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Correlated gene expression profiles in double insertional mutants of *Drosophila melanogaster*

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Abstract

In a qRT-PCR experiment, the expression levels of six genes from Drosophila melanogaster were studied in females of three transgenic lines symbolized l(3)S057302, DH2F31M1 and DH1F14M1. The considered genes are located in 3R chromosome and are as it follows: CG9805 (eIF3-S10), CG10272 (grappa), CG31349 (polychaetoid), CG11033, CG14709 and CG1528 (gammaCop). The transgenic line l(3)S057302 harbors a single P{lacW} in 5'UTR of gammaCop whereas DH2F31M1 and DH1F14M1 are double mutant lines, both of them containing the P{lacW} insertion of l(3)S057302. Additionally, DH2F31M1 has a second specific P{lacW} in the 5'UTR of eIF3-S10, while DH1F14M1 contains a specific insertion residing about 70 bp upstream of CG14709.

Comparative analyses revealed that eIF3-S10 is upregulated in l(3)S057302 and DH1F14M1 lines, but becomes downregulated in DH2F31M1, in correlation with a downregulation of gammaCop. By contrast, CG14709 is downregulated in l(3)S057302 and DH2F31M1, but upregulated in DH1F14M1, also in the context of a decreasing of gammaCop expression level. Conversely, grappa, polychaetoyd and CG11033 genes which are not insertionally mutated in the double mutant lines are upregulated in all of the described transgenic lines. It is tempting to consider that the second insertion hit genes co-regulated with gammaCop, also affecting other genes pertaining to a putative functional network. Our qRT-PCR results suggest that P transposons are involved in gene regulation mechanism upon a pattern orchestrating functionally related genes.

Keywords: transgenic lines, *P{lacW}*, qRT-PCR, gene expression modulation

Introduction

Since the beginning of the last century, *D. melanogaster* imposed itself as one of the major biological models. Nowadays, based on the many available genetic and molecular techniques and accumulated data, *D. melanogaster* is a fit model for studying genome's plasticity. The discovery of *P* transposable elements within *D. melanogaster* genome made possible a unique glimpse to the co-adaptive processes, which are a consequence of interactions between these two highly dynamic entities. Transposable elements are one of the main functional acquisitions of the eukaryotic genome. They increase the adaptive abilities of genomes and speed up their evolution, by acting on heterochromatin (DORER [1], DIMITRI [2]), determining chromosomal rearrangements (LIM [3], EGGLESTON [4]) and leading to the apparition of new genes and gene functions (BROSIUS [5], LONG [6], LONG [7]). There are also evidences that their massive presence influenced the regulatory mechanisms of transcription and the distribution of structural motifs within the genomes (ZHAO [8]).

There are many proofs that reveal a non-random distribution of the insertions of *P* element derived artificial transposons in *D. melanogaster*, which are biased toward certain genomic compartments, such as *5'UTR*'s of genes (SPRADLING [9]) and potential regulatory domains (ECOVOIU [10]).

Herein, we tested by qRT-PCR a model predicting that after the mobilization of a

certain artificial insertion associated with a specific gene, the transposon will tend to reinsert into or in the close vicinity of genes that are functionally related with the gene originally tagged by insertion (RATIU [11], ECOVOIU [12]). If the original insertion determines a severe phenotype, we expect that the transposition event will lead to the reconstruction of the original insertion (*primary local reinsertion* or PLR) concomitant with a reinsertional phenomenon (*secondary local reinsertion* or SLR). The SLR will also tend to determine a severe phenotype.

In order to test this hypothesis, we constructed a collection of mutant lines harboring reinsertions of *P{lacW}* transposon (BIER [13]) by mobilizing an insertion from *5'UTR* of *gammaCop* gene, located in the 100C chromosomal region of *D. melanogaster*'s 3R chromosomal arm. This insertion defines the transgenic line *l(3)S057302* and is symbolized $P\{lacW\}^{l(3)S057302}$. In the current study, we aimed to demonstrate that the relative expression of some genes is modulated by the coexistence of a PLR with a SLR within the same genome, in comparison to the relative expression of the same genes in lines free of SLRs.

