University of Glasgow | College of Medical, Veterinary & Life Sciences

Head of College Scholars List Scheme

Summer Studentship 2019

Report Form

This report should be completed by the student with his/her project supervisor. It should summarise the work undertaken during the project and, once completed, should be sent by email to: jill.morrison@glasgow.ac.uk **within four weeks of the end of the studentship**.

1. Student

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2. Supervisor:

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3. Research Project Report

   3.1 Project Title: ***Drosophila* as a model to study cancer cachexia**

   3.2 Project Lay Summary:

Cachexia – a syndrome characterised by an ongoing loss of skeletal muscle – is implicated in most advanced-stage cancers, enhancing their severity and diminishing the patient quality of life. The causes of and mechanisms mediating cancer cachexia are poorly understood and therefore represent an important target for research. This project aims to elucidate the roles of JAK/STAT and insulin signalling pathways – often found to be dysregulated in cachexia – in muscle wasting, tumour burden and systemic immune responses in a *Drosophila* model of cancer cachexia. The results will contribute to the understanding of cachexia aetiology, impacting the design of anti-cachectic therapies.

3.3 Start Date: 16/05/2018 Finish Date: 23/08/2018

3.4 Original project aims and objectives:

Previous work in the laboratory allowed the establishment of a larval *Drosophila* model of cachexia involving the loss of skeletal muscle as a response to tumour burden. Further experiments identified upregulation of JAK/STAT signalling in the skeletal muscle of cachectic animals. We hypothesized that JAK/Stat signalling contributes to muscle wasting phenotype as in tumour bearing animals.

Aims of the study:

- To assess the effect of muscle-specific knocking down of *Stat* and *Domeless*, transcription factor and receptor of the JAK/Stat signalling pathway, respectively, in the skeletal muscle wasting.
- To assess the effect of muscle-specific knocking down of *Stat* and *Domeless*, on tumour size, tumour cell death.
- To characterise immune signature of cachectic tumour-bearing larvae.

3.5 Methodology:

Quantification of Muscle Wasting, Tumour Volume and Tumour Cell Death in muscle-JAK/STAT-inhibited Larvae

To assess the effects of JAK/STAT signalling suppression on muscle wasting, we generated cachectic dlg40.2 tumour-bearing larvae with additional hyperactivation of the Ras oncogene (*dlg40.2, Ras^{v12}*) and carrying a muscle-specific gene driver and inducible RNAi transgenes of the JAK/STAT transmembrane receptor Domeless or the transcription factor Stat (*dome^{IR}* and *stat^{IR}*, respectively). We have then characterised the effects of such knockdowns on tumour volume (TV), tumour cell death (TCD) and muscle wasting (MW) in our models. The characterisation involved the following techniques: dissections, fixations (in 4% formaldehyde) and washings of larval tissues (cuticles, wing disks); tissue immunostainings with appropriate primary antibodies followed by washes and fluorophore-labelled secondary antibodies; tissue mounting and imaging using a Zeiss 710 confocal microscope; image analysis using Zen and ImageJ followed by quantification of muscle surface area, TV and TCD using ImageJ and Volocity 3D image analysis software. The data was statistically analysed using GraphPad Prism software. *dome^{IR}* and *stat^{IR}* knockdown would be confirmed by RT-qPCR

for the JAK/STAT pathway transcriptional target Socs36E, and Ras activation would be confirmed through immunostaining with pErk1/2 antibodies. All fly lines were kept at 25ºC. For information on the constructs, please see the Appendix Constructs note.

Characterisation of the Immune Signature in Cachectic Larvae

A new construct was generated to characterise the immune signature after muscle wasting was initiated. Using this construct, we attempted to induce haemocyte/macrophage depletion or upregulation in cachectic tumour-burdened larvae. The appropriate crosses were set and larvae periodically analysed under the fluorescence microscope to assess construct effectiveness. However, due to a number of issues with this model, we were unable to characterise the immune signature (see Discussion).

