Investigating the role of small non-coding RNAs (piRNA, miRNA and siRNA) in Transgenerational Epigenetic Inheritance in *Drosophila*

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Background

Transgenerational epigenetic inheritance (TEI) is the process by which epigenetic information is transmitted to subsequent generations via the germline of an organism. Small non-coding RNAs (sRNAs) have been implicated in many examples of TEI across a range of model organisms [3]. The transgenic *Drosophila* Fab2L line (Figure 1a for schematic) is a model for TEI as it shows heritable deposition of H3K27me3 by PRC2 at the transgenic *Fab-7* regulatory element. Inheritance of this epigenetic status does not occur spontaneously, rather it requires an *initiating event*, where the endogenous *Fab-7* locus on chromosome 3 forms an inter-chromosomal contact to the transgenic *Fab-7* locus on chromosome 2 (Figure 1b) [4]. This contact being formed in the nucleus of just one generation of *Drosophila* is then sufficient for the H3K27me3 present at the transgene to be transmitted to all subsequent generations – a form of TEI – rather than simply being stochastically created or removed during development [2]. In this project, we sought to identify whether siRNAs, piRNAs or miRNAs – the predominant sRNAs in *Drosophila* – were involved in either 1) maintaining the expression state of the transgene once TEI has been established or 2) generating these important inter-chromosomal contacts that enable the initiation of TEI in these Fab2L *Drosophila*.

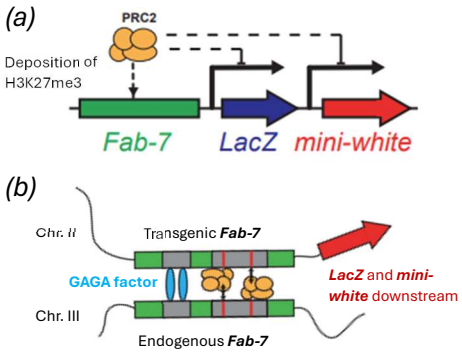

Figure 1 | (a) The *Drosophila* Fab2L line carries a 12.4kb transgene on chromosome 2 containing two reporter genes (*LacZ* and *mini-white*) and a regulatory element (*Fab-7*) which is also found endogenously on chromosome 3. (b) Initiation of TEI in the Fab2L line requires inter-chromosomal contact, mediated primarily by GAGA factor, between endogenous and transgenic *Fab-7* loci, allowing transfer of PRC2. Once this occurs in one generation, all subsequent generations will be able to inherit the epigenetic status of the transgenic locus.

Key Aims

1. To compare the transgene expression levels of sRNA mutant Fab2L *Drosophila* with wild-type Fab2L *Drosophila* and see **whether sRNAs are involved in maintaining the epigenetic state of the transgene**.
2. To see whether sRNAs facilitate inter-chromosomal contacts between endogenous and transgenic *Fab-7* loci and hence **contribute to the initiation of TEI in transgenic Fab2L *Drosophila***.
3. To see if *Sb[1]*, a mutation present on our *Drosophila* balancer chromosomes, was altering adult eye pigmentation through 1) *altering transgene expression* or 2) *altering H3K27me3 deposition* throughout the genome. Note that the *Sb[1]* mutant is unrelated to the sRNA pathways predominantly explored by this project but was also being investigated by the lab.

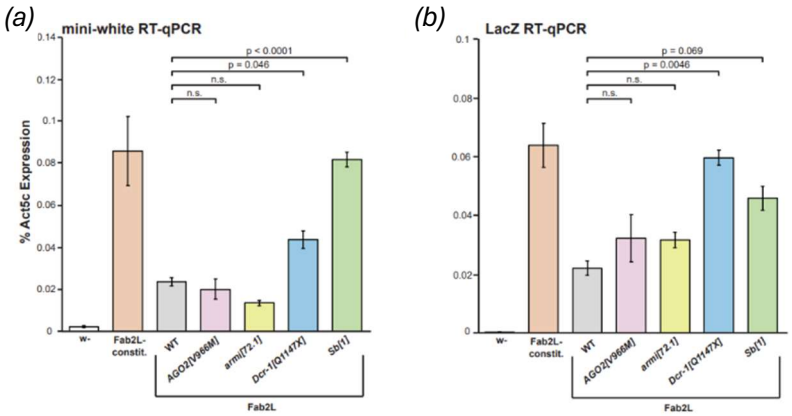

Figure 2 | RT-qPCR performed in triplicate with (a) *mini-white* and (b) *LacZ* primers to examine expression of the transgene in homozygous mutants of three major sRNA pathways. Expression in homozygous *Sb[1]* mutants was also analysed to see if this common balancer trait was impacting regulation of the transgene. Expression is normalised to Act5C, a major housekeeping gene, in each measurement. One-way ANOVA was used to test for significance.

Experiment 1: RT-qPCR

Rationale: We raised *homozygous* mutant *Ago2*, *Armi* and *Dcr-1* embryos (see methods) and performed reverse transcription quantitative PCR (RT-qPCR) to quantify the level of transcription at both of the reporter genes within the transgene (Figure 2). The wild type w- line (with no Fab2L transgene) was used as a negative control and the "Fab2L-constit." a positive control. Homozygous *Sb[1]* mutants were also included for Aim 3.