Materials and Methods

D. melanogaster lines that we used in this study were raised on a yeast-cornmeal-agar medium at 25^0C, and are as follows: ***l(3)S057302****/TM6TbHu* (*l(3)S057302* symbolized in Figure 1 as *gammaCop057302*), ***DH1F14M1****/ TM3SbSer*, ***DH2F31M1****/TM3SbSer* and wild-type ***Oregon***. Several days prior to RNA extraction, we transferred the flies in bottles with fresh medium and fresh yeast was added. For RNA extraction we selected two groups of ten adult females for each considered line, each group being a biological replicate. We performed whole body RNA extraction using NucleoSpin-RNA XS kit (Macherey-Nagel), following the manufacturer instructions. After eluting RNA we added 1.5 units of DNase (Promega) to each RNA sample, and subsequently treated them according to the provided protocol. We measured the concentration of our RNA sample using a micro-volume spectrophotometer ASP-2680 (Avans Biotechnology). Reverse Transcription System (Promega) was used for revers transcribing 1500 ng of RNA from each biological replicate in a final volume of 30 µl. The reactions were performed according to the manufacturer's recommendations on a PalmCycler PCR termocycler (Corbett Research).

To quantitate gene expression we performed quantitative Real-Time PCR (qRT-PCR) and the method of data analyzing was the $2^{-\Delta\Delta Ct}$ relative quantification method (LIVAK [14]). We considered six target genes for each line and one as an endogen reference, namely *RpL32*. Target genes were *CG9805* (*eIF3-S10*) from **82B1**, *CG10272* (*grappa*) from **83E6-7**, *CG31349* (*polychaetoid*) from **85B2-7**, *CG11033* from **85C3-4**, *CG14709* gene from **86E11** and *CG1528* (*gammaCop*) from **100C**. Both of *DH1F14M1* and *DH2F31M1* lines were obtained in our mutagenesis experiments and each of them harbors two insertions. *DH1F14M1* contains $P\{lacW\}^{l(3)S057302}$ in *CG1528* and $P\{lacW\}^{CG14709.DH1F14M1}$ in *CG14709* (GenBank/NCBI accession number *HM210946*), while *DH2F31M1* contains $P\{lacW\}^{l(3)S057302}$ in *CG1528* and $P\{lacW\}^{eIF3\text{-}S10.DH2F31M1}$ in *CG9805* (GenBank/NCBI accession number *GU814269*). The *l(3)S057302* transgenic line harbors only $P\{lacW\}^{l(3)S057302}$ and *Oregon* wild-type contains any transposon.

We designed primers complementary to the target genes using *Fast PCR* software (KALENDAR [15]) and manual annotation. For the target genes hit by insertions, the primer pairs are as it follows: *13F1gammaC* (5'tggtcgtgcaggccatttgc3') and *21R1gammaC* (5'ctcgcacagatgcgacaaacc3') determining a 191 bp amplicon; *eIF3-S10-F* (5'cgtgaagtggtgcgcttg3') and *eIF3-S10-R* (5'gatctgacgaatcagacgc3') determining a 213 bp amplicon; *CG14709-F* (5'gtatctcgagattggcatatc3') and *CG14709-R* (5'gtcatctccaccaccttgcc3')

determining a 229 bp amplicon; *CG11033-F* (5'ctcaccgtagctttcctcc3') and *CG11033-R* (5'ggactgtcatagtactgctgc3') determining a 218 bp amplicon; *grappa-F* (5'cacagacagaaacctcagtg3') and *grappa-R* (5'cgcgatcacgtgtggtgcg3') determining a 212 bp amplicon; *pyd-F* (5'ggcgacgacgttcgacgcac3') and *pyd-R* (5'catcctgcagtggagtcg3') determining a 225 bp amplicon. Excepting *gammaCop* primers, the forward primer of all the five other primer pairs is spanning an intron. The primers characteristic for *RpL32* were taken from literature (FIUMERA [16]) and they are *RPL32-F* (5'ccgcttcaagggacagtatc3') and *RPL32-R* (5'gacaatctccttgcgcttct3') and define a 196 bp amplicon.

