Generation of translational diseases in the *Drosophila melanogaster* model

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ABSTRACT

Aminoacyl-tRNA synthetases (aaRSs) are key enzymes involved in protein synthesis as they are responsible for adding a specific amino acid to their cognate tRNA. There are at least twenty aaRSs (one for each amino acid) and, generaly, eukaryotes have one cytosolic enzyme and another in organelles with their own genome. aaRSs have recently been reported to have roles in non-catalytic and non-canonical processes. Mutations in aaRSs can cause severe translation diseases, therefore, studies that help understand the causes and the effects of these diseases are crucial for adequate therapies. In this work, the seryl-tRNA synthetases (SRS) were investigated in *Drosophila melanogaster* with the aim to study the phenotypical effects produced by RNA interference of the three putative SRSs for flies: *m1-*, *m2-* and *c-SRS*.

RNAi transgenic fly lines were constructed in order to silence each SRS. This was done by crossing SRS transgenic flies with different GAL4 driver strains and through the UAS/GAL4 system. Constitutive and ubiquitous knock down of each SRS mRNA produced total lethality in flies. Translation of each SRS was also blocked in a tissue-specific pattern. In wing tissue, with drivers *nub-GAL4* and *MS1096-GAL4*, the silencing of the SRS resulted in flies with abnormal wings for all three SRS. Eye specific drivers were used and eye tissue damage was found in one month old transgenic-GAL4 flies. These results indicate essential functions for *m1-*, *m2-* and *c-SRS*. Furthermore, the RNAi system here used allows the generation of flies whose translation machinery can be impaired in a controlled manner and the study of the defects caused by it.

Aminoacyl-tRNA synthetases play a key role in protein synthesis by catalyzing the addition of amino acids to their cognate tRNAs. The tRNA aminoacylation reaction performed by aaRS consists of two steps. In the first step the enzyme activates the amino acid (aa) with adenosine-triphosphate (ATP) to form aminoacyl-adenylate (AMP), with release of pyrophosphate (PPi). Next, the amino acid is transferred via the formation of an ester bond, to a hydroxly group of the ribose of the terminal adenosine at the 3´-end of the tRNA, thus generating aminoacyl-tRNA (aa-tRNA). In this reaction there is also a release of AMP and aaRS. The aaRs will be available to be used to aminoacylate again. In general all organisms have at least twenty aaRSs enzymes – one for each amino acid – divided in two classes (class I and II) (Ribas de Pouplana *et al.* 2001 and Ibba e*t. al* 2005). Due to their essential role, aaRSs are considered to be housekeeping enzymes. However, several lines of evidences have demonstrated that these enzymes are multi-functional and play important roles in non-catalytic and non-canonical processes (Lee *et al.* 2004).

The seryl-tRNA synthetases from eukaryotic organisms (as well as the other aaRS) are coded by the nuclear genome, but some of them function in organelles that have their own genome. There are organisms that have only one gene that codes for a single SRS that has the capacity to act both in the cytoplasm and in other organelles, while other organisms can perform differential splicing and obtain two isoforms: one that functions in the cytosol and the second in the organelle. However, in most cases, there are two nuclear genes that code for two isoforms with different subcellular destinies. There is no description about the serylation system of *Drosophila melanogaster* and there is no characterization about its subcellular localization. It is known that in *Drosophila* the nuclear genome codes for the pool of $tRNAs^{Ser}$ that function in the cytosol, and the mitochondrial genome codes for its own pool of $tRNAs^{Ser}$ (Tomita *et al.*1999).

A large number of human diseases are caused by mutations that prevent the correct translation of mRNA. A drawback of some of aaRSs is that they may accept non-cognate amino acids that are smaller than the correct one. To avoid the misincorporation of these non-cognate amino acids into proteins, an editing function is performed by the same enzymes and the wrong amino acid is removed. If there is a deficiency in this function, the result would be the synthesis of misfolded proteins. Misfolding proteins and their aggregates are though to cause several neurodegenerative disorders. One of the most common inherited neurological disorders is the X-linked-Charcot-Marie-Tooth disease (CMT), presently incurable, and seems to be related with the loss of translation mediated by internal ribosome entry sites (IRESs). These IRESs are nucleotide sequences that allow translation initiation in the middle of a mRNA sequence. Several mutations in aaRSs cause human neuropathies including some types of the aforementioned Charcot-Marie-Tooth (Scheper *et al.* 2007).

With the use of bioinformatics tools, the Gene Translation Laboratory found three putative SRS for *Drosophila melanogaster*. This fact is known to occur only in insects and one invertebrate. This situation is exceptional, because organisms generally have one SRS in the cytosol to decode the nuclear genome and another SRSs in those organelles

which have their own genome. Therefore, *Drosophila melanogaster* was expected to have one SRS in the cytosol and another one working in the mitochondria.

