Do pseudouridine synthases regulate Drosophila intestinal stem cells and homeostasis?

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Abstract

Certain genes may increase the risk of developing cancer. Pseudouridine synthase (PUS) genes have been linked to increased risk of cancer developing in humans and mice in previous studies, as it can impact the structure of RNA molecules which are involved in regulation of stem cell replication and the immune system. This study investigates the function of PUS genes in the gut of *Drosophila melanogaster*, or the common fruit fly, which are often used to model the human gut due to shared features such as dynamic cell turnover and compartmentalisation. As a result, the expression of PUS in specific gut cells can then be manipulated to assess the effects on stem cells and tissue homeostasis. This is done through gene knockdown hrough inducible RNAi knockdown. Results suggest an increase in the proportion of stem cells in the PUS7 knockdown cells, which could mean that the PUS genes are directly involved in the regulation of epithelial stem cells, and subsequently involved in the development of cancer within the digestive system. These findings highlight the need for further research into PUS genes in the gut, as well as further studies to determine if this model of how they function in drosophila is true to their function in humans, as it could have significant implications for human cancer risk.

Key words: epithelial stem cells, pseudouridine synthase, genes, RNA, *Drosophila melanogaster*, cancer

Introduction

Stem cells are unspecialised cells with the potential to differentiate and give rise to other specialised types of cells. They can self-replicate indefinitely to create more stem cells while remaining undifferentiated, or they can specialise to form new tissues, or facilitate the repair of damaged specialised tissues. Consequently, stem cells are vital to tissue growth and repair. Epithelial stem cells line the surface of the gut, as the rate of cell turnover in the gut is high with the epithelial cells which line the surface of the gut being replaced every 4-5 days (van der Flier and Clevers, 2009). Overproduction of new cells leads to cancer, meaning the regulation of epithelial stem cells must be tightly controlled by local microenvironment or niche, which influences their gene expression.

Gene expression can be regulated at multiple levels, such as during the transcription of DNA into RNA and the translation of RNA into proteins. Additionally, this can be through the modification of RNA molecules, which affects their stability and rates of translation. Pseudouridine is the most common modified nucleoside, consisting of just the nitrogenous base and the sugar molecule, across all three kingdoms of life: eukaryota, prokaryota, and archaea (McKenney, Rubio and Alfonzo, 2017). It is prevalent in ribosomal RNA (rRNA), transfer RNA (tRNA) and small nuclear RNA (snRNA) (Vandivier and Gregory, 2017). RNA modifications significantly impact biological processes involved in immune cells, including activation,

development and migration, as well as stem cell regulation. This means changes made to RNA are related to disease prevalence. Pseudouridine synthase genes convert specific uridines into pseudouridines. The presence of these genes has been linked to a higher incidence of cancer in mice and humans (Haruehanroengra et al., 2020), such as colorectal cancer as well as gastrointestinal diseases like Crohn's disease and ulcerative colitis (Rodell, Robalin and Martinez, 2024).

Drosophila melanogaster is commonly used as a model organism due to their short regeneration time, low cost, and small, well-characterised genome. Moreover, the drosophila gut can be used to model human diseases due to shared features such as how dynamic cell turnover occurs (Jiang and Edgar, 2012) and compartmentalisation of epithelia to perform specific localised functions. A large range of drosophila mutants are commercially available and specific genetic manipulation can be performed. Genes are also conserved between humans and drosophila, with homologous genes relating to evolution, development, and cell regulation (Tolwinski, 2017). This includes direct orthologs for PUS genes which are expressed in the gut and have not been studied there. Consequently, an in vivo model to study these genes is lacking so searching for effects that might validate this system as a model that would enable a lot of further work. As a result of all these factors, the functions of PUS genes can be investigated through knockdown in drosophila to aid in identifying their roles in human health too.

Methods

Fly stocks and crosses

To set up crosses, virgin females were crossed with males of the desired genotype to produce the desired offspring. All adults were removed three to four days after introduction so there could be no confusion if progeny began to hatch. Stocks were maintained at 25°C and 60% humidity with a twelve-hour day/night cycle. Stocks were flipped every 4 days into new bottles. Females of the 5961GS drug-inducible driver line were crossed with male GD46747s, GD26030s, GD13479s, and W1118s. Each of the male lines were PUS7 RNAi. The other female lines used in the crosses were tubG4ts, which is a ubiquitous temperature inducible driver affecting all cells, and esgtsGFP, which is stem cell specific. TubG4ts females were crossed with male W1118s, GD46747s, and GD26030s. EsgtsGFP females were crossed with W1118 males and GD26030 males.