For each of the two biological replicates, we performed a distinct qRT-PCR reaction in a 96-wells plate. We worked each sample variant in three technical replicates, using Precision-2x qPCR Mastermix (Primerdesign) with SYBR Green and ROX, and 4 nmols of each primer/reaction, in a final volume of 25 µl. The quantity of cDNA template/reaction was 50 ng. The amplification reactions were performed with an ABI Prism 7500 analyzer and the cycling protocol was 1X (95^0C-10 min), 40X (95^0C-30 sec; 57^0C-30 sec; 72^0C-32 sec-data collection step), 1X dissociation stage (95^0C-15 sec; 60^0C-1 min; 95^0C-15 sec; 60^0C-15 sec).

After completion of runs, data were collected at a threshold value of 0.019004 and a baseline of 3-10.

We performed statistics on linearized ΔCt values, using ANOVA and Student's t-test from Minitab software (MCKENZIE [17]).

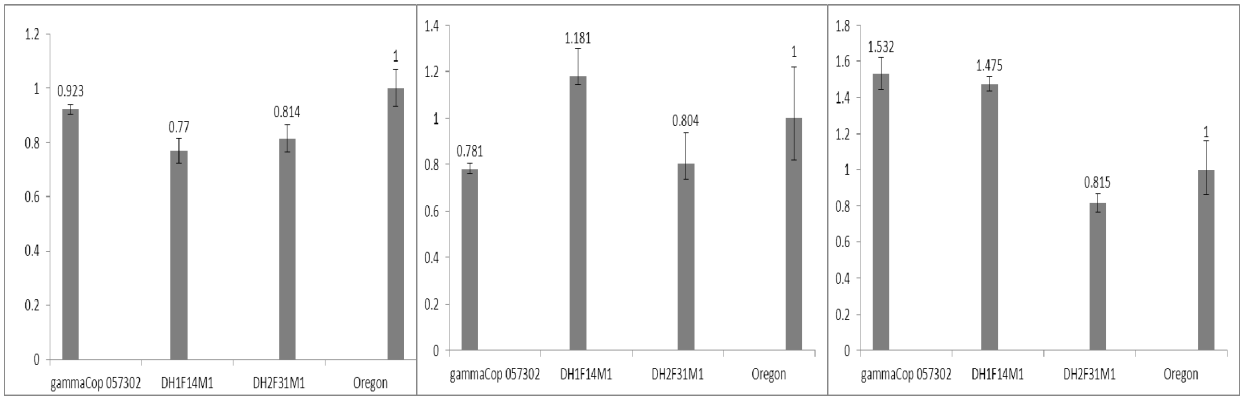
Results and Discussions

All qRT-PCR experiments were performed on templates derived from whole body RNA extracted from females since we induced the addressed insertional alleles $P\{lacW\}^{CG14709.DH1F14M1}$ and $P\{lacW\}^{eIF3\text{-}S10.DH2F31M1}$ in female germline.

For this study, we selected six genes hit by insertions in our experiments and for which bibliographic and bioinformatics data suggest a putative functional relationship excepting for *CG11033*. These genes are *gammaCop* from **100C**, known to be involved in triggering the immune response (CRONIN [18]), in tracheal system development (JAYRAM [19]) and presumably in oxidative stress response, and a group of genes that are relatively clustered in **82B1-86E11** chromosomal region. These are *CG14709* and *grappa*, both involved in oxidative stress response (AZAD [20], LUKE-GLASER [21]), *eIF3-S10*, activated when the organism is immunologically responsive (STUART [22]), *polychaetoid*, which helps the tracheal system development (JUNG [23]) and *CG11033*, with no apparently association with the other genes (for comparative analysis). We quantified their relative expression by qRT-PCR performed on females pertaining to four lines, namely *l(3)S057302*, DH2D31M1, DH1F14M1 and Oregon.