Using the National Center for Biotechnology Information (NCBI) web page for the search of proteins, there can be found three annotated putative SRS for *Drosophila melanogaster*: *c-SRS* (Accession number AAF51155, FlyBase code CG17259); *m1-SRS* (Accession number AAF55175; FlyBase code CG4938); *m2-SRS* (Accession number AAN13983, FlyBase code CG31133).

The experiments planed in this work consist on the suppression of the genes that encode for the putative *c-SRS*, *m1-* and *m2-SRS* from *Drosophila melanogaster*, using the RNAi machinery. In order to perform RNAi in flies (Fire *et. al* 2006), the use of the well established UAS/GAL4 system is necessary (Brand and Perrimon 2003). This system was first reported by Fischer *et al.* in 1998 when demonstrated that GAL4 expression can initiate transcription of a reporter gene under the upstream activator sequence (UAS) control in *Drosophila*. This feature allows the selective expression of any cloned gene in a wide variety of cell- and tissue-specific patterns in *Drosophila*. The long term objective of this project is to generate flies whose translation can be disrupted in a controlled manner in order to study the effect that it may cause to the organism.

MATHERIALS AND METHODS

Fly strains: Flies were raised in a standard medium and maintained at 18ºC or 25ºC and 60% humidity, in a 12 hour light/ 12 hour dark cycle. The *Drosophila* w^{1118} strain was microinjected with the transformation plasmid. The dominantly-marked balancer chromosomes *If/Cy; Ly/TM3* stock was used to map the chromosomal location of the transgene (TR) and to balance the transgenic lines (*CyO* was the balancer for the II chromosome and *TM3* for the III chromosome). The following GAL4 lines were used to drive the expression of the *UAS-m1*, *UAS-m2* or *UAS-c-SRS*: *Act-GAL4* (Laboratory of Dr. Ferran Azorín IBMB-CCSIC), *ey-GAL4*, *GMR-Gal4* (Laboratory of Dr. Marco Milán IRB), *sev-GAL4*, *nub-GAL4* and MS1096-GAL4 Laboratory of Dr Jordi Casanova IBMB-CCSIC). Dicer strains (from Laboratory of Dr Jordi Casanova IBMB-CCSIC) were used to insert the construct dicer-2 (*Dcr-2*) in the genome of the transgenic lines, in chromosome II or III. The *UAS-GFP* line was used to test the level of expression of a desired driver.

Cloning: Knowing the complete sequences of the genes that code for *m1*, *m2* and *c-SRS*, for each one of them, fragments with a size between 500 to 700 bp of cDNA were cloned into the vector pWIZ (or white intron zipper), under the control of an UAS sequence. The vector pWIZ also contained a reporter gene, *white* w^{+}, which allows the selection of the transgenic flies (the marker is colored eyes). The fragments of interest were cloned twice into the pWIZ in opposite orientations, obtaining what is called an inverted repeat (IR) construct separated by a function intron. The protocol used is described in *Lee et al, 2003*. The recombinant vectors constructed were injected, together with a Δ 2-3 helper plasmid, which contains the transposase coding sequence, into *Drosophila* w^{1118} developing embryos and transformant flies were generated by standard P element transformation.

Confocal flurescent images

The three different eye-specific GAL4 drivers (*ey-GAL4*, *sev-GAL4* and *GMR-GAL4*) were crossed with an *UAS-GFP* line. Third instar larvae were collected from the progeny, in order to analyse and compared by fluorescence the strength of expression of these drivers. Immunostaining protocol was used in order to intensify the GFP signal.

Immunostaining of imaginal discs: Larvae imaginal discs were dissected in PBS and fixed in 4% paraformaldehyde for 20 minutes at room temperature. They were subsequently washed in PBS, blocked in blocking buffer (PBS, 0.3% Triton, 1% BSA), and incubated overnight with the primary antibody (rabbit anti-GFP, "Molecular Probes, Invotrogen") diluted in blocking buffer at 4ºC. Washes were performed in blocking buffer, and the appropriate fluorescent secondary antibody was added for one hour at room temperature. Following further washes in blocking buffer, the discs were mounted in Vectashield and images were taken in a Leica SP2 confocal and subsequently processed using Adobe Photoshop.

***In vivo RNAi* experiments**

Transgenic lines *m1-*, *m2-* and *c-SRS-Dcr2* were crossed with ubiquitous and constitutive driver *Act-GAL4*. Crosses were maintained at 25ºC. Adult viable progeny was counted and phenotyped. Trangenic lines were also crossed at 25ºC with wing (*nub-GAL4*, *MS1096-GAL4*) and eye (*GMR-GAL4*, *sev-GAL4*) specific drivers. Adult progeny was screened for viability and tissue damage. With drivers *nub-GAL4* and *MS1096-GAL4*, crosses where also performed at 29ºC in order to test if the increase of temperature would cause an increase in the effect of the RNAi machinery.

