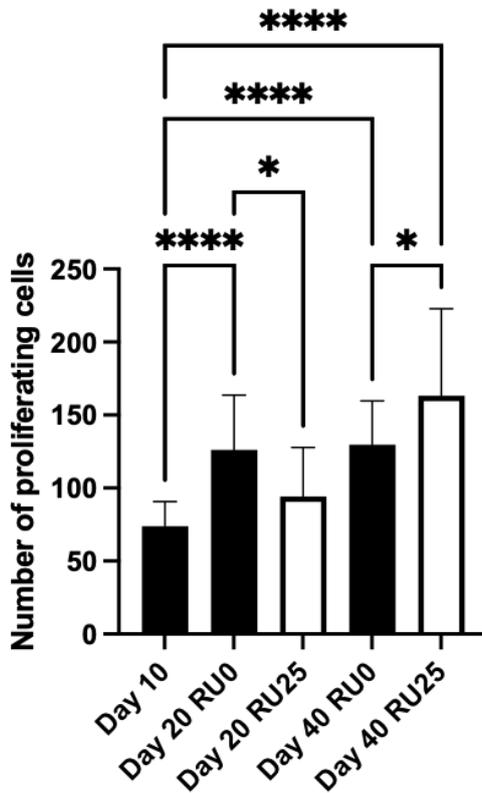
Figure 17: Full gut dissection ph3 counts in UAS-Muc55B.kk-IR flies. *All flies were female and aged for 10, 20 and 40 days respective of their sample group before undergoing dissection. Initial ANOVA showed significant difference in means (p<0.0001), so the post-hoc Tukey's test was subsequently performed. *p<0.05, ****p<0.0001.*

A one-way ANOVA revealed that there was a statistically significant difference in mean number of proliferating cells between timepoints ($F_{(4, 129)}$ = 2.883, p=0.0251) (Fig. 17). A Tukey HSD test for multiple comparisons was then completed to see which timepoints showed significance. There was a significant (p<0.0001) increase from day 10 to day 20 control during this time period. Muc55B expression data in the UAS-Muc55B.kk-IR line showed the knockdown did not work so it was expected there would be no change between conditions at day 20. However, at day 20, there was a significant decline in the number of proliferating cells between control and treated groups (0=0.0143). Between day 20 and day 40 controls there was no increase in proliferating cells which suggests there was no additional cell division or repair within control flies. However, treated flies showed a significant increase between day 20 and 40 (p<0.0001) which matches the increase in the number of deaths in the original UAS-Muc55B.kk-IR lifespan. This increase between day 20 and 40 in treated flies maybe caused by the reduction of proliferation in treated flies compared to controls at day 20 as an overactivation of repair pathways may have been triggered to compensate for this. The difference between day 40 control and treated flies were as expected as there was a significant increase (p=0.0345) in the number of proliferating cells which matched the lifespan extension. Given that the amount of proliferation at day 20 was significantly reduced between the two conditions, a similar outcome was expected at day 40. This was not seen, but it does correlate with the great number of deaths in the treated group at around the day 40 timepoint in the lifespan as well as the significant increase in the amount of smurf flies within the earlier shown UAS-Muc55B-IR population.

Control flies showed an extension of lifespan after antibiotic treatment in the UAS-Muc55B-IR line

Ageing related death in *Drosophila* is commonly associated with bacterial growth with age. A current model that has been proposed is as follows (Clark et al., 2015; Buchon et al., 2009; Guo et al., 2014): As the fly grows older a greater accumulation of bacteria within the gut occurs, when the gut barrier becomes damaged these bacteria can then reach receptors to cause an immune response. Data has shown this response can dangerously spiral out of control, causing the fly to inactivate vital metabolic pathways in favour of an immune response (Dionne et al., 2006; Reviewed in Dionne, 2014). We repeated previous lifespans including an antibiotic treatment to establish whether the

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Commented [CR50]: how do you relate this to the day 20 result where levels were reduced in RU25?

Commented [CR51]: This suggests direct causation. It correlates with the increased deaths and increased Smurfs.

change in lifespan was solely caused by the mucin 55B knockdown or whether this was coupled with bacterial growth.

Figure 18: UAS-Muc55B-IR antibiotic lifespan. *N, with respect to the order of the key = 276, 288, 290, 291 female flies. Statistical analysis shown in Table 1.*

Table 1: Statistical comparisons of the UAS-Muc55B-IR antibiotic lifespan. *All tests were conducted using the Mantel-Cox (log-rank) test. Significant comparisons are highlighted in red.*

Lifespan Comparison		Significant?	P-value
AB- RU0	AB- RU25	NO	0.9649
AB+ RU0	AB+ RU25	NO	0.1795
AB- RU0	AB+ RU0	YES	0.0023
AB- RU25	AB+ RU25	NO	0.0575

An antibiotic lifespan was therefore carried out using UAS-Muc55B-IR flies (Fig. 18). It was shown that the knockdown of mucin 55B was not enough to significantly change lifespan (Table 1, p=0.9649) and it was shown that antibiotic treatment did not affect this (Table 1, p=0.1795). The UAS-Muc55B-IR antibiotic line showed control flies lived significantly longer under antibiotic conditions (Table 1, p=0.0023) and the flies with knocked down mucin 55B showed no significance depending on antibiotics (Table 1, p=0.0575). As the increase in lifespan in response to antibiotic treatment was not consistent through control and treated groups and there was no significance in the comparison between mucin 55B knockdown and control, there can be no additional

details regarding the link between bacterial load and mucin 55B drawn from this data. It should be noted that there was a number of early deaths seen within this lifespan which could have affected the outcomes of this experiment.

Antibiotic treatment showed an extension of lifespan in UAS-Muc55B.kk-IR treated flies

