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Preliminary results of a forward genetic screen for X chromosomal dominant modifiers of *Drosophila melanogaster dfmr1*.

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Introduction

Fragile X syndrome is a neuro-developmental disease in humans. It is caused by a mutation in the gene *Fmr1,* which expands abnormally a CGG-repeat in its promoter region, thus leading to a subsequent hypermethylation and transcriptional inactivation of the gene (Sutcliffe *et al.*, 1992). *Fmr1* encodes the fragile X mental retardation protein (FMRP).

The most important clinical symptoms of the disease include mental retardation, sleep disturbances, autism, and impaired motor coordination. They reflect the key role of FMRP in the brain, where it is predominantly expressed (Devys *et al.*, 1993).

The lack of this protein is accompanied by defects in synaptic maturation and morphology, synaptic connectivity disturbances, and dysfunction throughout the nervous system (reviewed in Tessier and Broadie, 2009; Pfeiffer and Huber, 2009; Gatto and Broadie, 2011).

Research on animal models confirmed the main characteristics of fragile X syndrome – neuronal defects, synaptic abnormalities in synaptic development and function, circadian rhythms disturbances, impaired long term plasticity, abnormal mGluR signaling, and learning and memory deficits (reviewed in Bassell and Warren, 2008; Gatto and Broadie, 2009; Mercaldo *et al.*, 2009; Pfeiffer and Huber, 2009).

Drosophila studies have shown that dFMRP functions in axon growth, path finding, and activity dependent pruning and refinement of synaptic elaborations(Dockendorff *et al.*, 2002; Morales *et al.*, 2002; Michel *et al.*, 2004; Pan *et al.*, 2004; Tessier and Broadie, 2008).

FMRP is a selective mRNA-binding protein (with two KH domains and an the RGG box), which is a negative regulator of protein synthesis of its mRNA targets at synapses (Laggerbauer *et al.*, 2001; Li *et al.*, 2001; Sung *et al.*, 2003; Zalfa *et al.*, 2003; Qin *et al.*, 2005; Antar *et al.*, 2006; Price *et al.*, 2006). Accumulating evidence shows a role of FMRP in mRNA transport (Dictenberg *et al.*, 2008; Estes *et al.*, 2008) and in the regulation of mRNA stability (Zalfa *et al.*,2007; Gantois *et al.*, 2006; Miyashiro *et al.*, 2003; D'Hulst *et al.*, 2006; De Rubeis and Bagni, 2010).

Numerous candidate m-RNA targets, interacting with FMRP, were obtained by different approaches, though only a few of them have been validated *in vivo* (see the reviews: Zalfa and Bagni, 2004; Bassell and Warren, 2008; Callan and Zarnescu, 2011).

In order to exercise its multiple functions and to participate in different processes from the nucleus to the synapses, FMRP is thought to shuttle between nucleus and cytoplasm and to form different protein complexes. Models were created, suggesting that FMRP might take part in DNA

regulation, RNA transport, and translation by interacting with different subsets of mRNAs and proteins and by forming different RNP-complexes and protein-protein complexes (Zalfa and Bagni, 2004; Zarnescu *et al.*, 2005).

One way to identify functional partners of FMRP is to induce mutations in random genes and to screen for those of them, which interact with the gene, encoding the protein. In our study, we used the *Drosophila* Fragile X- model, which has a single FMRP ortholog - dFMRP, encoded by a single gene - *dfmr1* (Wan *et al.*, 2000). We designed a forward genetic screen to look for EMS-induced dominant enhancers and suppressors in a sensitized genetic background, where normal *dfmr1* function was disrupted by its GAL-4 over-expression in the wings. Such genetic interaction screens are a straightforward approach in identifying genes functioning in common biological pathways.

In our work we searched for X chromosomal interactors of *dfmr1*, which dominantly modified – enhanced or suppressed its mutant over-expression wing phenotype. We isolated 7 enhancers and 11 suppressors with recessive lethal effects on viability and determined their rough map locations on the X-chromosome.