Pictures of wings

Selected damaged flies were put in SH medium [glycerol: ethanol 1: 3] for at least 24 hours. The wings were dissected in water and mounted in Faurè medium [125ml H_2O; 125g chloral hydrate; 75g gum arabic; 50g glycerol] over a microscope slide. A small weight is put over the cover glass for 24 hours. Pictures were taken optical microscope Nikon Elipse E600 and saved with ACT software.

Pictures of flies

In order to take pictures of the flies, they were frozen for 30 minutes at -20ºC. The Stereomicroscope Leica MZ 16F with the DFC 300FX camera attached was used with amplitude

between 25X-32X to take pictures of the whole fly and between 40X-50X for details. Pictures were saved using the software Adobe Photoshop.

RESULTS

Generation of transgenic lines: several stocks were generated and mapped for the three transgenic lines *m1-*, *m2-* and *c-SRS-Dcr-2*. Each stock carried a single *UAS-TR* inverted repeat insertion, which allowed post-transcriptional gene silencing of the TR via RNAi, following its activation by GAL4 (Figure 1).

SRS	stock	chromosome	type
c-SRS	1	III	HO LETHAL
	2	III	HO LETHAL
	3	III	HO NOT LETHAL
	4	II	HO NOT LETHAL
	5	Not identified	HO NOT LETHAL
	6	Not identified	HO NOT LETHAL
	7	Not identified	HO LETHAL
	8	X	-
	9	X	-
	10	II	HO NOT LETHAL
m1-SRS	1	II	HO NOT LETHAL
	2	X	-
	3	II	HO LETHAL
	4	III	HO NOT LETHAL
	5	III	HO LETHAL
	6	II	HO LETHAL
	7	X	-
	8	X	-
	9	III	HO LETHAL
	10	II	HO NOT LETHAL
	11	II	HO NOT LETHAL
	12	III	HO NOT LETHAL
	13	II	HO NOT LETHAL
m2-SRS	1	II	HO NOT LETHAL
	2	III	HO LETHAL
	3	X	-
	4	III	HO NOT LETHAL
	5	III	HO NOT LETHAL
	6	III	HO NOT LETHAL
	7	III	HO NOT LETHAL
	8	III	HO NOT LETHAL

Figure 1 - Table of the obtained transgenic stocks followed by the result for the mapping of each one and the type of stock. For some cases it was not possible to map the insertion of the transgene due to the fact that the obtained progeny did not match any of the theoretically expected. Concerning the type of stock, since the location of the transgene could take place in an essential location of the fly genome, in future generations, the stock will tend to a heterozygous population in order to maintain always intact one copy of this essential gene. The flies with two copies of the SRS insertion in their genome will lose the essential and original fragment and result as non viable flies (homozygous lethal). On the other hand, if insertion happens in a non essential site of the genome, the transgene will not be disrupting the transcription of an essential gene or the proper functioning of regulatory sequences, and having two copies of it will not be lethal for the fly. In this case the stock will evolve to a heterozygous and homozygous mix (homozygous not lethal).

Figure 2 - Pictures of mounted wings; (A) Mounted *nub-GAL4-Dcr-2OE-c-SRS* 10 wing; (B) Mounted wild type wing. It is possible to observe the difference in more detail, between a wild type wing and one of the few damaged and non-expanded wing from a *nub-GAL4-Dcr-2OE-c-SRS* 10 fly.

Adult lethality in all *Act-GAL4* driven *SRS-Drc-2OE* individuals: All three transgenic lines when crossed with driver *Act-GAL4* resulted in total lethality for *Act-GAL4* driven *SRS-Drc-2OE* adult individuals. Stocks 10 and 11 from *m1-SRS* as well as 4 and 10 from *c-SRS* where tested. Progeny from both pair of stocks was analysed and average results are shown in Figure 3 (A and B). In this figure it is possible to observe that the proportion of *Act-GAL4*-driven *SRS* and *Act-GAL4*-Dcr-2-*SRS* is considerable much lower that all the other six types of phenotypes for both *m1-* and *c-SRS*. The same procedure was taken with *m2-SRS* transgenic flies. Stocks 4 and 7 were tested and the progeny was screened.

Average results are shown in Figure 3 (C). A similar pattern as the results for *m1-SRS* and *c-SRS* was observed in the case of *m2-SRS*: lethality occurs in all adult *Act-Gal4* driven Dcr-2-m2*SRS* individuals except that the proportion of *Act-GAL4*-driven *m2SRS* is not significantly lower than all the other six types of progeny.