We then completed the same antibiotic lifespan with the UAS-Muc55B.kk-IR (Fig. 19). The difference between the treated and control lifespans were shown to be insiginificant (Table 2, p=0.6350). The absence of the gut microbiome did not seem to effect this as control and treated flies fed antibiotics also observed no significant change (Table 2, p=0.1143). Contrary to available literature, antibiotic treatement had no significant effect on control flies (Table 2, p=0.4538). Although this differs from expected values, it matches the struggles in gaining bacterial load data through qPCR which suggested that there were low levels of bacterial load within the UAS-Muc55B.kk-IR fly line. Antibiotic treatment, however, was shown to significantly extend lifespan in treated flies (Table 2, p=0.0423). This experiment found no clear evidence that antibiotics had any effect on extending lifespan, which is in line with Yamada et al., 2015 as they found no alteration of lifespan in axenic condition flies.
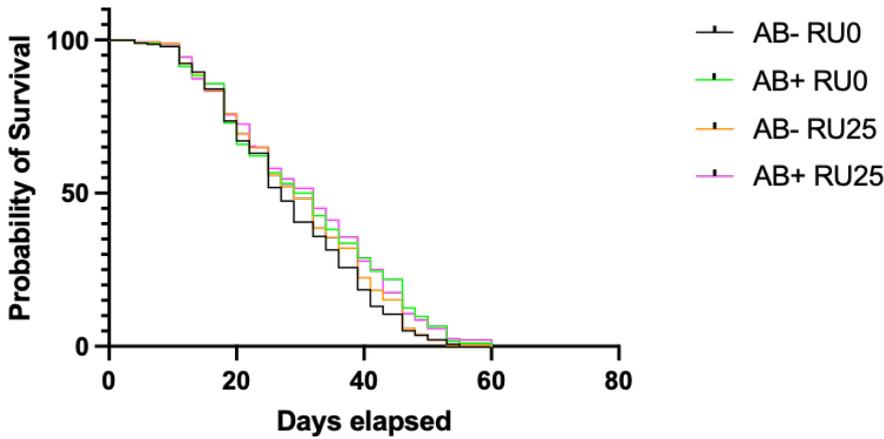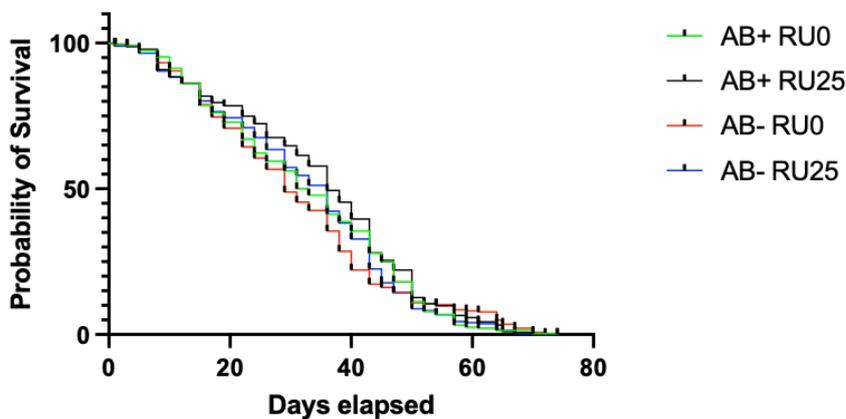

Figure 19: UAS-Muc55B.kk-IR antibiotic lifespan. *N, with respect to the order of the key = 276, 276, 279, 278 female flies. Statistical analysis shown in Table 2.*

Table 2: Statistical comparisons of the UAS-Muc55B.kk-IR antibiotic lifespan.
All tests were conducted using the Mantel-Cox (log-rank) test. Significant comparisons are highlighted in red.

Lifespan Comparison		Significant?	P-value
AB- RU0	AB- RU25	NO	0.6350
AB+ RU0	AB+ RU25	NO	0.1143
AB- RU0	AB+ RU0	NO	0.4538
AB- RU25	AB+ RU25	YES	0.0423

Mucin 55B expression is affected by the JNK pathway

Several key pathways were then investigated to attempt to find a regulatory pathway for mucin 55B. These were the JAK/STAT, the IMD and the JNK pathways. Firstly, the JAK/STAT pathway was investigated using a dominant negative system. Domeless was used for this as it is a transmembrane protein which, when bound by a ligand causes the activation of the JAK/STAT pathway. The dominant negative system removes the cytoplasmic end of the protein, shortening the receptor. This sequesters the ligand away from the functional native receptor and therefore reduces signalling, supressing the JAK/STAT pathway. It was shown that this reduction in JAK/STAT pathway activation had no significant effect on mucin 55B expression (Fig. 20A).

Upd3, a ligand which binds domeless, was then overexpressed with the aim of over activating the JAK/STAT pathway. By overexpressing UPD3, the JAK/STAT pathway was effectively always switched on, however, this had no effect on mucin 55B expression (Fig. 20B). This shows that the JAK/STAT pathway does not regulate mucin 55B expression.
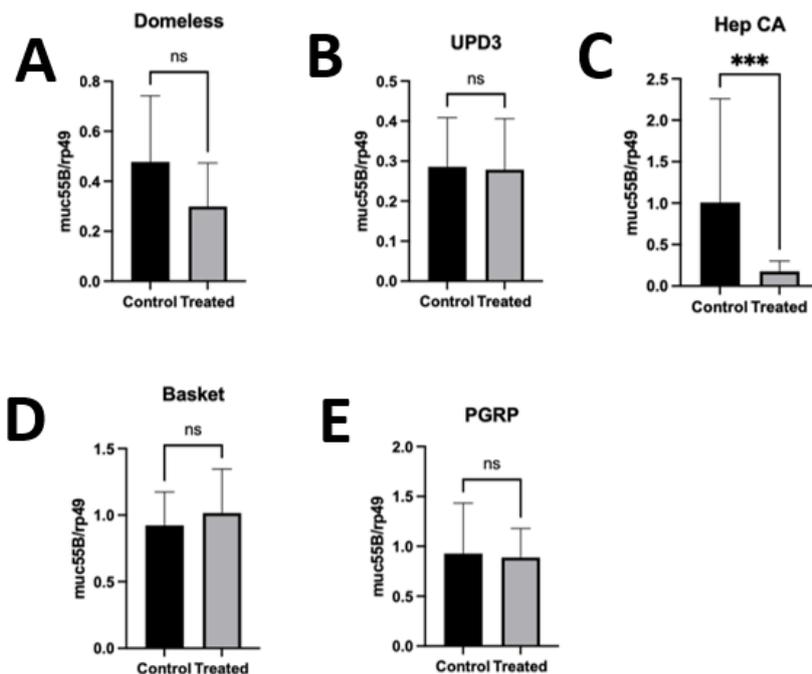
Figure 20: Possible regulators of mucin 55B. *(A-F) Muc55B expression levels in 20 day old female flies, N=6x5 for all conditions. (A) TIGS>UAS-Dme. (B) TIGS>UAS-UPD3. (C) TIGS>UAS-HepCA. (D) TIGS>UAS-Bsk. (E) TIGS>UAS-PGRP. Standard deviations are shown for each condition. ***p<0.001, Mann-Whitney U test.*

The JNK pathway was then investigated in a similar way. Hep is an activator of basket in the JNK pathway when phosphorylated. For this reason, a modified version of the Hep gene, named Hep CA, was overexpressed in order to activate the JNK pathway. This version of Hep was constitutively active so essentially allowed the JNK pathway to remain always switched on. Mucin 55B expression was significantly decreased by this, suggesting that it is regulated by the JNK pathway (Fig. 20C). A dominant negative version of Basket, JNK in mammals, was then used to confirm this finding. Contrary to the findings using Hep, the inactivation of the JNK pathway through this system showed no change in mucin 55B expression between control and treated groups (Fig. 20D).

The IMD pathway was the final pathway to be investigated. The receptor, PGRP, has been found to activate the IMD pathway when overexpressed (Takehana et al., 2002;

Clark et al., 2015). For this reason, it was used for this experiment. Mucin 55B is unlikely to be regulated by the IMD pathway as there was no significant change in expression levels when expression of PGRP receptors were overexpressed (Fig. 20E).