3.6 Results:

Domeless and Stat RNAi Knockdown Worsens Muscle Wasting Phenotype and Alters Tumour Volumes, but Does Not Affect Cell Death

Muscle wasting in tumour bearing animals was observed as early as 7 days after egg deposition (AED), and multiple dissections were performed with larvae of different ages to visually inspect the effects and severity of muscle wasting, which was consistently visibly worse in $dome^{IR}$ and $stat^{IR}$ flies when compared with controls (data not shown). To confirm these results, we reset the experimental crosses and then dissected, stained and quantified the parameters of interest in the dlg;QF/QS/GAL4 cachectic models at 10 days AED. While previous experiments in the lab suggested that the suppression of the JAK/STAT pathway via $dome^{IR}$ can rescue the muscle wasting phenotype, reduce tumour cell death and increase the tumour size in animals bearing *dlg40.2* tumours only (data not shown), our results showed that the introduction of activated Ras^{V12} oncogene to *dlg40.2* tumours leads to a different outcome . Firstly, we observed that the muscle wasting phenotype in *dlg40.2,* Ras^{v12} $dome^{IR}$ and $stat^{IR}$ flies (n=7) was significantly worse on average at 47.90% (p=0.001) and 44.06% (p=0.0001) of coverage, respectively, when compared with control larvae (n=7) with a mean 64.13% of coverage (Appendix Figure 1a). (The examples of the least wasted control and worst wasted $dome^{IR}$ and $stat^{IR}$ muscles are shown in Appendix Figure 2A-C). In addition, all three fly groups (n=13 in each) had similar levels of tumour cell death (Appendix Figure 1c) and similar numbers of *Ras*-activated cells as percentages of total tumour volumes (Appendix Figure 1b). At the same time, $stat^{IR}$ fly tumours were overall significantly smaller than the control tumours (p=0.001), while $dome^{IR}$ fly tumours displayed a trend towards tumour size decrease (Figure

1d). Representative examples of the different fly tumour phenotypes can be seen in Figure 2A'-C'.

3.7 Discussion:

At the moment, it is difficult to draw definitive conclusions based on our current results. More experimental samples and independent experiments are required. Importantly, the efficiency of *Stat* and *Domeless* knockdown is yet to be confirmed by RT-qPCR as, regrettably, it was not possible to carry it out during the internship due to the lack of larvae that could be used as sources of RNA (the experiment had to be re-set multiple times and the student ran out of time). Thus, it remains an essential requirement to validate our results. In addition, it is important to confirm and quantify Ras^{V12} activation, comparing it with the activation levels inferred from the expressed levels of the QUAS-driven Tomato-3xHA. I have attempted to use two types of pErk antibodies and two different protocols for immunostainings, but still failed to get it to work as expected, possibly due to the antibodies' age. After the efficiency of *Stat* and *Domeless* knockdown is confirmed and Ras^{V12} activation quantified, further experiments should establish the relationship between muscle wasting phenotypes and several variables that need to be considered and controlled for when analysing the experimental outcomes. For instance, variables such as fly bottle/vial infections, high (25ºC+) stock storage temperatures, media composition, addition of different concentrations of D-Quinic acid (necessary for the action of the genetic construct in question) etc. may affect the phenotypic outcomes. Despite the best efforts to keep close to the original experimental protocol, some changes in conditions were unavoidable and so might have impacted the outcomes.

Another important missing piece in the cachexia puzzle is the role of the immune system in affecting the severity of muscle wasting. The proposed genetic construct to analyse the immune signature changes in tumour-bearing larvae did not work as expected. The complex construct that involved the usage of *Hemese*-GAL4 driver system to drive UAS-Bax, Tub-$GAL80^{TS}$ gene transcription – which was supposed to show the effects of fly haemocyte depletion – induced significant larval developmental delay, thus making it impossible for us to use appropriate controls. In addition, we noted general *Hemese* driver leakiness into larval salivary glands and Malpighian tubules, whereas originally it was only supposed to act in the imaginal disks and fly brain (data not shown). Because of that, the system had to be adjusted with *Hemese* replaced by an alternative Hml^{delta}-based driver system that had to be localised

to the second chromosome. The establishment of this line, which requires a large number of crosses, is still in progress.