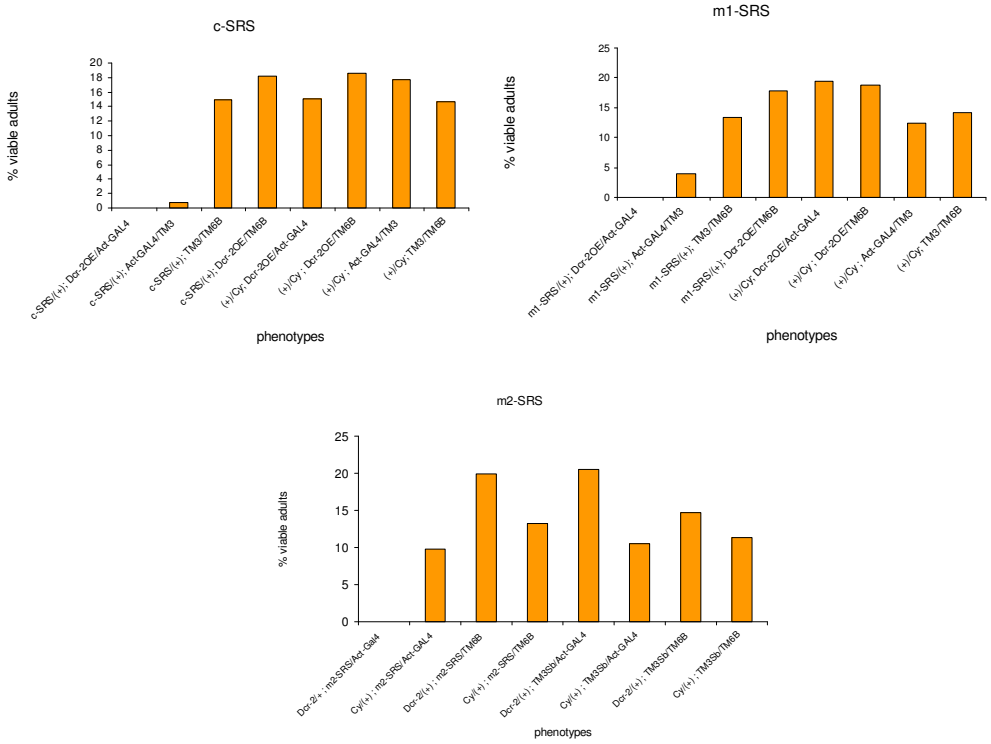

Figure 3 – Results in percentage for the crosses between the transgenic lines and the driver *Act-GAL4*. In the axis the different genotypes are shown; note that the *SRS-Act-GAL4* driven individuals are represented in the two first columns, being the first column for the ones that have the *Dcr-2* construct in the genome and the second for the ones that do not. (A) Percentage of adult viability, obtained by the average of two independent experiments using stocks 4 and 10 from *c-SRS*. (B) Percentage of adult viability, obtained by the average of two independent experiments using two stocks 10 and 11 from *m1-SRS*. (C) Percentage of adult viability obtained by the average of two independent experiments using two different stocks 4 and 7 from *m2-SRS*.

Low level of expression for *ey-GAL4* driver: Before starting with the experiments, the strength of expression of these three eye-specific drivers were crossed with the *UAS-GFP* and larvae form the progeny was analysed and compared by fluorescence. Figure 4 shows the level of expression of the marked eye protein in larvae eye imaginal discs. *ey-GAL4* is the one with the lowest expression and for this reason it was not used in further experiments. *sev-GAL4* and *GMR-GAL4* seem to have similar strengths of expression, and were both used in the RNAi experiments.

Using drivers *sev-GAL4* and *GMR-GAL4* No eye damage observed in new born flies from the progeny: Stocks from the *c-* and *m1*-SRS transgenic lines were crossed with the *sev-GAL4* and *GMR-GAL4* drivers separately and a large number of progeny was phenotyped. In the progeny all the theoretically expected phenotypes were obtained and in similar proportions. However, from all the progeny flies observed, neither for *c-* or *m1*-SRS stocks, were flies found with morphologically affected eyes, or with any other part of the body visibly affected.

Four week old *GMR-GAL4-Dcr-2OE-c-SRS* flies show damage in eye tissue: Since no damage was observed for new born flies from *sev-GAL4* and *GMR-GAL4* crossed with the transgenic lines, a new strategy was then undertaken. The only tested stock for this experiment was *c-SRS* 10. Among the progeny from the cross between transgenic flies and *GMR-GAL4* flies, adult flies that corresponded to the transgenic driven GAL4 individuals *(GMR-GAL4-Dcr-2OE-c-SRS* and *GMR-GAL4-c-SRS)* were selected, kept at 25ºC and analysed every week. At the forth week, a damage affecting ommatidia organization was seen in some of these flies. Figure 5 shows the observed phenotype.