Materials and Methods

Drosophila stocks and fly rearing

In our work we used the following *Drosophila* stocks:
w[1118]; P{w[+mc] = UAS – Fmr.Z}3, w[*];P{w[+m]=GAL4vg.M}2;TM2/TB6B,Tb[1];
w[67c23]P{w[+mC]=lacW}dlg1[GO456]/FM7c; C96-GAL4; *UAS-MamN; y ct v*; *y f* .
They all were obtained from the Bloomington Drosophila Stock Center at Indiana University. Additional information on the above stocks can be found at the website of the Bloomington Drosophila Stock Center (www.flybase.org).

We also used the stocks: *y* w^a N^{54l9}*//FM6* and rst^{CT} N^{54l9} *rb//C(1)DX y w f; Dp(1;2)w+51b//+*, which were kindly gifted to us by Prof. S. Artavanis-Tsakonas.

They all were maintained on corn meal/ yeast extract/raisins at the standard temperature of 25°C.

Genetic interaction experiments were performed at two temperatures: at 21°, when we looked for dominant enhancers of the over-expression wing phenotype of *dfmr1*, and at 27°, when we looked for dominant suppressors of the same phenotype (see below).

UAS/GAL4 system and over-expression phenotype of the gene dfmr1

In our study we used the UAS/GAL4 system, which enables expression of a particular gene, driven by a selected promoter in a tissue of choice (Brand and Perrimon, 1993).

We induced over-expression of *dfmr1* in the wing imaginal discs of *D. melanogaster* by means of the following cross:

$$\text{P: } \female \ \frac{w}{w}\frac{GAL4-vg.M}{GAL4-vg.M}\frac{TM2}{TM6B} \times \male \ \frac{w}{+}\frac{+}{UAS-fmr1}\frac{UAS-Fmr1}{}$$

$$\downarrow$$

$$F_1: \quad \frac{w}{w}\frac{GAL4-vg.M}{+}\frac{UAS-Fmr1}{TM2}$$

The F_1 progeny was maintained at a set of temperatures - 18º; 21º; 25º, and 27º, in order to determine the optimal *dfmr1* over-expression phenotype, designated by us as control wing phenotype or control phenotype.

EMS mutagenesis and screening for dominant modifiers

To induce mutations dominantly modifying the over-expression phenotype of *dfmr1*, we applied the mutagen EMS (ethyl-methane sulfonate) according to the method of Lewis and Bacher (1968) with modifications. The principle of the method is in directly exposing the *UAS*- bearing stock to mutagenesis and then in expressing the trangene in F1 generation (Guichard *et al.*, 2002; Penton *et al.*, 2002).

We used 0-48 hours old males of the genotype *w[1118]; P{w[+mc] = UAS – Fmr.Z}3*, which were starved for 12 hours. Cohorts of 20 flies were put into vials with a piece of kitchen paper, soaked with a 25 mM EMS in 1% sucrose and were exposed to the mutagen for 6 hours. After the recovery of the treated flies on fresh media for one hour, each male was crossed individually to several virgin females of the genotype *w[*]; P {w [+m] = GAL4-vg.M}2; TM2/TB6B, Tb[1]* and allowed to mate for 3 days.

The progeny of these crosses were kept either at 21º or at 27º and screened for wing phenotypes different from the *dfmr1*-over-expression phenotype in the control crosses, cultured at the same temperatures.

These new phenotypes were considered to result from genetic interactions between *dfmr1* and the EMS-induced dominant modifier mutations.

Selection of X chromosomal enhancers and suppressors with recessive lethality

In order to select for X chromosomal modifiers - suppressors and enhancers with recessive lethal effects, only female mutants from F_1, bearing the mutation either on the autosomes or on the X chromosome, were used for further analysis.