For each of the lines, we analyzed two biological replicates, and we computed the relative expression of target genes from three technical replicates/gene/biological replicate. The method for calculating quantitative relative expression was $2^{-\Delta\Delta Ct}$ (LIVAK [14]), using *RpL32* as a referential gene and gene expression in *Oregon* as calibrator. The amplification profiles revealed that all of the genes have very similar amplification efficiencies, being appropriate the use of $2^{-\Delta\Delta Ct}$ method (SCHMITTGEN [24]). The amplifications efficiencies were previously calculated for *gammaCop* and *RpL32* genes using serial dilutions and both efficiencies are close to 2 (data not shown).

We tested the statistical significance of the results describing relative expressions of *gammaCop*, *CG14709* and *eIF3-S10* (Figure 1) with both ANOVA and Student's t-test. For these analyses, we employed all ΔCt combinations possible for a particular gene in any mutant line.



A B C

Figure 1. The figure shows the graphics of relative expression of ***gammaCop*** (A), ***CG14709*** (B) and ***eIF3-S10*** (C) genes in the four lines that we analyzed. On the y-axis is the amplitude of relative gene expression. All the comparisons were made against expression in *Oregon* line, which was used as a calibrator, thus it has the value of 1 in all considered mutant lines; **1A**. In all the mutant lines, harboring one or two *P{lacW}* insertions, the relative expression of *gammaCop* is downregulated in comparison with its expression in *Oregon*. ANOVA analysis for all the lines taken together indicated that the model of expression variation is statistically robust ($p < 0.001$), but it could not provide a significant difference for the relative expression of *gammaCop* between *l(3)S057302* (symbolized as *gammaCop057302* in the figures 1A-C) and *Oregon* lines. When performing Student's t-test for *gammaCop* in *l(3)S057302* vs. *Oregon* the difference was significant ($p = 0.015$). The expression of *gammaCop* is also significantly different in *DH1D14M1* and *DH2F31M1* vs. *Oregon* ($p < 0.05$). The relative expression of *gammaCop* in *DH1F14M1* was significantly different from that in *l(3)S057302* ($p = 0.001$), but not different from that in *DH2F31M1* ($p = 0.241$). There are no consistent differences between relative expression of *gammaCop* between *l(3)S057302* and *DH2F31M1* ($p = 0.271$), a condition explainable by the large standard error among the linearized ΔCt values used in the analysis of *DH2F31M1* line; **1B**. Both ANOVA and Student's t-test indicate statistically relevant differences between the relative expression in each of the mutant lines against *Oregon* ($p < 0.01$). Interestingly, the relative expression of *CG14709* in *DH1F14M1*, which harbors a *P{lacW}* reinsertion near *CG14709*, besides the insertion in 5'UTR of *gammaCop*, is upregulated, at a level statistically different from that in both other mutant lines, which do not harbor insertions directly associated with *CG14709* ($p < 0.001$). Between *l(3)S057302* and *DH2F31M1* there were no statistically relevant differences ($p = 0.412$); **1C**. Both ANOVA an Student's t-test provided statistically relevant differences between the relative gene expression of *eIF3-S10* in the mutant lines comparative with the relative gene expression in *Oregon* ($p < 0.01$). When *gammaCop* and *eIF3-S10* were simultaneously affected by *P{lacW}* insertions in the *DH2F31M1* line the relative expression of *eIF3-S10* was severely downregulated comparative with its relative expression in the other two mutant lines, which harbor other combination of insertions ($p < 0.001$). The levels of upregulation of *eIF3-S10* in *l(3)S057302* and *DH1F14M1* mutant lines are not statistically different when performing ANOVA, but Student's t-test shows that there are statistically consistent differences between them ($p = 0.011$). For all the statistics, we used linearized Ct values and performed ANOVA and Student's t-test assuming unequal variance between the groups compared.