Discussion

The functional decline of the gut with age has become a prominent area of research in recent years. This study has used the model organism, *Drosophila melanogaster*, in order to investigate the role of the protein, mucin 55B, and its effect on ageing. As well as this we sought to discover how mucin 55B is regulated. To achieve this, we used non-invasive approaches, namely lifespans and smurf assays, to test intestinal function as well as invasive strategy, dissections, to explore the metabolic and inflammatory responses to ageing. We show that intestinal barrier function does decrease with age and mucin 55B suppression does slow down this deterioration.

Mucin 55B suppression strengthens the gut barrier

Data from both the UAS-Muc55B-IR and UAS-Muc55B.kk-IR lines showed an increase in smurf proportion at each time point. This is in line with other studies which have shown that the gut becomes 'leakier' with age (Rera et al., 2012). Unusually, the UAS-Muc55B.kk-IR line was unable to sustain this significance through to the end timepoint as there was only significantly more smurfs at day 20 compared to day 10. This is contrary to previous publications which commonly show the proportion of individuals displaying barrier loss continues to increase as the population ages (Rera et al., 2012; Clark et al., 2015).

This smurf assay then showed, in the UAS-Muc55B-IR line, that mucin 55B suppression causes the integrity of the gut barrier to remain intact, strengthening evidence that intestinal dysfunction increases with age and mucin 55B suppression slows this (Rera et al., 2012: Fasano, 2020). Unfortunately, the UAS-Muc55B.kk-IR line was unable to cause a knockdown of mucin 55B. This leads us to conclude that the UAS-Muc55B.kk-IR line is not effective. The ineffective knockdown was not ideal, however, the expression data did indicate a significant increase in expression at day 40 in the treated group, allowing us to view this effect in each experiment.

In the UAS-Muc55B.kk-IR line, there was no difference between control and treated groups, which is to be expected. However, at day 40 expression levels showed the levels of Mucin 55B were higher in the treated group but this did not cause an increase in the number of smurfs present. We expected to see a correlation between higher mucin 55B levels and higher smurf numbers as the UAS-Muc55B-IR knockdown line showed a decrease in smurfs. A possible reason we did not see this is because an increase in the expression from control was not large enough to cause further disruption to the intestinal barrier. This is most likely due to the 'overexpression' happening as a result of a faulty knockdown. Other studies have shown that overexpression of genes has the reverse effect of a knockdown, for example, the miR-340 micro-RNA gene showed that an overexpression inhibited the proliferation and a knockdown of the same gene reversed the effect (Zhu et al., 2019)

The mucin 55B knockdown showed evidence for lifespan extension

Lifespan data was consistent with the results of the smurf assay and further supported the link between intestinal dysfunction and mortality. The UAS-Muc55B-IR lifespan showed that the knockdown of mucin 55B caused a small but significant extension in lifespan. Like the human mucin, Muc5B, mucin 55B is a lowly expressed gene within the gut (Grondin et al., 2020). This low level of expression could be a reason why we do not see a large gap between lifespan curves in the UAS-Muc55B-IR line as there is only so much change knocking down a lowly expressed protein can cause. A rise in mucin 55B expression levels at late life correlated with a reduction in lifespan in the UAS-Muc55B.kk-IR line. Smurf data, however, did not match this finding as there was no significant difference between treated and control flies. These findings highlight that the changes found in the UAS-Muc55B.kk-IR line are likely not related to the change in Muc55B expression.

One reason we repeated the UAS-Muc55B-IR lifespan in another line of flies was due to the high number of early deaths. The UAS-Muc55B.kk-IR line did not show early deaths. It has been suggested that the early deaths could have been due to the UAS/GAL4 system as flies using this system can have issues with early deaths. This is due to the fact that both the UAS and GAL4 coding regions are randomly inserted into the genome. In very rare instances these random inserts can cause problems as they can be inserted within important genes and coding regions. For example, in a

2010 study, researchers found that one third of 1,332 homozygous *P*-element insertion lines had quantitative effects on lifespan, with mutations reducing lifespan being twice as likely as those extending (Magwire et al., 2010). Our data suggests that the UAS or GAL4 regions may have disrupted a key coding region as early deaths should not be common within the first 20 days of a lifespan (Linford et al., 2013).

Drosomycin and diptericin expression is not altered by a mucin 55B knockdown

Our data confirms that the expression of mucin 55B is linked to the integrity of the gut barrier and lifespan. The role of the microbiota was suggested to be key to these findings so the response to antibiotic treatment as well as AMP biomarkers were investigated.

We were unable to show conclusive evidence that the antibiotic treatment improved lifespan in either fly line. Although we did see a significant increase in lifespan after antibiotic treatment in control flies, this was not consistent across all experiments nor was the extension as long as expected. This is in direct contradiction with other publications which have shown antibiotic treatment greatly extends lifespan (Ren et al., 2007; Ridley et al., 2012; Iatsenko et al., 2018). There was no significant lifespan extension between control and treated flies, regardless of antibiotic treatment, suggesting that the presence of the gut microbiota is not necessary for lifespan extension. Antibiotic treatment is known to cause other changes within the fly, so these small changes could also be affecting the lifespans of flies within this antibiotic experiment. For example, a study found that tetracyclines, even at low levels, induce mitochondrial proteotoxic stress which can lead to changes in nuclear gene expression and alter the mitochondrial dynamics and function in *Drosophila*, impacting lifespan (Moullan et al., 2015).

This lack of effect by the antibiotic treatment is backed up throughout the AMP response data. Within the gut, diptericin showed no significant change with age in the UAS-Muc55B-IR line. This is unusual as it has been found that as the fly ages, the bacterial load increases with age (Ren et al., 2007) which would suggest that the AMP response would be greater. However, the UAS-Muc55B.kk-IR did show this increase in Dpt response in the gut with age. This is consistent with past publication which show that IMD pathway, the driver of diptericin, is activation increases with age (Chambers et al., 2019; Tanji et al., 2007; Kleino and Silverman, 2014). Unusually, drosomycin

expression levels showed no change in the gut with age in either fly line. Drosomycin is driven by the Toll pathway in other parts of the of the fly, however, publications have shown that the Toll pathway is not involved in the gut immune response (Buchon et al., 2009). Instead it appears that Drs expression in the gut is regulated by the JAK/STAT signalling pathway (Myllymaki and Ramet, 2014) which contributes to the antimicrobial defence. The main role of JAK/STAT signalling seems to be to maintain gut homeostasis by regulating the epithelial layer which is stimulated by indigenous gut microbiota (Buchon et al, 2009). Given that we believe that we were unable to obtain bacterial data due to a low bacterial load, it would therefore follow why we no significant increase of drs was seen with age.

Future research could focus on axenic flies as little research has been done in axenic culture on the link between mucin genes and the gut microbiota, with most of this work being performed on the mucin, *muc68D* (Kim et al., 2020). Previously, Clark et al (2015) found that axenic flies showed improved markers of intestinal homeostasis which is shown to be correlated with improved lifespan. An axenic study could add to this research. As our findings have suggested the gut microbiota is necessary to cause an extension of lifespan in mucin 55B knockdown mutants, it would be expected that there would be no difference in lifespan between axenically and normally reared. It therefore follows that there would be no difference in smurf flies, confirming our results.