Each newly emerged female fly with a modified wing phenotype was crossed individually to several males from the stock *w[1118]* and their progeny was inspected for a male/female ratio and for the segregation of the mutant and the control wing phenotypes. During this isogenization step, only families where the progeny (F_2) showed a sex ratio 2:1 in favor of the females, instead of the regular 1:1, were taken for further studies. We assumed that they contained an X chromosomal mutation, which dominantly affected the *dfmr1*-over-expression phenotype and recessively influenced viability.

Such mutations were put over the balancer chromosome *FM7c*, and balancer stocks were generated [*w* l(Su or Eh)/FM7c; +/+; +/+*, where "*l*" is the lethal mutation].

The ability of the isolated mutations to modify specifically the over-expression wing phenotype of *dfmr1* was tested by using the stocks *C96-GAL4* and *UAS-MamN* (previously described in Helms *et al.*, 1999). When these stocks are crossed, their progeny exhibits a similar wing nicking phenotype, due to over-expression of the dominant negative MamN – a truncated version of Mam (Notch transcriptional co-activator mastermind) at the wing margins.

Meiotic mapping

Meiotic mapping of each X-linked modifier mutation with a recessive lethality was performed by crossing females from the balanced mutant stock to male flies *y ct v*. Some modifier stocks were crossed in another experiment to males from the stock *y f*.

In the F_1 generation females *w* l(Su or En)/y ct v* were mated to their brothers *FM7c//Y* from the same generation, and in F_2 only the male progeny was analyzed. Over 1200 male flies from F_2 generation were scored, and recombinant flies were counted for each mutation.

Recombination distances were calculated between the lethal mutation "*l*" and the recessive markers "*y*" and "*ct*" or "*y*" and "*f*", and these distances served to determine the region of its map position.

Drosophila wing processing

Wings were dissected from adult flies of interest and mounted in DePeX - Mounting medium for histology (SERVA). Pictures were taken using Stereomicroscope *CARL ZEISS JENA TECHNIVAL* with Nikon Coolpix L10 5MP Digital Camera with 3× Optical Zoom.

Results and Discussion

Screening for EMS-induced dominant modifiers of dfmr1

In our work we used the *UAS/GAL4* system for targeted gene expression (Brand and Perrimon, 1993). We crossed flies from the stocks *GAL4-vg.M* and *UAS – Fmr.Z* to drive in their F_1 - progeny over-expression of dFMRP in the wing imaginal discs of developing *Drosophila* larvae. As a result, we observed notched wing phenotypes, due to apoptotic cell loss at the wing margins, where the *vg*-promoter functions (Wan *et al.*, 2000).

On the background of these wing phenotypes we looked for EMS-induced mutations, dominantly modifying them. We were interested in those of them, which were X-linked, as there are so far no data on experimentally induced *dfmr1*- modifier mutations.

We first optimized the conditions for the application of the *GAL4-vg.M/UAS – Fmr.Z* and investigated the penetrance of the over-expression notched wing phenotype (control phenotype) at different temperatures, as it is known that transcription activation by *GAL-4* is strongly influenced by temperature (Brand *et al.*, 1994). On the other hand, expression of genes downstream of the *UAS*-sequence influences *Drosophila* development/viability, and this also correlates with the temperature of rearing the flies (Allemand *et al.*, 2001).