As depicted in Figure 1, all monitored genes exhibit a significant difference between the levels of relative gene expression in the transgenic lines comparative with their relative expression in *Oregon*. These results suggest that an insertional mutation affecting *5'UTR* region of *gammaCop* may determine relevant expression changes of more than one gene, leading to downregulation (*gammaCop* and *CG14709* in *l(3)S057302*) or upregulation (*eIF3-S10* in *l(3)S057302*) comparative to the same genes profiles in a wild-type genomic environment. When individually targeted by *P{lacW}* reinsertion in the double insertional mutant lines, both *CG14709* and *eIF3-S10* dramatically shifted their expression pattern from downregulation to upregulation and respectively from upregulation to downregulation. When not directly targeted by transposon insertions, the levels of relative expression of *CG14709* in those respective mutant lines (*DH2F31M1* and *l(3)S057302*) are quasi-equal (0.804 vs. 0.781). A similar feature is observable also for *eIF3-S10* gene. Comparing the average levels

of expression of *eIF-3S10* wild-type alleles in the double mutant line *DH1F14M1* and in *l(3)S057302* single mutant line, we observed almost equal specific levels of expression (1.475 vs. 1.532). These similarities are proving that the differences in the genetic backgrounds due to *TM3SbSer* and *TM6TbHu* balancer chromosomes do not influence the expression profiles (see Materials and Methods). In comparison with the situation when only *gammaCop* is hit by *P{lacW}*, if there is a supplementary insertion targeting other gene the expression level of *gammaCop* is subsequently downregulated. The difference is statistically significant only between *l(3)S057302* and *DH1F14M1*, probably because of the relative large standard deviation between the groups of linearized ΔCt values obtained from the two *DH2F31M1* biological replicates. The other three genes analyzed in this study, not directly affected by *P{lacW}* insertions, also exhibit dynamic expression profiles (data not shown). *CG11033*, *grappa* (*gpp*) and *polychaetoid* (*pyd*) are upregulated in all of the transgenic lines. Their relative expressions significantly differ in *l(3)S057302* line compared to Oregon ($p < 0.005$ for ANOVA and $p \leq 0.001$for t-test) as well as between *l(3)S057302* and the double insertional mutant lines ($p < 0.005$ for ANOVA), excepting *pyd* in *DH2F31M1*. Both ANOVA and Student's t-test failed to reveal significant differences between the expression of these three genes between *DH1F14M1* and *DH2F31M1* lines.

When comparing *DH1F14M1* with *Oregon*, only Student's t-test calculates significant differences for all the considered genes (maximum $p < 0.03$), but it fails to score a significant difference when comparing *DH2F31M1* and *Oregon*. The amplitude of standard deviations calculated for genes expressions in *DH2F31M1*, higher than for the other lines, may explain these results.

The above described expression features suggest that affecting genes that are supposed to collaborate may influence the level of relative expression of other genes pertaining to a putative functional network. In addition, it appears that the number of concomitantly mutated genes could be an important factor in determining the amplitude and the sign of gene expression variation. A similar approach focusing on expression fluctuations of several genes pertaining to a biological meaningful module was performed on *D. melanogaster* lines harboring P insertions (HARBISON [25]).

When comparing expression profiles in transgenic lines vs. *Oregon* we could score significant differences for almost all of the considered genes, the single notable exception being the relative expression of *CG11033*, *gpp* and *pyd* in *DH2F14M1*. Excepting for *CG14709* in *DH2F31M1* and for *eIF3-S10* in *DH1F14M1*, the described genes clearly changed their expression profile in double insertional mutants comparative to the single insertional line (*l(3)S057302*). Analysis of the expression profiles among the double insertional mutants did not reveal significant differences for any particular gene, but only when they were directly hit by *P{lacW}* artificial transposon (the situations of *CG14709* in *DH1F14M1* and of *eIF3-S10* in *DH2F31M1*). These results indicate that a co-regulation mechanism might exist between the genes subjected into this relative expression profile study. The fact that *CG14709* and *eIF3-S10* do not significantly influence each other's expression in double insertional mutant background is a sort of a deviation from the model that we are proposing, but there may be an explanation. More accurately, *CG14709* is the only gene from the group that is specifically biased to be expressed in males, while all other genes tend to express mainly in females, excepting for *gpp* that has no expression biases (GNAD [26]). Since *eIF3-S10* has a very robust expression in females but *CG14709* have a male-biased expression preference, we can speculate that the changes in *CG14709* expression cannot influence the expression of *eIF3-S10*. In addition, the apparent level of downregulation is milder in *DH1D14M1* (harboring the second insertion associated with *CG14709*) than in *DH2F31M1* (harboring the second insertion associated with *eIF3-S10*).