4. Reflection by the student on the experience and value of the studentship:

The main premise of my project was to further extend on previous data from the Cordero lab, suggesting a crucial role of muscle-derived JAK/STAT signalling in cancer cachexia. It became very clear through my experiments that the role of JAK/STAT signalling in cancer cachexia is key. However, this is likely to involve a complex role, which may be influenced by the genetic characteristics of the tumour. Further studies are clearly required to fully elucidate these processes. However, results will likely illuminate a very important aspect of cancer research of relevance beyond *Drosophila*. This experience, to me, is a great reminder that science does not always happen as expected – however it shows how unexpected results can also lead to very interesting discoveries. This is the exact reason why this project and science in general is interesting and intellectually stimulating. I thoroughly enjoyed working in the lab and, importantly, I was able to gain confidence in my abilities because I was actively encouraged to organise my own schedule, plan the experiments and carry them out independently. I am now more informed and confident about a career in research, and even more set on obtaining a PhD.

5. Dissemination: (note any presentations/publications submitted/planned from the work):

These findings (among others pertaining to a different project) will be included in two independent papers currently underway at the Cordero lab.

6. Signatures:

Supervisor  Date 29/09/2018

Student  Date 23/09/2018

APPENDIX

Constructs:

1) Muscle wasting: We used a previously established dual-driver model of cancer cachexia, which combines the Q-system with GAL4/UAS system in a *discs large* (*dlg*$^{40.2}$) mutant background that models non-invasive neoplastic tumour growth. In this model – referred to as dlg;QF/QS/GAL4 onwards – it is possible to induce muscle wasting upon addition of D-Quinic acid (QA) – an agent that deactivates the Q-system repressor, thus activating the transcription of QF, which drives the expression of genes linked to QUAS (in our case, Tomato-HA, which acts like RFP, and RasV12, which makes tumours more aggressive and thus amplifies wasting).
2) Immune signature changes: Similarly to the above, the construct was a combination of the abovementioned Q-system and the Hemese-GAL4 driver system, and the experiments were planned so that the effects of haemocyte depletion (through UAS-Bax, Tub-GAL80TS-driven gene transcription) or upregulation (through UAS-RasV12-driven gene transcription) could be analysed in cachectic tumour-burdened larvae.