Results: No significant increase in reporter expression was found in either Ago2 or Armi mutants. We did, however, observe significant increases in expression of the reporter genes for the *Dcr-1[Q1147X]* mutant which warranted

further investigation into the role that miRNAs were playing. A similar significant result was obtained for the *Sb[1]* mutant. We also selected three genes (*Mus308*, *Blood* and *Kah*), each of which are under the control of a different sRNA, to confirm that our sRNA mutants caused elevated expression in these loci. Unfortunately due to DNA contamination during the extraction process, I could not obtain enough repeat results for these three loci during my project, so after my departure the lab repeated these controls with RNase-free DNase treatment and obtained sufficient data.

Experiment 2: Fluorescence in Situ Hybridisation (FISH)

Rationale: Previous studies had implicated sRNAs in mediating contacts between *Fab-7* elements, so we reasoned that they could be necessary for the triggering mechanism that enables TEI in this Fab2L *Drosophila* line [5]. We performed FISH on our three homozygous sRNA mutant embryos with probes targeting regions *surrounding* the endogenous (89E) and transgenic (37B) *Fab-7* loci to see if knocking down these pathways ablates the chromosomal contacts necessary for TEI initiation.

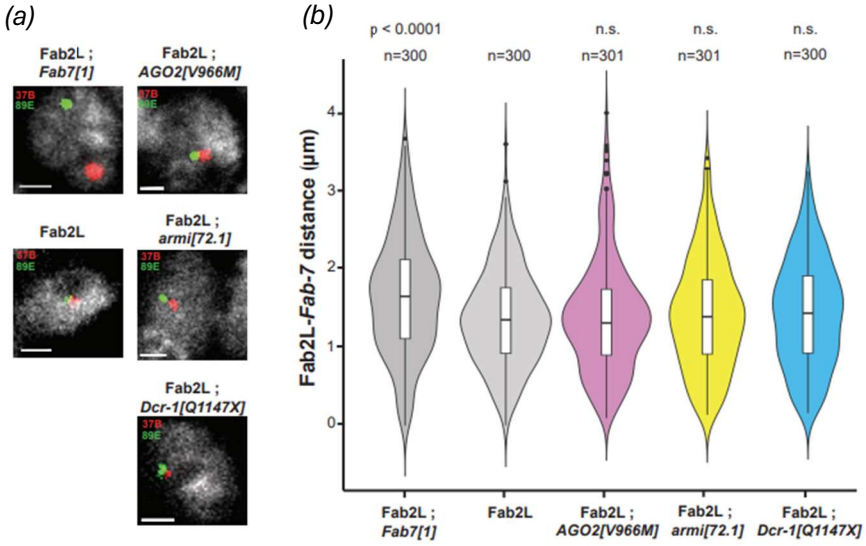

Figure 3 | (a) Fluorescence in situ hybridisation (FISH) reveals that Ago2, Armi and Dcr-1 homozygous mutants show no significant difference in the distance between endogenous *Fab-7* (89E) and the Fab2L transgene (37B) compared to the WT (Fab2L). This suggests that initiation of TEI is still possible in these homozygous mutants, so sRNAs must play no role in the recruitment of GAF and in mediating inter-chromosomal contacts. (b) Violin plots representing the distribution of distance between probes in ~100 nuclei from each of our three repeat samples.

Results: Our Fab2L ; *Fab-7[1]* negative control (Figure 3a, top left) has no contact between the probes as the lack of an endogenous *Fab-7* locus prevents homologous pairing and hence prevents initiation of TEI. Each of our three sRNA mutants showed no significant increase in the distance between the probes compared to WT Fab2L embryos (Figure 3b), suggesting that sRNAs do not impact the frequency of inter-chromosomal contacts or the initiation process of TEI.

Experiment 3: Cut and Run

Rationale: As previous work by the lab had identified a potential contribution of the *Sb[1]* mutation to adult eye pigmentation, we wanted to explore this further and see if *Sb[1]* mutants had any significant differences in the distribution of H3K27me3 throughout their genome compared to wild type Fab2L cells.

Results: After aligning the data we received from Illumina sequencing we did not find any significant changes in H3K27me3 deposition at relevant loci in the *Sb[1]* mutants. This indicated to us that the *Sb[1]* mutant, like we suspected with the *Dcr-1* mutant, may be *indirectly* impacting the regulation of the transgene.