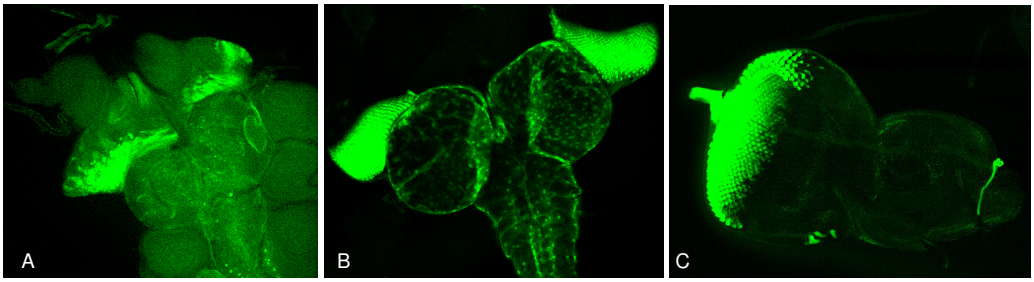

Figure 4 - Pictures obtained for the expression of GFP for the three eye tissue specific drivers, in larvae form the progeny of the cross between the eye specific drivers and the UAS-GFP line. (A) Result for the driver *ey-GAL4*. (B) Result for the driver *sev-GAL4*. (C) Result for the driver *GMR-GAL4*. Pictures A and B represent the two eye imaginal discs, and picture C only one of the discs. *ey-GAL4* is the one with the lowest expression and for this reason it was not used in further experiments.

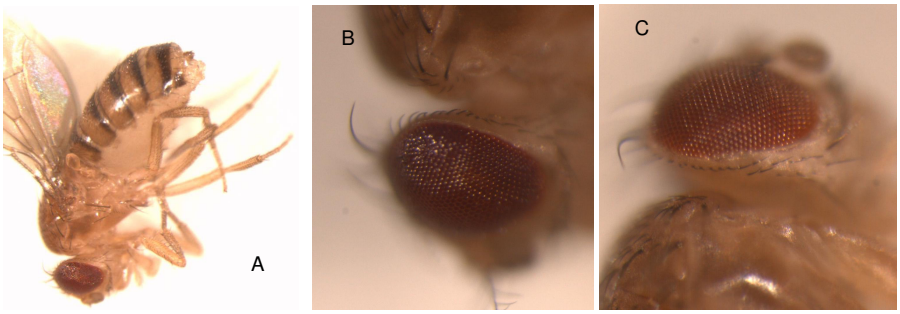

Figure 5 - Result for the 4 week-old *GMR-GAL4-Dcr-2OE-c-SRS* fly; (A) *GMR-GAL4-Dcr-2OE-c-SRS* fly; (B) *GMR-GAL4-Dcr-2OE-c-SRS*, zoom of the eye; (C) normal red eye. A normal adult eye is characterized by an organized and homogeneous layer of photoreceptors cells. In picture B it is noticeable the ommatidia disorganization.

***c-SRS* stocks tested with *nub-GAL4* resulted in an extremely low percentage of *nub-Gal4-Dcr-2OE-c-SRS* individuals:** Stocks *c-SRS* 4 and 10 where crossed with wing specific driver *nub-GAL4*, having a cross at 25ºC and another at 29ºC. Unexpectedly, observing the *c-SRS* graph in Figure 6 it is noticeable that the percentage of *nub-GAL4-Dcr-2OE-c-SRS* individuals born is extremely low, either for those that have affected wings, or for those that do not, at both tested temperatures. Furthermore, the percentage of flies that do not have the *nub-GAL4* insertion is very similar for both temperatures. Figure 2 (A) and Figures 6 (A and B) show some of the few examples of the damage observed in the wing tissue.

***m1-SRS* 1 stock tested with *nub-GAL4* resulted in an increase of wing damaged individuals from 25ºC to 29ºC:** Stock *m1-SRS* 1 was crossed with wing specific driver *nub-GAL4* at both 25º and 29ºC. The *m1*-SRS graph in Figure 6 shows that the obtained adult flies with wings damaged is around 20% at 25ºC and this percentage increases almost double at 29ºC. Notice that the percentage of flies that do not have the *nub-GAL4* insertion is maintained stable in both temperatures (close to 45%). Figures 6 (C and D) show some examples of the damage observed in the wing tissue.

***m2-SRS* stocks tested show different patterns of results:** The stocks *m2-SRS* 4, 5 and 7 where crossed with the *nub-GAL4* at both 25ºC and 29ºC. The *m2-SRS* graphs in Figure 6 show that, firstly even though they do not share the same pattern, all three stocks have an increase on the number of adult flies with affected wings when the experiment is done at a higher temperature. Figures 6 (E and F) show some examples of the damage observed in the wing tissue of flies from the obtained progeny.