Drosomycin and diptericin expression is linked to smurf fly proportions

Given the smurf data showed that a knockdown of mucin 55B caused strengthening of gut barrier and the 'leaky gut' hypothesis suggesting that bacterial particles emerge from the gut when it becomes damaged, we expected to see a decreased immune response within the abdomen after the knockdown and an increased immune response with age. However, this was not shown in either circumstance. This is unusual as elevated immune responses in response to ageing are common within animals, for example, human syndromes such as atherosclerosis and Alzheimer's disease and are associated with this (Bruunsgaard and Pedersen, 2000). So, this result, paired with the lack of coherent antibiotic data shows that it is likely that the amount of bacterial load in these flies was low.

Mucin 55B suppression showed no significant impact on Dpt or Drs expression during the UAS-Muc55B-IR lifespan in either the gut or the abdomen suggesting that the gene

is not linked to the amount of bacteria present within the gut nor does it protect from damage responded to be diptericin or drosomycin. This is in contrast to other genes linked to the disruption of barrier integrity, which do impact AMP response when inhibited. For example, excess of Upd3 caused by a release by haemocytes after injury to the gut caused an increase in the expression of *Drosomycin* in the intestine (Chakrabarti et al., 2016).

Insulin pathway activation was increased in regards to intestinal barrier dysfunction

It has been shown in the past that metabolic regulation is tightly linked with immune responses and inflammation (Hotamisligil, 2006). Here we were unable to show this consistent link even though we showed mucin 55B suppression reduced insulin receptor expression in midlife. Although we found that insulin suppression was increased in the UAS-Muc55B.kk-IR line, it was not reproduced in the UAS-Muc55B-IR line. This is most likely down to, as explained earlier, the lack of immune activation systemically across these flies so it is unlikely we would see their metabolic status change. Previous studies have shown that chronic immune activation can cause the fly to lose metabolic stores in the form of fat and glycogen causing wasting (Dionne et al., 2006). Smurf flies have also been shown to have reduced metabolic stores and increased inflammatory markers compared to their healthy counterparts (Rera et al., 2012). It may be an interesting follow-up project to investigate whether the reduction in insulin receptor expression at the day 20 timepoint in the UAS-Muc55B-IR line is large enough to increase metabolic stores and thus slow wastage in flies with the mucin 55B knockdown.

Evidence to suggest mucin 55B is regulated by the JNK pathway

After confirming that mucin 55B impacted lifespan and the activation of the insulin signalling pathway, it was key we found which pathway the gene is regulated by. Here we give evidence that mucin 55B is likely regulated through the JNK pathway. Overexpression of HepCA showed a clear suppression of mucin 55B expression in the gut. This link to the JNK pathway aligns with the theory that reduced expression of the stress response pathways can cause a downward spiral in terms of health (Onyango, 2018). In humans, changes in the expression of the JNK pathway can result in inflammatory skin disorders and cancer (Sabapathy, 2012). The human mucin gene,

46

muc5AC, has also been shown regulated by the JNK signalling pathway (Podder et al., 2014), which matches these findings. Unusually the suppression of basket did not cause any increase in the expression of Muc55B. However, we did not investigate the effectiveness of the intervention. In order to further test the intervention used in this study, we could use expression expression levels of the gene encoding the protein, puc. This is because it is already known how this protein is affected with changes in basket and hep expression so we would be able to see whether the JNK pathway has been sufficiently supressed.

Conclusion

This study has given strong indications that mucin 55B is a key factor in the maintenance of intestinal barrier function. Although certain experimentation did not work as planned, it demonstrated that the knockdown of mucin 55B results in a lower number of smurf flies and an extension of lifespan. This project therefore lays the groundwork for future studies to take place and hopefully be used to impact ageing on a larger scale in humans.

References

Akasaka-Manya, K. and Manya, H. (2020) The role of APP O-glycosylation in Alzheimer's disease. *Biomolecules*, 10(11), 1569.

Alessi, D.R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P. and Hemmings, B.A. (1996) Mechanism of activation of protein kinase B by insulin and IGF-1. *The EMBO journal*. 15(23), 6541-6551.

Alzheimer Disease International (2022) Dementia statistics. [Online] Available at: <https://www.alzint.org/about/dementia-facts-figures/dementia-statistics/> [Accessed October 2022]

Alzheimer's UK (2014) Dementia UK report. [Online] Available at: <https://www.alzheimers.org.uk/about-us/policy-and-influencing/dementia-uk-report> [Accessed October 2022]

Annaert, P., Brouwers, J., Bijnens, A., Lammert, F., Tack, J. and Augustijns, P. (2010) Ex vivo permeability experiments in excised rat intestinal tissue and in vitro solubility measurements in aspirated human intestinal fluids support age dependent oral drug absorption. *European journal of pharmacology*, 39, 15-22.

Apidianakis, Y. and Rahme, L.G. (2011) Drosophila melanogaster as a model for human intestinal infection and pathology. *Disease models and mechanisms*, 4(1), 21-30.

Arias-Rojas, A. and Iatsenko, I. (2022) The role of microbiota in Drosophila melanogaster aging. *Frontiers in aging*, 909509.

Badinloo, M., Nguyen, E., Suh, W., Alzahrani, F., Castellanos, J., Klichko, V.I., Orr, W.C. and Radyuk, S.N. (2018) Overexpression of the antimicrobial peptide contributes to ageing through cytotoxic effects in Drosophila tissues. *Archives of insect biochemistry and physiology*. 98(4), e21464.

Baeuerle, P.A. and Baltimore, D. (1996) NF-kappa B: ten years after. *Cell*, 87(1), 13-20.

Bansil, R. and Turner, B.S. (2006) Mucin structure, aggregation, physiological functions and biomedical applications. *Current Opinion in Colloid and Interface Science*, 11(2-3), 164-170.

Baril, P., Martin-Duque, P. and Vassaux, G. (2010) Visualization of gene expression in the live subject using the Na/I symporter as a reporter gene: applications in biotherapy. *British Journal of Phramacology*, 159(4), 761-771.

Bartosch, S., Fite, A., Macfarlane, G.T. and McMurdo, M.E.T. (2004) Characterisation of bacterial communities in feces from healthy elderly volunteers and hospitalised elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microbiota. *Applied and environmental microbiology*, 70(6), 3575-3581.

Bitner, K., Shahrestani, P., Pardue, E. and Mueller, L.D. (2020) Predicting death by the loss of intestinal function. *PLOS ONE*, 15(4), e0230970.

Brummel, T., Ching, A., Seroude, L., Simon, A.F. and Benzer, S. (2004) *Drosophila* lifespan enhancement by exogenous bacteria. *PNAS*, 101(35), 12974-12979.

Brummel, T., Ching, A., Seroude, L., Simon, A.F. and Benzer, S. (2004) Drosophila lifespan enhancement by exogenous bacteria. *PNAS*, 101(35), 12974-12979.