Conclusions

We performed qRT-PCR for evaluating the relative expression levels of several genes for which we previously identified putative functional relationships. Three transgenic lines harboring *P{lacW}* insertions were compared to wild-type *Oregon* regarding to the modulation of relative expression of *gammaCop*, *CG14709*, *eIF3-S10*, *pyd*, *gpp* and *CG11033* genes. The outcome of our experiments demonstrated that when a single *P{lacW}* insertion affects *gammaCop* gene in line *l(3)S057302*, the expression profile for all the monitored genes seems to be significantly modified. In the double insertional mutant lines *DH1F14M1* and *DH2F31M1* the gene expression profiles are different from those particular for *l(3)S057302* or *Oregon* and all differences are statistically relevant. These results suggest that there is a sort of co-regulation between the levels of expression of different genes pertaining to a functional network, which is revealed when one or two of them are affected by insertions.

In conclusion, the study of specific expression profiles may reveal situations when mobilized *P{lacW}* artificial transposons reinsert in genes that are amenable to have functional associations with the gene harboring the original insertion.

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References

1. D.R DORER, S. HENIKOFF, Expansions of transgene repeats cause heterochromatin formation and gene silencing in Drosophila, *Cell* 77(7), 993-1002 (1994).
2. P. DIMITRI, N. JUNAKOVIC, Revising the selfish DNA hypothesis: new evidence on accumulation of transposable elements in heterochromatin, *Trends Genet* 15, 123-124 (1999).
3. J.K. LIM, M.J. SIMMONS, Gross chromosome rearrangements mediated by transposable elements in Drosophila melanogaster, *Bioessays* 16, 269-275 (1994).
4. W.B. EGGLESTON, N.R. RIM, J.K. LIM, Molecular Characterization of Hobo- Mediated Inversions in Drosophila Melanogaster, *Genetics* 144(2), 647-656 (1996).
5. J. BROSIUS, Retroposons–seeds of evolution, *Science* 251, 753 (1991).
6. M. LONG, Evolution of novel genes, *Curr Opin Genet Dev* 11, 673-680 (2001).
7. M. LONG, Preface, Origin and evolution of new gene functions, *Contemporary Issues In Genetics and Evolution* 10, 97 (2003).
8. H. ZHAO, G. BOURQUE, Recovering Genome Rearrangements in the Mammalian Phylogeny, *Genome Res.* 19, 934-942 (2009).
9. A.C. SPRADLING, D. STERN, I. KISS, J. ROOTE, T. LAVERTY, G.M. RUBIN, Gene Disruptions Using P Transposable Elements, PNAS 92, 10824-10830 (1995).
10. A.A. ECOVOIU, A.C. RATIU, M. GRAUR, L. SAVU, High-Frequency of P{lacW} Double Local Reinsertions in Chromosomal Region 100C of Drosophila melanogaster are Amenable to a Homing-Like Mechanism, *Bulletin UASVM Animal Science and Biotechnologies* 67(1-2), 381-387 (2010).
11. A.C. RATIU, A.A. ECOVOIU, M. GRAUR, L. SAVU, L. GAVRILA, Transposition of P{lacW}gammaCop057302 into the germline of Drosophila melanogaster correlates with retaining of the original insertion, *Roumanian Biotechnological Letters* 13(5), 3891-3900 (2008).
12. A.A. ECOVOIU, A.C. RATIU, M. GRAUR, L. SAVU, Mapping of multiple P{lacW} insertions into the germline of Drosophila melanogaster, *Bulletin USAMV Animal Sciences and Biotechnologies* 66 (1-2), 424-429 (2009).
13. E. BIER, H. VAESSIN, S. SHEPHERD, K. LEE, K. McCALL, K. *et al*., Searching for pattern and mutation in the Drosophila genome with a PlacZ vector, *Genes Dev*. 3, 1273-1287 (1989).
14. K.J. LIVAK, T.D. SCHMITTGEN, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, *Methods* 25(4), 402-8 (2001).
15. R. KALENDAR, FastPCR: a PCR primer and probe design and repeat sequence searching software with