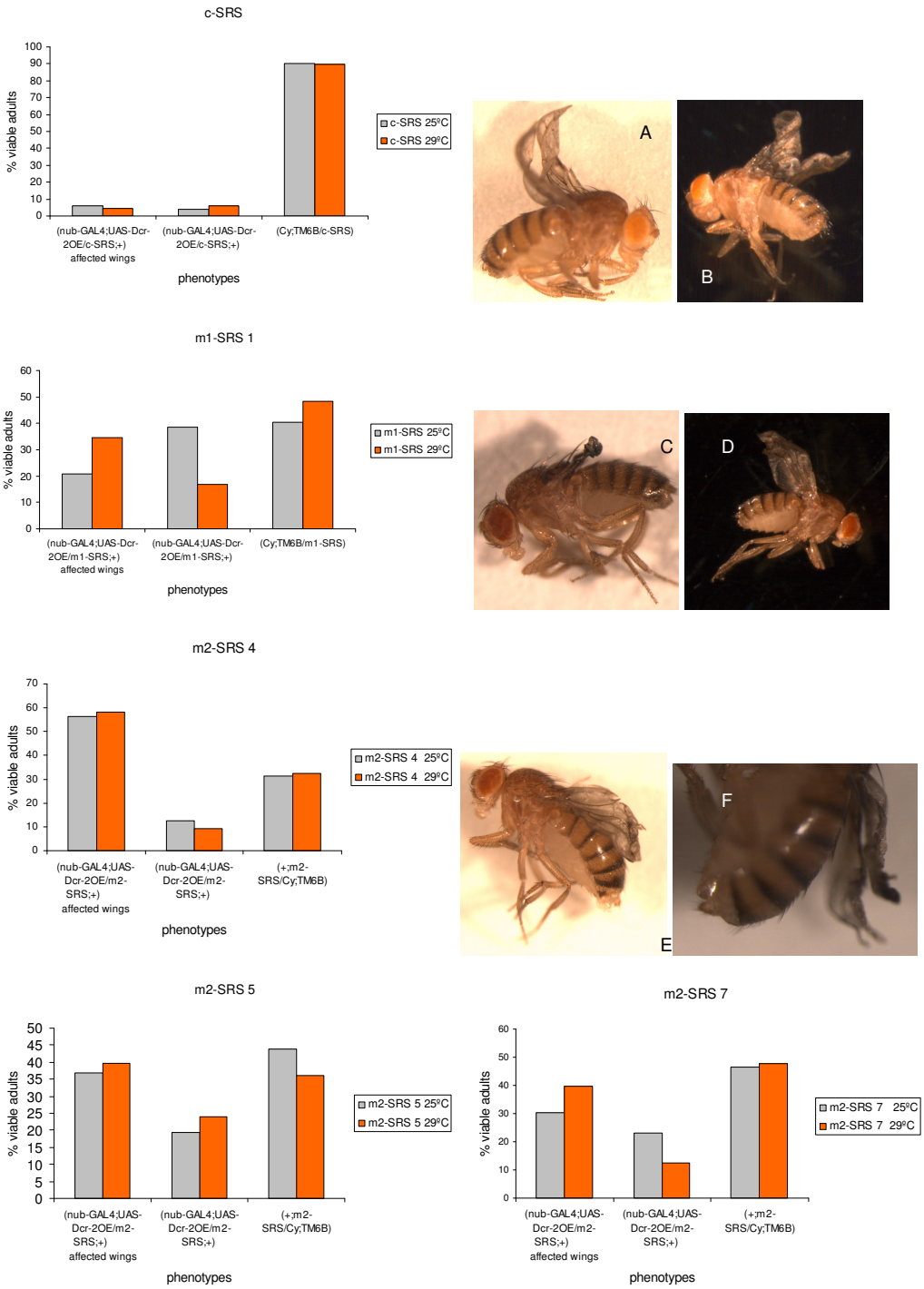

Figure 5 – Percentage of viable adult flies obtained from the cross between *m1-*, *m2-* and *c-SRS* transgenic flies and *nub-GAL4-UAS-Dcr-2* driver. Two genotypes are possible for the progeny: *UAS-TR-UAS-Dcr-2 nub-GAL4* driven individuals and the transgenic non *nub-GAL4* individuals. The graphs show the progeny divided in three since among the *TR-nub-GAL4* driven individuals there were both flies with affected and non affected wings. All transgenic lines tested were crossed at 25ºC and 29ºC. Pictures show example of some of the observed damage in *nub-GAL4-Dcr-2OE--SRS* wings. (A and B) *nub-GAL4-Dcr-2OE-c-SRS* flies from stock 10 at 25ºC; (C) *nub-GAL4-Dcr-2OE-m1-SRS* fly from stock 1 at 29ºC; (D) *nub-GAL4-Dcr-2OE-m1-SRS* fly from stock 1 at 25ºC; (E) *nub-Gal4-Dcr-2OE-m2-*SRS 4 individual obtained from a cross at 25ºC; (F) *nub-GAL4-Dcr-2OE-m2-SRS* fly from stock 5 at 29ºC. The morphologic effects observed include for example non-expanded wings, curly wings and incomplete or broken wing tips. The damages are noticeable when compared with the wild type wing shown in Figure 2.