Food and temperature

For each of the 5961GS crosses, the progeny were aged for seven days at 25^{o}C before the female progeny were collected, which allowed for time for them to mature and for all the females to mate, which increases the size of the gut. The females collected were then split into two equal groups, one being placed on RU+ food - meaning it contains a drug which activates the knockdown of the gene - and the other group placed onto RU-, or control, food for another

seven days before dissection, being flipped into vials with 4mls of fresh food every two days. This meant each individual cross was independent and had a self-contained control group to compare against.
In the tubG4ts and esgtsGFP crosses, the knockdown in the progeny was temperature activated, meaning progeny were kept at 18^{o}C for seven days to mature and mate until being placed at 29^{o}C for seven days before dissection, again being flipped onto fresh food every two days. The progeny produced by crosses with W1118s acted as the controls in this knockdown.

Collecting and dissecting

A stereo microscope with an attached pad which diffuses CO_2 is used for working with live flies. The CO2 anaesthetises the flies to keep them still for fly pushing and the stereo microscope uses light which does not harm the flies. The magnification can also be adjusted so that dissections can also be performed under the same microscope and the flies for dissection can be kept anaesthetised on the CO_2 pad.
Dissections are performed using forceps to remove the head and then the gut of the fly. This must be performed in liquid otherwise the gut will stick to the dissection surface. Therefore, the dissections are performed in wells filled with PBS buffer. The PBS is kept in an autoclaved bottle to prevent any microbes or spores which could affect the results, meaning to use the PBS, an aliquot must be prepared and then pipetted using sterile pipette tips.
Once dissected, the guts must be fixed in 4% paraformaldehyde (PFA). This must be added in the fume cupboard as it can cause serious irritation and is suspected to be carcinogenic. They are then covered with parafilm and foil to prevent evaporation and light exposure while being shaken on a rocker for thirty minutes. The guts are then washed three times before being blocked with blocking buffer and NGS on the rocker again for thirty minutes. Primary antibodies - which attach to specific proteins found in certain cells - can then be prepared in PBS + 0.5% Triton X-100. For the 5961GS and tubG4ts crosses, these primary antibodies were PH3 at a concentration of 1/500 and Prospero at 1/100. For esgtsGFP, the primary antibodies were GFP at 1/2000 and Prospero at 1/100. The primary antibodies were left on overnight while the samples were covered in parafilm and foil on a rocker in a cold room. The next day, the primary antibodies were removed and three washes with PBS where the samples were placed on the rocker for ten minutes in between were performed before corresponding secondary antibodies - which attach to the primary antibodies and label them with a fluorescent tag - in PBS + Triton X-100 were added and left covered on the rocker for two hours. DAPI, which stains nuclei, was then also prepared in PBS + Triton X-100 and added after the secondary antibodies were removed and the samples were placed, covered, back on the rocker for another ten minutes. Three final ten-minute PBS washes were then performed before the samples could be mounted in mounting medium onto slides.
The slides were then viewed and imaged under a confocal microscope, which uses lasers corresponding to the wavelengths of the antibodies used. The confocal microscope produces z stacks – a series of images taken through the 3D structure of the gut. The images produced were used to compare the cells in knocked down versus control guts; count the number of specific cell types found in the gut such as stem cells and enteroendocrine cells; and count

proliferating cells. This helps to determine the specific impact of these genes being knocked down on the gut.

Data analysis and statistics

The images taken using the confocal microscope must be processed to compare the cell types and counts present. A software called Fiji allows the layers within the images (z stacks) to be viewed. Colour settings can be applied so that the staining of nuclei and different cells can be seen and counted using the Cell Counter within Fiji. The number of and proportion of certain cell types can be counted and compared between the genotypes, which can provide information about rates of replication and active mitosis. If there was a significant difference in the rate of replication, it could suggest the genes had an impact on regulating replication. As well, the cell shapes and sizes could be compared to each other, again having implications about regulation of replication and cell structures.