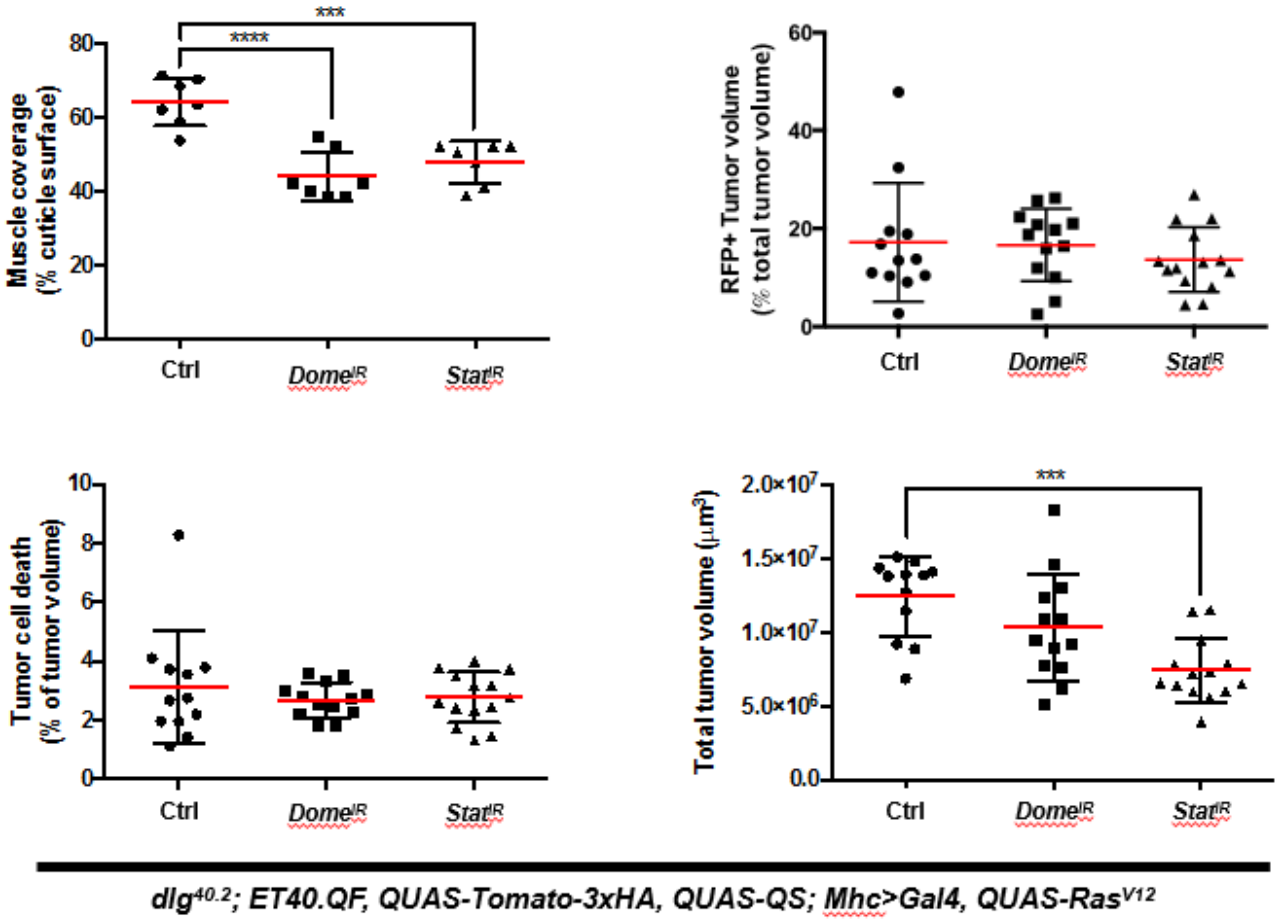

Figure 1. *Domeless* and *Stat* RNAi knockdown effects on muscle wasting, tumour volume and tumour cell death in dlg;QF/QS/GAL4 10-days-old larvae. (a.) Quantification of muscle coverage. **(b.)** Quantification of *Ras*^{V12} activated RFP-positive tumour cells, expressed as the percentage of the total tumour volume**. (c.)** Quantification of tumour cell death, expressed as the percentage of the total volume of the tumour positive for the Dcp-1 cell death marker. **(d.)** Quantification of the total tumour volumes in µm^3.