A "curly-like" phenotype found in progeny, disabled results with driver *MS1096-GAL4*: The crosses between the transgenic lines and the wing specific driver *MS1096-GAL4* were constructed based on the initial idea that it would be possible to distinguish among the progeny, the transgenic flies from the non transgenic selecting the flies with curly wings. However, observing the progeny from the performed crosses, a "curly – like" phenotype was found, for experiments at both temperatures. This fact made the expected separation of non-curly from curly flies impossible, as some flies showed a clearly different phenotype from the one given by the *Cy* marker, but others showed a very dubious phenotype, not allowing to identify if it the wings were curly because the fly was transgenic and the RNAi was working or if the flies simply possessed the *Cy* marker and were non transgenic.

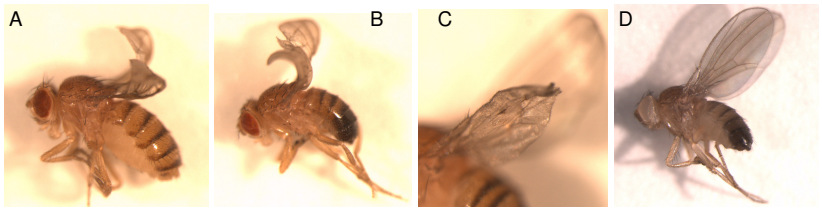

Figure 6 - Some of the damage seen in *MS1096-Gal4-Dcr-2OE-SRS* individuals including the "curly-like" phenotype. (A) *MS1096-GAL4-Dcr-2OE-m1-SRS* 11 fly from a cross at 29ºC; (B) *MS1096-GAL4-Dcr-2OE-c-SRS* 10 fly from a cross at 29º; (C) *MS1096-GAL4-Dcr-2OE-m2-SRS* 5 fly from a cross at 25ºC; (D) w^{1118} with wild type wing.

DISCUSSION

This work aimed to study the consequences of the post-transcriptional silencing of the three annotated putative SRS for *Drosophila*: *m1-*, *m2-* and *c-SRS*. Post-transcriptional silencing was obtained by the expression of the IR constructs that where inserted in the *Drosophila* genome which led to the formation of double stranded RNA (dsRNA) and the switch on of the RNAi process. The UAS/GAL4 system was used to drive the knockdown of the target gene under the control of several promoters, namely ubiquitous and tissue specific drivers.

Regarding the cross of the transgenic lines with the driver *Act-GAL4*, total lethality was the result observed for *Dcr-2-SRS-Act-GAL4* individuals from the progeny, for all three transgenic SRS lines. All the other theoretically expected phenotypes appeared in the progeny and were viable adults. Taken together, this data suggest several conclusions. Firstly, an extremely important conclusion is that *c-*, *m1-* and *m2-*SRS are three essential proteins for the fly. The results indicate that in each case, the RNAi is functioning, and that the strength of the machinery, when an extra copy of *Dcr-2* is present, is enough to cause severe damage in all cells resulting in complete lethality. It is also possible to conclude that the *UAS-Dcr-2* construct is functional and that the overexpression of the enzyme is increasing the effect of the silencing. This is demonstrated by the fact that that for *c-* and *m1-SRS*, the addition of *Dcr-2* to the *SRS-Act-GAL4* system is crucial to produce lethality, since the absence of this extra *Dcr-2*, allows the viability of a low percentage of the flies that contain *SRS-Act-GAL4.* However, this was not the case for *m2-SRS*. Several bioinformatics analyses (unpublished data) suggest that the *m2-SRS* is a putative SRS that may have lost its ability to aminoacylate, and since the *Dcr-2-SRS-Act-GAL4* individuals were lethal, if it is not functioning as an SRS enzyme, it is responsible for some other essential function. The fact that the percentage of flies containing *m2-SRS-Act-GAL4* is higher than in the canonical SRSs, for all the stocks tested, may also suggest that the *m2-SRS* has a different function, since the partial silencing of this protein allows adult viability.

Since lethality was the result obtained with the ubiquitous driver, the use of tissue specific drivers allowed the SRS transcripts to be knocked down only in a certain tissue, thus theoretically allowing the generation of defects in only part of the living organism.