Results

Control Knockdown

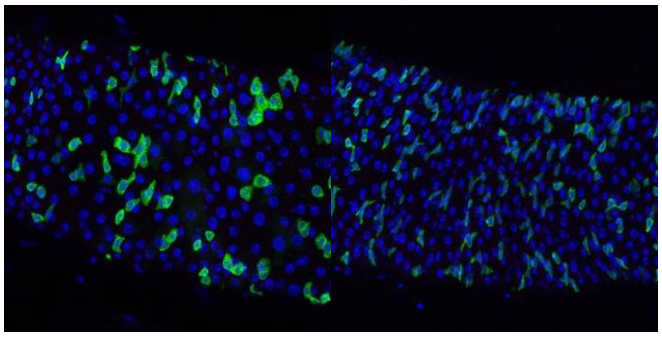


Fig. 1 a

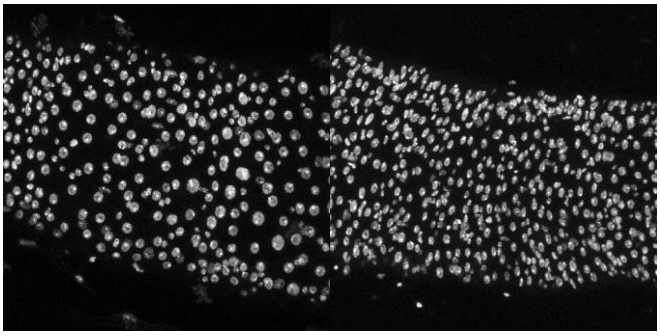


Fig. 1 b

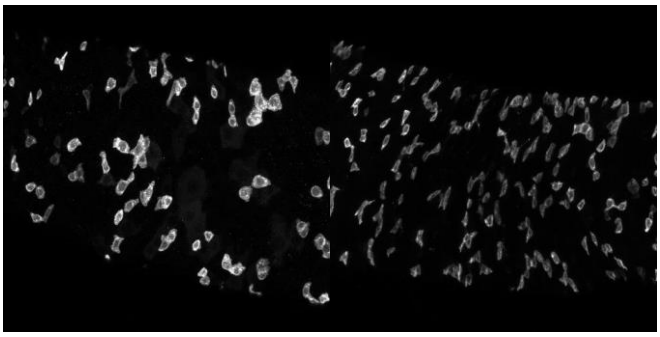
Fig. 1 c 

Figure 1 – **Representative projected Z stacks of the control and intestinal stem cell PUS7 knockdown posterior midguts showing different cell types within the gut.** Figure 1 a shows intestinal stem cells (green) and all nuclei (blue) in the control (left) versus in the knockdown (right). The isolated nuclei (Figure 1 b) and stem cells (Figure 1 c) are also shown in greyscale, allowing for comparison of cell count, size, and shape. Figures prepared by Dr David Doupé and Aaryn McDonald-Brown.

Table 1 – Numbers of DAPI, GFP, and Prospero-stained cells and the percentage of cells stained with GFP and Prospero in each gut from a single dissection session. Table prepared by Dr David Doupé and Aaryn McDonald-Brown.

Sample	DAPI	GFP	Prospero	% GFP	% Prospero
esgtsGFP x W1118 gut 1	328	86	12	26.2195122	3.6585366
esgtsGFP x W1118 gut 2	416	110	10	26.4423077	2.4038462
esgtsGFP x W1118 gut 3	140	37	6	26.4285714	4.2857143
esgtsGFP x W1118 gut 4	380	100	10	26.3157895	2.6315789
esgtsGFP x W1118 gut 5	372	89	24	23.9247312	6.4516129
esgtsGFP x GD26030 gut 1	505	190	1	37.6237624	0.1980198
esgtsGFP x GD26030 gut 2	299	118	22	39.4648829	7.3578595
esgtsGFP x GD26030 gut 3	424	108	0	25.4716981	0

esgtsGFP x GD26030 gut 4	211	71	12	33.6492891	5.6872038
esgtsGFP x GD26030 gut 5	386	103	23	26.6839378	5.9585492
esgtsGFP x GD26030 gut 6	324	110	25	33.9506173	7.7160494

DAPI labels nuclei giving the total number of cells in the sample, therefore the number of GFP-stained cells and Prospero-stained cells can then be used to calculate the proportion of the total number of cells they make up.

Table 2 – Averages of the percentages of GFP-stained cells and Prospero-stained cells for the two genotypes, esgtsGFP x W1118 and esgtsGFP x GD26030. Prepared by Dr David Doupé and Aaryn McDonald-Brown.