Bruunsgaard, H. and Pedersen, B.K. (2000) Effects of exercise on the immune system in the elderly population. *Immunology and cell biology*, 78(5), 523-531.

Buchon, N., Broderick, N.A., Poidevin, M., Prandervand, S. and Lemaitre, B. (2009) *Drosophila* intestinal response to bacterial infection: activation of host defense and stem cell proliferation. *Cell host and microbe*, 5(2), 200-211.

Burchell, J.M., Beatson, R., Graham, R., Taylor-Papadimitriou, J. and Tajadura-Ortega, V. (2018) O-linked mucin-type glycosylation in breat cancer. *Biochemical Society transactions*, 46(4), 779-788.

Castle, W.E. (1906) Inbreeding, cross-breeding and sterility in Drosophila. *Science*, 23(578), 153.

Catterson, J.H., Khericha, M., Dyson, M.C., Vincent, A.J., Callard, R., Haveron, S.M., Rajasingam, A., Ahmad, M. and Partridge, L. (2018) Short term, intermittent fasting induces long lasting gut health and TOR dependent lifespan extension. *Current biology*, 28(11), 1714-1724.

Chakrabarti, S., Dudzic, J.P., Li, X., Collas, E.J., Boquete, J-P. and Lemaitre, B. (2016) Remote control of intestinal stem cell activity by haemocytes in Drosophila. *PLOS Genetics*, 12(5), e1006089.

Chambers, M.C., Jacobson, E., Khalil, S. and Lazarro, B.P. (2019) Consequences of chronic bacterial infection in Drosopila melanogaster. *PLOS ONE*, 14(10), e0224440.

Chandra, M., Liniger, M., Tetley, L., Roditi. I. and Barry, J.D. (2004) TsetseEP, a gut protein from the tsetse *Glossina morsitans*, is related to a major surface glycoprotein of trypanosomes transmitted by the fly and to the products of a *Drosophila* gene family. *Insect biochemistry and molecular biology*, 34(11), 1163-1173.

Clark, R.I. and Walker, D.W. (2017) Role of gut microbiota in ageing-related health decline: insights from invertebrate models. *Cellular and molecular life sciences*, 75(1), 93-101.

Clark, R.I., Salazar, A., Yamada, R., Fitz-Gibbon, S., Morselli, M., Alcaraz, J., Rana, A., Rera, M., Pellegrini, M., Ja, W.W. and Walker, D.W. (2015) Distinct shift in microbiota composition during *drosophila* aging impair intestinal function and drive mortality. *Cell Reports*, 12(10), 1656-1667.

Crapser, J., Ritzel, R., Verma, R., Venna, V.R., Liu, F., Chauhan, A., Koellhoffer, E., Patel, A., Ricker, A., Maas, K., Graf, J. and McCullough, L.D. (2016) Ischemic stroke

induces gut permeability and enhances bacterial translocation leading to sepsis in aged mice. *Aging*, 8(5), 1049-1063.

De Sousa, H.S.P. and Fiocchi, C. (2016) Immunopathogenesis of IBD: current state of the art. *Nature reviews. Gastroenterology and hepatology*, 13(1), 13-27.

Denny, P.A., Klauser, D.K., Villa, B.J., Hong, S.H. and Denny, P.C. (1991) The effect of ageing on mucin contents in mouse submandibular glands. *Archives of Oral Biology*, 36(7), 477-481.

Derrien, M., van Passel, M.W., van de Bovenkamp, J.H., Schipper, R.G., de Vos, W.M. and Dekker, J. (2010) Mucin-bacterial interactions in the human oral cavity and digestive tract. *Gut microbes*, 1(4), 254-268.

Dhanisha, S.S., Guruvayoorappan, C., Drishya, S. and Abeesh, P. (2018) Mucins: Structural diversity, biosynthesis, its role in pathogenesis and as possible therapeutic targets. *Critical Reviews in Oncology/Hematology*, 122, 98-122.

Dionne, M. (2014) Immune-Metabolic interaction in Drosophila. *Fly (Austin)*, 8(2), 75-79.

Dionne, M.S., Pham, L.N., Shirasu-Hiza, M. and Schneider, D.S. (2006) Akt and FOXO dysregulation contribute to infection-induced wasting in Drosophila. *Current biology*, 16(20), 1977-1985.

Elderman, M., Sovran, B., Hugenholtz, F., Graversen, K., Huijskes, M., Houtsma, E., Belzer, C., Boekschoten, M., de Vos, P., Dekker, J., Wells, J. and Faas, M. (2017) The effect of age on the intestinal mucus thickness, microbiota composition and immunity in relation to sex in mice. *PLOS ONE*, 12(9), e0184274.

Engl, T., Michalkova, V., Weiss, B.L., Uzel, G.D., Takac, P., Miller, W.J., Abd-Alla, A.M.M., Aksoy, S. and Kaltenpoth, M. (2018) Effect of antibiotic treatment and gamma-irradiation on cuticular hydrocarbon profiles and mate choice in tsetse flies (Glossina m. morsitans). *BMC Microbiology*, 18, 145.

Etienne-Mesmim, L., Chaissaing, B., Desvaux, M., Paepe, K.D., Gresse, R., Sauvaitre, T., Forano, E., Van de Wiele, T., Schuller, S., Juge, N. Blanquet-Diot, S. (2019) Experimental models to study intestinal microbes-mucus interactions in health and disease. *FEMS microbiology reviews*, 43(5), 457-489.

Fasano, A. (2020) all disease begins in the (leaky) gut: role of zonulin-mediated gut permeability in the pathogenesis of some chronic inflammatory diseases. *F1000 Research*. 9.

Franzka, P., Kruger, L., Schurig, M.K., Olecka, M., Hoffman, S., Blanchard, V. and Hubner, C.A. (2021) Altered glycosylation in the aging heart. *Molecular Biosciences*, 8, 673044.

Gan, T., Fan, L., Zhao, L., Misra, M., Liu, M., Zhang, M. and Su, Y. (2021) JNK signaling in Drosophila aging and longevity. *Int J Mol Sci*, 22(17), 9649.

Giannakou, M.E., Goss, M., Junger, M.A., Hafen, E., Leevers, S.J. and Partridge, L. (2004) Long lived Drosophila with overexpressed dFOXO in adult fat body. *Science*, 305(5682), 361.

Grondin, J.A., Kwon, Y.H., Far, P.M., Haq, S. and Khan, W.I. (2020) Mucins in intestinal mucosal defense and inflammation: learning from clinical and experimental studies. *Frontiers in Immunology*, 11, 2054.

Guarner, F. (2015) The gut microbiome: What do we know?. *Clinical Liver Disease*, 5(4), 86-90.

Guo, X., Pace, R.G., Stonebraker, J.R. Commander, C.W., Dang, A.T. and Drumm, M.L. (2014) Mucin variable number tandem repeat polymorphisms and severity of cystic fibrosis lung disease: significant association with MUC5AC. *PLoS ONE*, 6(10), e25452.