Concerning the specific eye tissue specific drivers, *ey-GAL4*, *sev-GAL4* and *GMR-GAL4* drivers where crossed with an *UAS-GFP* in order to compare the level of expression of each. It was possible to conclude that the *ey-GAL4* is the driver that shows the lowest level of expression, and was consequently excluded from the tissue specific driver experiments.

Stocks from the *c-* and *m1-*SRS transgenic lines were crossed with the *sev-GAL4* and *GMR-GAL4* and since no flies were found with morphologically affected eyes, a new strategy was then undertaken: the *SRS-GMR* driven flies were selected and kept at 25ºC to allow their ageing. In the case of the tested *c-SRS* 10 stock, in some four week old flies, the eye tissue appeared damaged, more specifically, part of the cell layer was disorganized and degenerated. The hypothesis is that in photoreceptors the level of protein

synthesis is low. Consequently, the accumulation of deficient proteins, resulting from the decrease of SRS and problems in the translation process, would not be immediate, but rather, would manifest itself later in the life as severe cell damage. There is also the possibility that the SRS turnover is slow, having a long half-life and being only necessary after a certain number of days, and as the *GMR-GAL4* is not expressed in early stages, the cells have synthesised normally SRS up until then.

The second tissue chosen for RNAi analysis was the wing. Different stocks were crossed with two wing drivers, *nub-GAL4* and *MS1096-GAL4*, in order to silence the three SRSs.

For experiments with driver *nub-GAL4*, crosses were done at 25ºC and 29ºC.

The results for the line *c-SRS* unexpectedly showed an extremely low percentage of *nub-GAL4-Dcr-2OE-c-SRS* individuals, for both temperatures. For the case of the *m1-*SRS 1, the number of *nub-GAL4*-Dcr-2OE-*m1-SRS* individuals with damaged wings doubles from the experiment at 25ºC to the one done at 29ºC. For *m2-SRS*, the results for the three stocks with *nub-GAL4* presented different patterns, on the other had, all of them show an increase on the number of wing affected flies from 25ºC to 29ºC. There are in fact variations for the other types of flies between stocks *m2-SRS* 4, 5 and 7. However, it is important to note that each transgenic stock constructed has the insertion in a random and different location of the fly's genome. It is also known that when comparing transgenic stocks for the same protein, the location of the transgene can affect the strength of the RNAi machinery due to different transcription levels for the transgene, depending on the chromatin accessibility by the transcription machinery. Thus it is not surprising that such different results were obtained between the tested *m2-SRS* stocks, it demonstrates the distinct levels of RNAi efficiency.

Considering the graph resulted from the average of *c-SRS* stocks showing a drastically low amount of *nub-GAL4*-Dcr-2OE-c-*SRS* flies, a possible explanation would be that the driver is leaking, in other words, a very small amount of GAL4 in another expression pattern, apart from the wing specific. *nub* is expressed in early stages in other tissues besides the wing, thus it is possible that for transgenic *nub-GAL4* driven flies, the target mRNA is being suppressed also in other parts of the organism affecting severally its normal development, leading to the extremely low percentage of adult individuals in the case of the *c-SRS*. It would be natural to ask why this effect is not seen with *m1-* or *m2-SRS*. Note that the *nub-GAL4* experiment was done for only two stocks from *c-SRS* collection, only one from *m1-SRS* and three from *m2-SRS*. More replicas need to be done in order to have more data. Additionally, it is important to mention that many different wing aberrations were observed, but that there was a clear relation between severity and temperature, as seen in the defects present in the progeny that came from a cross at 29ºC. Taken together, it is possible to conclude that the *nub-GAL4* driver caused a noticeable effect in the wing tissue the three putative SRS, demonstrating that suppression of these genes is detrimental to normal development and maintenance of the cell.

The last tested driver was the *MS1096-GAL4*. Only stocks *c-SRS* 4, *m1-SRS* 11 and *m2-SRS* 5 were tested with this driver. The observed curly-like phenotype in the progeny made the visual separation between the transgenic and non transgenic flies impossible. Thus, concerning the experiments for the *c-*, *m1-* and *m2-SRS* with the *MS1096-GAL4* driver, it is possible to conclude that the RNAi was working and the suppression of the two genes cause visible alterations in wings. However, a different kind of cross would have to be planned with this driver to overcome this technical problem and collect data.

The differences seen between the results for each putative SRS, in particular in *nub-GAL4* experiments, which are the ones which gave us more variety of results, might reflect the different threshold level of each putative SRS need to maintain normal translation activity and/or the difference that the location of the transgene causes to RNAi strength.

As a final remark, it is important to mention that up to date the role that the *m2-SRS* is playing in the cell is unknown, but the essentiality of this protein has been confirmed in the present work and the effects observed in the experiments are caused by he impaired functioning of this non-canonical organellar SRS.

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