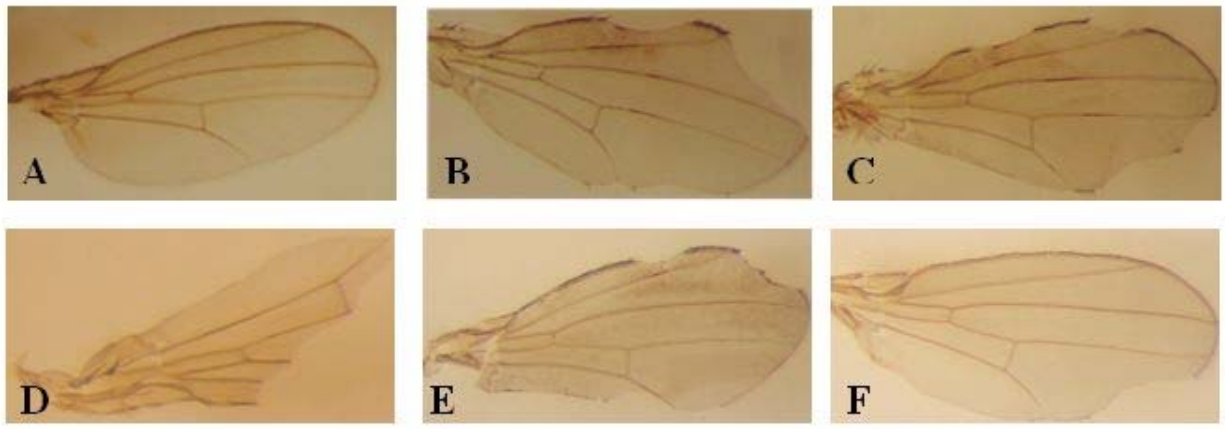

Figure 1. Wing phenotypes of flies. A – wild type wing (♀ and ♂); B - over-expression of *dfmr1* in the adult wings at 21°(♀, control phenotype); C, over-expression of *dfmr1* in the adult wings at 27° (♀ and ♂, control phenotype); D - strong enhancer wing phenotype; E – moderate enhancer wing phenotype; F- weak enhancer wing phenotype. *Note: Suppressor wing phenotype is not shown, as it looks like the wild type.*

We reared *GAL4-vg.M/+; UAS – Fmr.Z/+ flies* (F_1- generation, see the cross in Materials and Methods) at several different temperatures and found that the notched wing phenotype, induced by the over-expression of *dfmr1,* was temperature sensitive. It showed a complete penetrance in the male flies from F_1, reared at 21°C and no penetrance in their sibling females. At 27°C both sexes had notched wing phenotypes (Figure 1).

We also observed, that these two temperatures ensured about 70 – 80% adult eclosion and viability, which was high enough to allow a large-scale genetic screen.

The difference in the wing phenotype penetrance between the male and female F_1 progeny at 21°C, as well as the uniform over-expression wing phenotype at 27°C, was used by us to set up a two-part screen for *dfmr1* dominant modifier mutations induced by EMS-mutagenesis (for details of the EMS-procedure, see Materials and Methods).

In one part of it we set up 1250 individual crosses, which consisted of one mutagen-treated *UAS – Fmr.Z* - male and several *GAL4-vg.M*- females, and cultured the progeny at 21°C. As we screened for X-linked modifier mutations, according to our cross procedure, we needed to isolate from F_1 only female flies with modified phenotypes. At this temperature, we easily visualized and scored for an appearance of female flies with notched wing phenotypes. We defined such phenotypes as enhancer phenotypes. They represented "worsened" control wing phenotypes, the latter being indistinguishable from the wild type at this particular temperature. Mutated genes, causing this "worsened" phenotype, were designated as enhancers.

Altogether we examined about 18,000 female flies over-expressing *dfmr1* under the control of *GAL4-vg.M* and found 126 enhancer mutations, dominantly modifying the control wing phenotype at 21°C.

We divided them into four groups, according to an arbitrary scale of their modifying effect on the control wing phenotype: very strong, strong, moderate, and weak enhancers. As very strong enhancers (7) we considered those having extremely "worsened" wing phenotype – one or two wings were completely absent. The strong enhancers (53) showed missing wing blades. As moderate enhancers (57) were determined those having one or two small notched areas- anterior and posterior wing margin loss on both wings, and the weak enhancers (9) had only one small notched area on one of the wings. Enhancer wing phenotypes are presented in Figure 1.