Conclusion and Further Experiments

My experiments, taken alongside previous RNA-seq work completed by the lab (not reported here), lead to the conclusion that none of the three sRNAs explored here are involved in either the maintenance or initiation of TEI in this transgenic *Drosophila* Fab2L line. The unexpected increases in transgene expression that my project uncovered in the *Dcr-1* and *Sb[1]* mutants was further investigated by the lab and led to the finding that both mutants have significantly decreased expression of Pleiohomeotic, a protein which recruits PRC2 to the *Fab-7* regulatory element. Though this does not fully explain how these two mutants are impacting regulation of the transgene, it does confirm to us that the effects are not likely to be caused by a direct interaction of an miRNA with the transgene, hence we can now conclude that sRNAs are not involved in TEI. My cut and run experiments similarly confirmed that *Sb[1]* was affecting eye pigmentation without directly interacting with the transgene, as H3K27me3 levels were unchanged. Overall, my experiments support the idea that H3K27me3, found at the *Fab-7* element of the transgene, may actually be a directly heritable modification in the TEI process, rather than a modification that is recreated in the offspring via an intermediate such as an sRNA, an exciting prospect that can hopefully be explored further.

Photo 1 | Myself on the left with Maximilian Fitz-James on the right outside the Dorothy Crowfoot Hodgkin Building in Oxford where the Sarkies lab is currently based.

Value of Studentship

In this 6-week long studentship I learnt a range of transferrable skills on top of performing all the experiments listed above. I presented a journal club to the lab, extending my knowledge of other research groups within the field and attended regular meetings to practice presenting my results professionally to our PI. I also had the opportunity to attend a departmental chromosome and RNA biology symposium day which allowed me to discuss my research with other researchers from Oxford and external institutions, forming valuable connections in the process. This project also improved my statistical analysis and enabled me to write my own scripts in R to analyse and present my data with clear and professional figures. My data also enabled the lab to draw firm conclusions about the role of siRNAs and piRNAs into TEI and opened a new line of enquiry after I left for the role of miRNAs. Though the outcome of this project was a negative result, the process of obtaining clear and reliable data to form robust conclusions was still an incredibly valuable experience and I am very grateful to have had this opportunity.

Methods

Mutant Generation

- Three genes involved in the synthesis of each sRNA were chosen to be knocked down (*Ago2* for siRNA, *Armi* for piRNA and *Dcr-1* for miRNA). Mutants were crossed with lines containing the Fab2L transgene and the *TM3, Sb, Kr-GFP (TKG) balancer of chromosome 3* to keep the mutant stable over many generations of crosses.

Embryo Collection and sorting (for RT-qPCR and FISH)

- Embryos of sRNA mutant flies were raised overnight at 18oC and collected between stages 12-13 of embryogenesis for sorting. Embryos were dechorionated in bleach before being sorted for the homozygous mutant phenotype. This involved select against the GFP-containing TKG balancer (Photo 2) and keeping flies that did not fluoresce. Embryos would reach stage 14-15 by the end of sorting.

Total RNA extraction, reverse transcription and qPCR.

- A minimum of 20 embryos were used for total RNA extraction using TRIzol (Invitrogen), chloroform and isopropanol. 200ng of RNA was converted to cDNA with a High-Capacity RNA-to-cDNA kit (Applied Biosystems) with each RNA sample *also* being added to a supermix with no reverse transcriptase so that DNA contamination could be seen. RT-qPCR was performed in triplicate with 5µl cDNA and 5µl SYBR green supermix (with 1µl of primer per well).
- The primers used for RT-qPCR included: 1) *Act5C*: normalising control, 2) *LacZ* and *mini-white*: to measure expression at the Fab2L transgene, 3) *Mus308, Blood and Kah*: to confirm the *Ago2*, *Armi* and *Dcr-1* mutants had no expression of their relevant sRNAs.

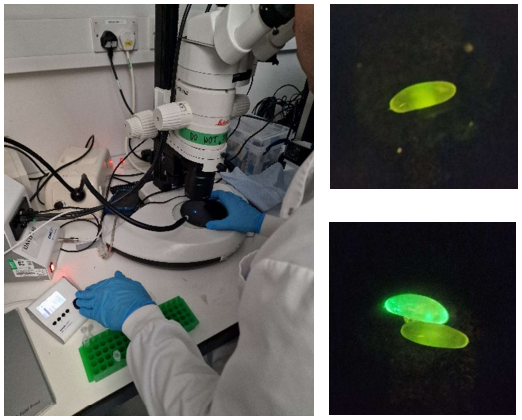

Photo 2 | Embryo sorting process using a fluorescence microscope.. The balancer chromosome contains GFP under the control of a Krüppel promoter which leads to a distinct band of GFP during embryogenesis (top right). These embryos were selected *against* so our collected embryos were known to be homozygous mutants (i.e. without the balancer chromosome). Some embryos had very intense fluorescence whilst others exhibited only mild but still detectable fluorescence (bottom right).

Fluorescence in situ hybridisation (FISH)

- Probes against the regions surrounding the endogenous and transgenic *Fab-7* loci were made during the project with a nick translation kit and Alexa Fluor 555 and Alexa Fluor 647 dyes (Invitrogen Life Technologies). Embryos were fixed at stages 14-15 (following sorting) using 4% paraformaldehyde in heptane. See Bantignies, F., Cavalli, G. (2014) [1] for full protocol.

Cut and Run

- 20 Adult *Sb[1]* and WT heads were dissected and homogenised. Anti-H3K27me3 antibodies and anti-IgG antibodies (negative control) were used and the protocol followed as in Kami Ahmad (2022) [6].

References

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