Halpern, M.E., Rhee, J., Goll, M.G., Akitake, C.M., Parsons, M. and Leach, S.D. (2008) Gal4/ UAS transgenic tools and their application to zebrafish. *Zebrafish*, 5(2), 97-110.

Han, Z.S., Enslen, H., Hu, X., Meng, X., Wu, I., Tamera, B., Davis, R.J. and Ip, Y.T. (1998) A conserved p38 mitogen-activated protein kinase pathway regulates Drosophila immunity gene expression. *Molecular and cell biology*, 18(6), 3527-3539.

Hanson, M.A., Dostalova, A., Ceroni, C., Poidevin, M., Kondo, S. and Lemaitre, B. (2019) Synergy and remarkable specificity of antimicrobial peptides in vivo using a systematic knockout approach. *Elife*, 8, e44341.

Hansson, G.C. (2020) Mucins and the microbiome. *Annual Review of Biochemistry*, 89, 769-793.

Heintz, C. and Mair, W. (2014) You are what you host: microbiome modulation of the aging process. *Cell*, 156(3), 408-411.

Hollander, D. and Tarnawski, A. (1989). *Gastric Cytoprotection*. Boston, MA: Springer US.

Hollingsworth, M.A. and Swanson, B.J. (2004) Mucins in cancer: protection and control of the cell surface. *Nature reviews, Cancer*, 4(1), 45-60.

Hotamisligil, G.S. (2006) Inflammation and metabolic disorders. *Nature*, 444, 860-867.

Iatsenko, I., Boquete, J. and Lemaitre, B. (2018) Microbiota-derived lactate activates production of reactive oxygen species by the intestinal NADPH oxidase Nox and shortens Drosophila lifespan. *Immunity*, 49(5), 929-942.

Ikeya, T., Broughton, S., Alic, N., Grandison, R., Partridge, L. (2009). The endosymbiont Wolbachia increases insulin/IGF-like signalling in Drosophila. *Proc. Biol. Sci.,* 276(1674), 3799-3807.

Imler, J.L. and Bulet, P. (2005) Antimicrobial peptides in Drosophila: structures, activities and gene regulation. *Chem Immuno Allergy*, 86, 1-21.

Johansson, M.E.V. and Hansson, G.C. (2016) Immunological aspects of intestinal mucus and mucins. *Nature reviews, Immunology*. 16(10), 639-649.

Johansson, M.E.V., Gustafsson, J.K., Holmen-Larsson, J., Jabbar, K.S., Xia, L., Xu, H., Ghishan, F.K., Carvalho, F.A., Gewirtz, A.T., Sjovall, H. and Hansson, G.C. (2014) Bacteria penetrate the normally impenetrable inner colon mucus layer in both murine colitis models and patients with ulcerative colitis. *Gut*, 63(2), 281-291.

Johansson, M.E.V., Larsson, J.M.H. and Hansson, G.C. (2011) The two mucus layers of colon of organised by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions. *PNAS USA*, 108, 4659-4665.

Johansson, M.E.V., Sjovall, H. and Hansson, G.C. (2013) The gastrointestinal mucus system in health and disease. *Nature reviews, Gastroenterology and hepetology*, 10(6), 352-361.

Kambris, Z., Hoffman, J.A., Imler, J.L., Capovilla, M. (2002). Tissue and stage-specific expression of the Tolls in Drosophila embryos. *Gene Expr. Patterns,* 2(3-4), 311-317.

Kameyama, A., Tin, W.W.T., Nishijima, R. And Yamakoshi, K. (2021) Alteration of mucins in the submandibular gland during ageing in mice. *Archives of Oral Biology*, 121, 104967.

Karpowicz, P., Zhang, Y., Hogenesch, J.B., Emery, P. and Perrimon, N. (2013) The circadian clock gates the intestinal stem cell regenerative state. *Cell reports*, 3(4), 996-1004.

Kim, K., Lane, E.A., Saftien, A., Wang, H., Xu, Y., Wirtz-Peitz, F. and Perrimon, N. (2020) Drosophila as a model for studying cystic fibrosis pathophysiology of the gastrointestinal system. *PNAS USA*, 117(19), 10357-10367.

Kim, S. and Jazwinski, S.M. (2019) The gut microbiota and healthy aging. *Gerontology*, 64(6), 513-520.

Kleino, A. and Silverman, N. (2014) The Drosophila IMD pathway in the activation of the humoral immune response. *Developmental and comparative immunology*, 42(1), 25-35.

Kounatidis, I., Chtarbanova, S., Cao, Y., Hayne, M., Jayanth, D., Ganetzky, B. and Ligoxygakis, P. (2017) NF-kB immunity in the brain determines fly lifespan in healthy ageing and age-related neurodegeneration. *Cell reports*, 19(4), 836-848.

Kumar, S., Cruz, E., Joshi, S., Patel, A., Jahan, R., Batra, S.K. and Jain, M. (2017) Genetic variants of mucins: unexplored conundrum. *Carcinogenesis*, 38(7), 671-679.

Kuraishi, T., Hori, A. and Kurata, S. (2013) Host-microbe interactions in the gut of *Drosophila melanogaster*, *Frontal Physiology*, 4, 375.

Lang, T., Hansson, G.C. and Samuelsson, T. (2007) Gel-forming mucins appeared early in metazoan evolution. *PNAS*, 104(41), 16209-16214.

Lee, H-Y., Lee, S-H., Lee, J-H., Lee, W-J. and Min, K-J. The role of commensal microbes in the lifespan of Drosophila melanogaster. *Aging (Albany NY)*, 11(13), 4611-4640.

Lemaitre, B. and Hoffman, J. (2007) The host defense of *Drosophila melanogaster*. *Annual review of Immunology*, 25, 697-743.

Lemaitre, B. and Miguel-Aliaga, I. (2013) The digestive tract of *Drosophila melanogaster*. *Annual review of genetics*, 47, 377-404.

Lemaitre, B., Reichhart, J. and Hoffman, J.A. (1997) Drosophila host defense: Differential induction of antimicrobial peptide genes after infection by various classes of microorganism. *PNAS*, 94(26), 14614-14619.

Linden, S.K., Sutton, P., Karlsson, N.G. and McGuckin, M.A. (2008) Mucins in the mucosal barrier to infection. *Mucosal Immunology*, 1(3), 183-197.

Linford, N.J., Bilgir, C., Ro, J. and Pletcher, S.D. (2013) Measurement of lifespan in Drosophila melanogaster. *Journal of visualized experiments*, 71, 50068.

Loeb, J. and Northrop, J.H. (1916) Is there a temperature coefficient for the duration of life?. *PNAS USA*, 2(8), 456-457.

Magwire, M.M., Yamamoto, A., Carbone, M.A., Roshina, N.V., Symonenko, A.V., Pasyukova, E.G., Morozova, T.V., Mackay, T.F. (2010). Quantitative and molecular genetic analyses of mutations increasing Drosophila life span. *PLoS Genet.*, 6(7), e1001037.