In the second part of our screen, we set up 1900 individual crosses, designed as above, but reared the progeny at 27°C. At this temperature, we scored for an appearance of female F_1 flies with normal wings among the uniformly present notched wing phenotypes. Such flies with an "improved" wing phenotype were considered as having suppressor phenotypes, and their mutated genes were designated as suppressors.

We scored about 21,000 female flies, over-expressing *dfmr1* in the developing wing tissue and found 128 suppressor mutations, which dominantly modified the over-expression wing phenotype at 27°C. To avoid false weak suppressors, we isolated only those of them which modified this phenotype to wild type appearance.

Meiotic mapping of the modifier mutations with recessive lethality

Each enhancer or suppressor mutation was isogenized, by crossing the mutant female to male flies *w[1118]*, and the male/female ratio was determined as well as the segregation of the notched wing and the control wing phenotypes. In this way, we were able to define and select only those modifier mutations, which were X-linked and recessively lethal, so that we could further map them genetically.

Among the 126 modifier enhancer mutations isolated in our screen, we found 8 X-chromosomal lethal mutations. We tested them for their specificity to modify the specific abnormal wing phenotype induced by the gene *dfmr1*. For that we combined each mutation in a common

genotype with the trans-genes *C96-GAL4* and *UAS-MamN,* which produce a wing phenotype similar to our control wing phenotype but driven by a different gene and expressed under a different *GAL4* promoter. Neither of our mutations was able to modify the latter wing phenotype, in contrast to a control experiment with the mutation *y* w^a N^{54l9}*//FM76*, which enhanced the MamN-driven wing phenotype (Helms *et al.*, 1999).

Among the 128 suppressor mutations we found 11 to be located on the X-chromosome and to be recessively lethal. They specifically modified the control over-expression wing phenotype at 27°C.

All these modifier mutations were mapped by means of the recombination analysis, and their positions on the X chromosome were calculated by their recombination distances from the genes *y* and *ct* (see Materials and Methods). For those of them, which showed a closer proximity to *y* than to *ct,* genetic distances from *y* and *f* were determined as well.

The results of our recombination analysis are shown in Table 1.

Table 1. Recombination analysis and map position of the X-linked modifier mutations.

Mutation signature	Total number of F_2 – males scored	Map region
Eh 5	1705	1 – 53.0 - 59.0
Eh 25	1175	1 – 49.8 - 55.8
Eh 40	1244	1 – 31.7 - 37.7
Eh 44	1175	1 – 01.0 - 05.0
Eh 50	1380	1 – 00.0 - 04.1
Eh 53	1364	1 – 35.0 - 41.0
Eh 109	1546	1 – 37.9 - 44.9
Eh 134	1382	1 – 38.2 - 44.2
Su 1	1320	1 – 34.6 - 40.6
Su 5	1202	1 – 52.7 - 58.7
Su 10	1340	1 – 48.9 - 54.9
Su 37	1319	1 – 45.2 - 51.2
Su 55	1381	1 – 38.3 - 44.3
Su 71	1238	1 – 46.1 - 52.1
Su 87	1396	1 – 53.0 - 59.0
Su 104	1319	1 – 03.1 - 10.1
Su 121	1263	1 – 31.9 - 37.9
Su 123	1283	1 – 00.0 - 05.0

Note: As recombination distances to known chromosomal markers give only a crude location of a gene, we determined in this way the map region of each lethal mutation, which acted as a dominant enhancer/suppressor of the over-expression dfmr1 wing phenotype.

We undertook our large scale forward genetic screen in order to find new genes, which interact with *dfmr1* and function in a common biological pathway.

So far most modifier screens are conducted by over-expression the gene of interest in the adult retina. Zarnescu and co-authors looked for functional partners of *dfmr1* and performed a dominant modifier screen for the autosomal *Drosophila* genome (Zarnescu *et al.*, 2005). They found 19 mutations in the tumor suppressor gene *l(2)gl* and 90 mutations in other autosomal loci, which modified the retinal over-expression of *dfmr1* in *Drosophila.* Later, Cziko and co-authors screened mutations in 43 candidate genes for their ability to modify the "rough" eye phenotype, induced by over-expression of *dfmr1* in the adult retina, and identified several suppressors of this mutant phenotype (Cziko *et al.*, 2009). In their experiments, new translational repressor proteins were characterized, which interacted with the protein dFMRP.