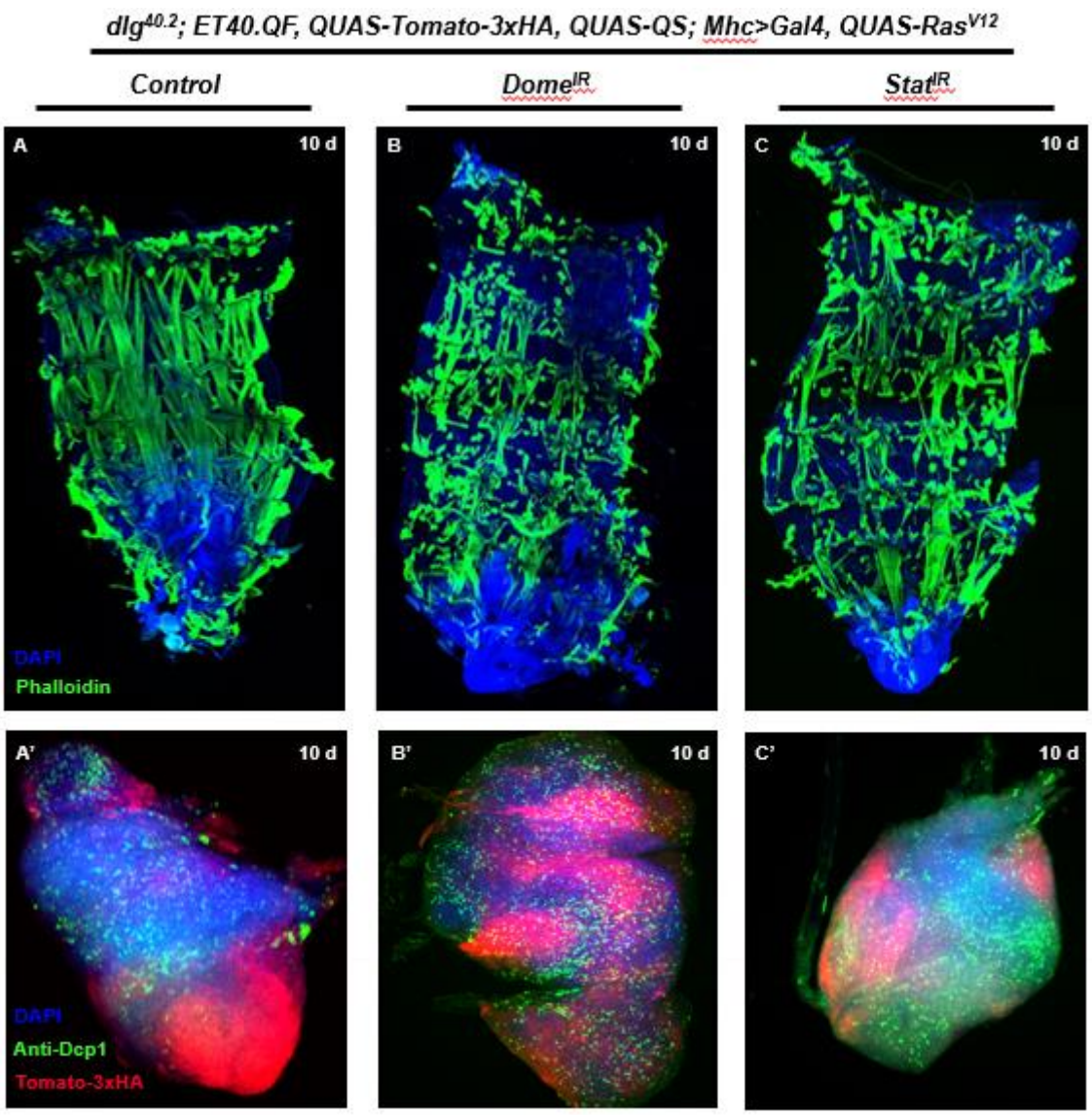

Figure 2. Confocal microscopy images of fixed dlg;QF/QS/UAS cachectic larval cuticles and tumours. (A-C) The figure shows cachectic larval cuticles 10 days AED stained with Phalloidin (green) to highlight muscle actin and DAPI (blue) to highlight DNA. (**A**) An example of a Dlg;QF/QS/GAL4 larval cuticle with 68.58% muscle coverage – one of the least wasted larva. (**B**) Dlg;QF/QS/GAL4,UAS-*Dome*^{IR} larval cuticle with 38.93% muscle coverage – the most wasted one. (**C**) dlg;QF/QS/GAL4,UAS-*Stat*^{IR} with 38.56% muscle coverage – the most wasted one. **(A'-C')** Examples of tumours from different dlg;QF/QS/GAL4 10-days-old larvae stained with DAPI to highlight DNA (blue) and Anti-Dcp1 to highlight cell death (green); the suspected Ras-activated cells are shown in red, highlighted by the UAS-driven Tomato-3xHA.