Man, A.L., Bertelli, E., Rentini, S., Regoli, M., Briars, G., Marini, M., Watson, A.J.M. and Nicoletti, C. (2015) Age associated modifications of intestinal permeability and innate immunity in human small intestine. *Clinical science (London)*, 129(7), 515-527.

Maynard, C. and Weinkove, D. (2018) The gut microbiota and ageing. *Sub-cellular biochemistry*, 90, 351-371.

Maynard, C. and Weinkove, D. (2020) Bacteria increase host micro nutrient availability: mechanisms revealed by studies in C. elegans. *Genes and nutrition*, 15(1), 4.

Menzel, F., Schmitt, T. and Blaimer, B.B. (2017) The evolution of a complex trait: cuticular hydrocarbons in ants evolve independent from phylogenetic constraints. *Journal of evolutionary biology*, 30(7), 1372-1385.

Michielan, A. and D'Inca, Renata. (2015) Intestinal permeability in inflammatory bowel disease: Pathogenesis, clinical evaluation and therapy of leaky gut. *Mediators of inflammation*, 628157.

Miguel-Aliaga, I., Jasper, H. and Lemaitre, B. (2018) Anatomy and physiology of the digestive tract of Drosophila melanogaster. *Genetics*, 210(2), 357-396.

Moullan, N., Mouchiroud, L., Wang, X., Ryu, D., Williams, E.G., Mottis, A., Jovaisaite, V., Frochaux, M.V., Quiros, P.M., Deplancke, B., Houtkooper, R.H. and Auwerx, J.

(2015) Tetracyclines Disturb Mitochondrial Function across Eukaryotic Models: A Call for Caution in Biomedical Research. *Cell Reports*, 10(10), 1681-1691.

Myllymaki, H. and Ramet, M. (2014) JAK/STAT pathway in Drosophila immunity. *Scandinavian Journal of Immunology*, 79(6), 377-385.

Neu, A. T., Allen, E. E. and Roy, K. (2021) Defining and quantifying the core microbiome: Challenges and prospects. *PNAS*, 118(51), e2104429118.

Nielsen, H., Engelbrecht, J., Brunak, S. and von Heijne, G. (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein engineering*, 10, 1-6.

Onyango, A.N. (2018) Cellular stresses and stress responses in the pathogenesis of insulin resistance. *Oxidative medicine and cellular longevity*, 2018, 4321714.

Osterwalder, T., Yoon, K.S., White, B.H. and Keshishian, H. (2001) A conditional tissue-specific transgene expression system using inducible GAL4. *PNAS USA*, 98(22), 12596-12601.

Pajares, M., Rojo, A.I., Manda, G, Bosca, L. and Cuadrado, A. (2020) Inflammation in Parkinson's Disease: Mechanisms and Therapeutic Implications. *Cells*, 9(7), 1687.

Paone, P. and Cani, P.D. (2020) Mucus barrier, mucins and gut microbiota: the expected slimy partners?. *Gut*, 69(12), 2232-2243.

Paredes, J.C., Welchman, D.P., Poidevin, M. and Lemaitre, B. (2011). Negative Regulation by Amidase PGRPs Shapes the Drosophila Antibacterial Response and Protects the Fly from Innocuous Infection. *Immunity*, 35(5), 770-779.

Partridge, L., Alic, N., Bjedov, I. and Piper, M.D.W. (2011) Ageing in Drosophila: the role of the insulin/Igf and TOR signalling network. *Experimental gerontology*, 46(5), 376-381.

Pelaseyed, T. and Hansson, G.C. (2020) Membrane mucins of the intestine at a glance. *Jounral of cell science*, 133(5), jcs240929.

Perez-Vilar, J. and Hill, R.L. (1999) The structure and assembly of secreted mucins. *Journal of biological chemistry*, 274(45), 31751-31754.

Perkins, L.A., Holderbaum, L., Tao, R., Hu, Y., Sopko, R., McCall, K., Yang-Zhou, D., Flockhart, I., Binari, R., Shim, H.S., Miller, A., Housden, A., Foos, M., Randkelv, S., Kelley, C., Namgyal, P., Villalta, C., Liu, L.P., Jiang, X., Huan-Huan, Q., Wang, X., Fujiyama, A., Toyoda, A., Ayers, K., Blum, A., Czech, B., Neumuller, R., Yan, D., Cavallaro, A., Hibbard, K., Hall, D., Cooley, L., Hannon, G.J., Lehmann, R., Parks, A., Mohr, S.E., Ueda, R., Kondo, S., Ni, J.Q. and Perrimon, N. (2015). The Transgenic RNAi Project at Harvard Medical School: Resources and Validation. *Genetics*, 201(3), 843-852.

Petkau, T.L. and Leavitt, B.R. (2014) Progranulin in neurodegenerative disease. *Trends in neuroscience*, 37(7), 388-398.

Piper, M.D.W. and Partridge, L. (2018) Drosophila as a model for ageing. *Biochimica et biophysica acta. Molecular basis of disease*, 1864(9), 2707-2717.

Podder, B., Song, K.S., Song, H. And Kim, Y. (2014) Cytoprotective effect of Kaempferol on paraquat-exposed BEAS-2B cells via modulating expression of MUC5B. *Biological and pharmaceutical bulletin*, 37(9), 1486-1494.

Puig, O., Marr, M.T., Ruhf, M.L. and Tijan, R. (2003) Control of cell number by Drosophila FOXO: downstream and feedback regulation of the insulin receptor pathway. *Genes and development*, 17, 2006-2020.

Raimondi, S., Musmeci, E., Candeliere, F., Amaretti, A. and Rossi, M. (2021) Identification of mucin degraders of the human gut microbiota. *Scientific Reports*, 11, 11094.

Ren, C., Webster, P., Finkel, S.E. and Tower, J. (2007) Increased internal and external bacterial load during *Drosophila* aging without life-span trade-off. *Cell metabolism*, 6(2), 91-93.

Rera, M., Clark, R. I., and Walker, D. W. (2012) Intestinal barrier dysfunction links metabolic and inflammatory markers of aging to death in *Drosophila*. *PNAS*, 109(52), 21528-21533.

Resnik-Docampo, M., Koehler, C.L., Clark, R.I., Schinaman, J.M., Sauer, V., Wong, D.M., Lewis, S., D'Alterio, C., Walker, D.W. and Jones, D.L. (2017) Tricellular junctions regulate intestinal stem cell behaviour to maintain homeostasis. *Nature cell biology*, 19(1), 52-59.

Ridley, E.V., Wong, A.C.N., Westmiller, S. and Douglas, A.E. (2012) Impact of the resident microbiota on the nutritional phenotype of Drosophila melanogaster. *PLOS ONE*, 7(5), e36765.

Riedel, S., Pheiffer, C., Johnson, R., Louw, J. and Muller, C.J.F. (2021) Intestinal barrier function and immune homeostasis are missing links in obesity and type 2 diabetes development. *Frontiers of endocrinology*, 12, 833544.