In our work we drove over-expression of *dfmr1* in the wing imaginal discs. Previous studies have shown that such over-expression leads to notched wing phenotypes, due to apoptotic cell loss at the wing margins (Wan *et al.*, 2000).

We have chosen the wing tissue as a place to conduct our screen, as it very easy to score and save for the organism's viability and fertility. *Drosophila* wing is also a suitable model to study genetic interactions for another reason. Differentiation of distinct cell types within the growing wing tissue depends on their interactions with adjacent cells, and mutations in specific genes affect differently behavior of the different cell subpopulations (Craig *et al.*, 1997). Important signaling pathways - Notch, Wingless, Decapentaplegic, and others take part in the wing development (reviewed in Artavanis-Tsakonas *et al.*, 1995; Klingensmith and Nusse,

1994). Looking for dominant modifiers of *dfmr1* that function in these pathways would elucidate novel developmental roles of this gene, which might be related to the wing tissue (or to other tissues as well).

In our study we found modifiers – enhancers and suppressors, interacting with *dfmr1*, whose product levels were crucial for the signal transduction efficiency in the adult wings, so that their mutations were recessively lethal. To identify and characterize these mutations we plan to carry out fine duplication/deficiency genetic mapping and complementation analysis with selected candidate genes from the same cytological map locations.

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Genetic analysis of body color polymorphism in *Drosophila melanogaster* through selection experiment.

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Abstract

Drosophila melanogaster is a widespread species that exhibits enormous variation in abdominal melanisation throughout its range. To gain insight into this variation, present work involves selection for abdominal melanisation. In 38 generations of selection for melanisation, an increase of ~2.5 fold in dark selected strain and a decrease of ~6-7 folds in light strain was observed in both the sexes as compared to control populations of *D. melanogaster*. Genetic crosses between dark and light strains obtained through selection produced intermediate offspring, but a clear maternal effect differentiated the reciprocal F_1's. F_1 flies showed higher plastic effect as compared to selected dark and light strains across various growth temperatures. Our results are novel in the occurrence of five body color phenotypes in ratio of 1:4:6:4:1 (two-gene) in both sexes of *D. melanogaster*.

Keywords: Abdominal melanisation, dark and light selected strains, *Drosophila melanogaster,* selection experiment.

Introduction

Body melanisation exhibits a large amount of variability in Drosophilids, resulting either from genetic polymorphism or phenotypic plasticity (Gibert *et al.,* 1998; Rajpurohit *et al.,* 2008; Parkash *et al.,* 2011). The color polymorphism in abdominal tergites was reported by da Cunha (1949) for *D. polymorpha.* In this species, both males and females show any one of the three types of abdominal tergite coloration: dark, intermediate or light. In four species of montium subgroup, discrete polymorphism for 6^{th} and 7^{th} abdominal segments occurs only in female individuals, but the dominance level of darker and lighter phenotypes vary between species (Ohnishi and Watanabe, 1985). Recent studies in montium species *D. jambulina* and *D. punjabiensis* have shown genetic polymorphism for body color morphs (Parkash *et al.,* 2009; Singh, 2011). *D. melanogaster* is known for its sexual dimorphism. Males possess a black abdomen (tergites 5 and 6) while females exhibit yellow tergites with black stripe at their posterior margin.

Several studies on melanisation include analyses of phenotypic plasticity (David *et al.,* 1990; Gibert *et al.,* 1998, 2000; Parkash *et al.,* 2011), evolutionary developmental basis of intra- and interspecific differences (Kopp *et al.,* 2000; Wittkopp *et al.,* 2002a, 2002b), a phylogenetic and