	% W1118 GFP	% W1118 Prospero	% GD26030 GFP	% GD26030 Prospero
Mean	25.8661824	3.8862578	32.8073646	4.4862803
Lower Quartile	26.2195122	2.6315789	28.4252756	1.5703158
Median	26.3157895	3.6585366	33.7999532	5.8228765
Upper Quartile	26.4285714	4.2857143	36.7054761	7.008032
Interquartile Range	0.2090592	1.6541354	8.2802005	5.4377162

The spread of data can be observed using the lower quartile, median, and upper quartile. The interquartile ranges for both GFP and Prospero-stained cells are much larger in the esgtsGFP x GD26030 group than in the esgtsGFP x W1118 group.

The means can be compared using a two-tailed t-test, given that the sample groups are normally distributed, independent of each other, and the sample size and variability are appropriate. The null hypothesis is that the means would be the same, and the alternative hypothesis is that the means will be significantly different, as a two-tailed test is being carried out to investigate whether the knockdown mean could be significantly greater than or less than the control. The percentage of GFP-stained cells in the knockdown group is significantly different to the percentage in the control group (two-tailed independent t-test, critical t value = 2.262, $p = 0.02552 < 0.05$). However, the means for the Prospero-stained cells are not significantly different between the two groups (two-tailed independent t-test, critical t value = 2.262, $p = 0.7330 > 0.05$).

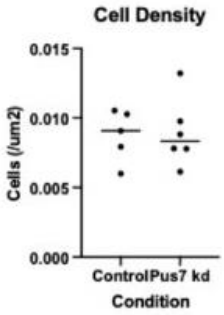


Figure 2 – **Cell density showing individual data points and mean (horizontal line) and SD (whiskers).** Cell density is not significantly different between the control and the PUS7 knockdown ($p>0.05$, two-tailed t-test). Figure prepared by Dr David Doupé.

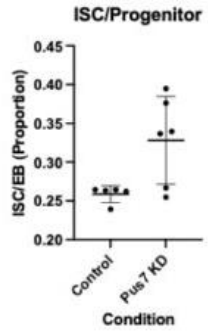


Figure 3 – **Intestinal stem cell proportion quantification showing individual data points and mean (horizontal line) and SD (whiskers).** Intestinal stem cell proportion is significantly different between the control and the PUS7 knockdown ($p<0.05$, two tailed t-test). Figure prepared by Dr David Doupé.

Discussion

Stem cells

When knockdown of PUS7 has occurred, the proportion of GFP-stained cells, which in this experiment are stem cells, increases compared to the control guts. Additionally, as can be seen in Figure 2 compared to Figure 1, there is a consistently a difference in the appearance of the stem cells between the control guts and the knockdown guts, although there are inconsistencies

in exactly how they differ, as seen in Figure 3. As a result, this is likely to have contributed to the disparity between the control group interquartile range and the knockdown group interquartile range, which was much larger, meaning much less consistent. The difference in the percentage of the stem cells in the gut they make up and their structures suggests that there is a link between PUS and regulation of the stem cells within the gut of drosophila.

Homeostasis

Stem cells have a role in homeostasis with the maintenance of the gut epithelial tissue; however, the enteroendocrine cells also contribute, with functions relating to hormonal secretion and signalling, gut motility, and digestion. The lack of significant difference in the mean percentage of Prospero-stained cells, or enteroendocrine cells, could suggest PUS7 does not control regulation in this capacity. However, as the knockdowns in esgtsGFP were stem cell specific, it could be that they do have effects on enteroendocrine cells that are not currently visible. This could also suggest that the knockdown does not affect the differentiation of stem cells into enteroendocrine cells.

Further Research

Data from the other genotypes (5961GS and tubG4ts) were limited by time and resources, as well as human error in the duration of the experiment, meaning that further repeats of these dissections could reveal further data. As well, more repeats of esgtsGFP with larger sample sizes could help to reduce the interquartile range of the knockdown group and give a clearer view of the typical impact of knocking down the gene on the stem cell percentage.

Furthermore, while this has provided evidence of an impact on the regulation of stem cells in *Drosophila melanogaster*, how much this would directly translate into an impact on human epithelial stem cells is not yet clear, meaning that research there could definitively support the evidence that there is a link between PUS and cancer in the digestive system.

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