additional tools for the manipulation and analysis of DNA and protein, (www.biocenter.helsinki.fi/bi/programs/fastpcr.htm) (2008).
16. A.C. FIUMERA, B.L. DUMONT, A.G. CLARK, Sperm Competitive Ability in Drosophila melanogaster Associated With Variation in Male Reproductive Proteins, *Genetics* 169, 243-257 (2005).
17. J.D. MCKENZIE, R. GOLDMAN, The student edition of Minitab for Windows user's manual: release 12, New York: Addison-Wesley, 464 (1999).
18. S.J.F. CRONIN, N.T. NEHME, S. LIMMER, S. LIEGEOIS, J.A. POSPISILIK, D. SCHRAMEK, A. LEIBBRANDT, R. SIMOES, S. GRUBER, U. PUC, I. EBERSBERGER, T. ZORANOVIC, G.G. NEELY, A. HAESELER, D. FERRANDON, J.M. PENNINGER, Genome-wide RNAi screen identifies genes involved in intestinal pathogenic bacterial infection, *Science* 325 (5938), 340-343 (2009).
19. S.A. JAYARAM, K.A. SENTI, K. TIKLOVA, V. TSAROUHAS, J. HEMPHALA, C. SAMAKOVLIS, COPI vesicle transport is a common requirement for tube expansion in Drosophila, *PLoS ONE* 3(4): e1964, 1-11 (2008).
20. P. AZAD, D. ZHOU, E. RUSSO, G.G. HADDAD, Distinct mechanisms underlying tolerance to intermittent and constant hypoxia in Drosophila melanogaster, *PLoS One* 4(4):e5371 (2009).
21. S. LUKE-GLASER, M. ROY, B. LARSEN, T. LE BIHAN, P. METALNIKOV, M. TYERS, M. PETER, L. PINTARD, CIF-1, a shared subunit of the COP9/signalosome and eukaryotic initiation factor 3 complexes, regulates MEL-26 levels in the Caenorhabditis elegans embryo. *Mol Cell Biol.* 27(12), 4526–4540 (2007).
22. L.M. STUART, J. BOULAIS, G.M. CHARRIERE, E.J. HENNESSY, S. BRUNET, I. JUTRAS, G. GOYETTE, C. RONDEAU, S. LETARTE, H. HUANG, P. YE, F. MORALES, C. KOCKS, J.S. BADER, M. DESJARDINS, R.A.B. EZEKOWITZ, A systems biology analysis of the Drosophila phagosome, *Nature* 445, 95-101(2007).
23. A.C. JUNG, C. RIBEIRO, L. MICHAUT, U. CERTA, M. AFFOLTER, Polychaetoid/ZO-1 is required for cell specification and rearrangement during Drosophila tracheal morphogenesis, *Curr. Biol.* 16(12), 1224-1231 (2006).
24. T.D. SCHMITTGEN, K.J. LIVAK, Analyzing real-time PCR data by the comparative C_T method, *Nature Protocols* 3, 1101-1108 (2008).
25. S.T. HARBISON, M.A. CARBONE, J.F. AYROLES, E.A. STONE, R.F. LYMAN, T.F.C. MACKAY, Co-regulated transcriptional networks contribute to natural genetic variation in Drosophila sleep, *Nature Genetics* 41(3), 371-375 (2009).
26. F. GNAD, J. PARSCH, Sebida: a database for the functional and evolutionary analysis of genes with sex-biased expression, *Bioinformatics* 22, 2577-2579 (2006).