Rubin, D.C. (2007) Intestinal morphogenesis. *Current Opinions in Gastroenterology*, 23, 111-114.

Sabapathy, K. (2012) Role of the JNK pathway in human diseases. *Progress in molecular biology and translational science*, 106, 145-169.

Salem, F., Kindt, N., Marchesi, J.R., Netter, P., Lopez, A., Kokten, T., Danese, S., Jouzeau, J., Peyrin-Biroulet, L. and Moulin, D. (2019) Gut microbiome in chronic rheumatic and inflammatory bowel diseases: similarities and differences. *United European Gastroenterology Journal*, 7(8), 1008-1032.

Salim, S.Y. and Soderholm, J.D. (2011) Importance of disrupted intestinal barrier in inflammatory bowel disease. *Inflammatory bowel diseases*, 17(1), 362-381.

Saltzman, J.R., Kowdley, K.V., Perrone, G. and Russell, R.M. (1995) Changes in small-intestine permeability with aging. *Journal of Am Geriatr Society*, 43, 160-164.

Sanz, A., Fernandez-Ayala, D.J.M., Stefanatos, R.K. and Jacobs, H.T. (2010) Mitochondrial ROS production correlates with, but does not directly regulate lifespan in Drosophila. *Ageing*, 2(4), 200-223.

Schneider, H., Pelaseyed, T., Svensson, F. and Johansson, M.E.V. (2018) Study of mucin turnover in the small intestine by *in vivo* labelling. *Scientific Reports*, 8, 5760.

Scialo, F., Sriram, A., Stefanatos, R. and Sanz, A. (2016) Practical recommendations for the use of the Geneswitch gal4 system to knockdown genes in Drosophila melanogaster. *PLOS ONE*, 11(8), e0161817.

Sciambra, N. and Chtarbanova, S. (2021) The impact of age on response to infection in Drosophila. *Microorganisms*, 9(5), 958.

Selman, M., Lopez-Otin, C. and Pardo, A. (2016) Age-driven developmental drift in the pathogenesis of idiopathic pulmonary fibrosis. *European Respiratory Journal*, 48(2), 538-552.

Shaposhnikov, M.V., Guvatova, Z.G., Zemskaya, N.V., Koval, L.A., Schegoleva, E.V., Gorbunova, A.A., Golubev, D.A., Pakshina, N.R., Ulyasheva, N.S., Solovev, I.A., Bobrovskikh, M.A., Gruntenko, N.E., Menshanov, P.N., Krasnov, G.S, Kudryavseva, A.V. and Moskalev, A.A. (2022) Molecular mechanisms of exceptiona lifespan increase of Drosophila melanogaster with different genotypes after combinations of pro longevity interventions. *Communications biology*, 5, 566.

Sheffield, L., Sciabra, N., Evans, A., Hagedorn, E., Goltz, C., Delfield, M., Kuhns, H., Fierst and Chtarbanova, S. (2021) Age-dependent impairment of disease tolerance is associated with a robust transcriptional response following RNA virus infection in Drosophila. *G3*, 11(7).

Silverman, H.S., Sutton-Smith, M., McDermott, K. and Heal, P. (2003) The contribution of tandem repeat number to the O-glycosylation of mucins. *Glycobiology*, 13(4), 265-277.

Sommer, F. and Backhed, F. (2013) The gut microbiota – masters of host development and physiology. *Nature reviews, Microbiology*, 11, 227-238.

Sovran, B., Hugenholtz, F., Elderman, M., van Beek, A.A., Graversen, K., Huijskes, M., Boekschoten, M.V., Savelkoul, H.F.J., de Vos, P., Dekker, J. and Wells, J.M. (2019) Age-associated impairment of the mucus barrier function is associated with profound changes in microbiota and immunity. *Scientific reports*, 9, 1437.

Syed, Z.A., Hard, T., Uv, A. and van Dijk-Hard, I.F. (2008) A potential role for *Drosophila* mucins in development and physiology. *PLoS ONE*, 3(8), e3041.

Tanabe, K., Itoh, M. and Tonoki, A. (2017) Age-Related Changes in Insulin-like Signaling Lead to Intermediate-Term Memory Impairment in *Drosophila. Cell reports*, 18(7), 1598-1605.

Tanji, T., Hu, X., Weber, A.N.R., Ip, Y.T. (2007). Toll and IMD pathways synergistically activate an innate immune response in Drosophila melanogaster. *Mol. Cell. Biol.*, 27(12), 4578-4588.

Taormina, G., Ferrante, F., Vieni, S., Grassi, N., Russo, A. and Mirisola, M.G. (2019) Longevity: Lesson from model organisms. *Genes (Basel)*, 10(7), 518.

Tatar, M., Kopelman, A., Epstein, D., Tu, M.P., Yin, C.M. and Garofalo, R.S. (2001) A mutant Drosophila insulin receptor homolog that extends lifespan and impairs neuroendocrine function. *Science*, 292(5514), 107-110.

Tauszig, S., Jouanguy, E., Hoffmann, J.A. and Imler, J.L. (2000). From the cover: toll-related receptors and the control of antimicrobial peptide expression in Drosophila. *PNAS USA*, 97(19), 10520-10525.

Tsurumi, A. and Li, W.X. (2020) Aging mechanisms – Aperspective mostly from *Drosophila*. *Advanced Genentics,* 1(1), e10026.

Turnbaugh, P.J., Ley, R.E., Hamady, M., Fraser-Liggett, C.M., Knight, R. and Gordon, J.I. (2007) The human microbiome project. *Nature*, 449, 804-810.

US Department of Health and Human Services (2020) Demographics and human development. [Online] Available at: <https://www.dni.gov/index.php/gt2040-home/gt2040-structural-forces/demographics-and-human-development> [Accessed September 2022]

Van Beek, A.A., Sovran, B., Hugenholtz, F., Meijer, B., Hoogerland, J.A., Mihailova, V., van der Ploeg, C., Belzer, C., Boekschoten, M.V., Hoeijmakers, J.H.J., Vermeij, W.P., de Vos, P., Wells, J.M., Leenen, P.J.M., Nicoletti, C., Hendriks. R.W. and Savelkoul. H.F.J. (2016) Supplementation with Lactobacillus plantarum WCFS1 prevents decline of mucus barrier in colon of accelerated ageing Ercc1$^{-/\Delta 7}$ mice. *Frontiers in immunology*, 7, 408.

Verma, I.M., Stevenson, J.K., Schwarz, E.M., van Antwerp, D. and Miyamoto, S. (1995) Rel/NF-kB/IkB family: intimate tales of association and dissociation. *Genes and development*, 9, 2723-2735.

Zerofsky, M., Harel, E., Silverman, N. and Tatar, M. (2005) Ageing of the innate immune response in Drosophila melanogaster. *Ageing cell*, 4(2), 103-108.

Zhu, X., Tian, G., Quan, J., He, P. and Liu, J. (2019) Effects of miR-340 overexpression and knockdown on the proliferation and metastasis of NSCLC cell lines. *International journal of molecular medicine*, 44(2), 643-651.