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Reduced Expression of *Glycerol-3-phosphate dehydrogenase* (*Gpdh*) in *Drosophila melanogaster Pasha*-mutants Suggests A miRNA-dependency in Its Regulation

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Running title: *Gpdh* regulation by miRNA in *Drosophila*

Abstract. In *Drosophila*, the Glycerol-3-phosphate dehydrogenase (Gpdh) enzyme plays an active role in many pathways, including the glycerol metabolic pathway and the alpha-glycerophosphate cycle. It is also important for ethanol metabolism, as well as flight muscle development. Recent years have exposed small RNAs as a major posttranscriptional regulator of multiple metabolic-pathway genes. Of the many kinds of these RNAs at work, micro RNAs (miRNAs) are the most widely implicated and well understood. However, the roles they may play in regulating *Gpdh* has never been shown in any model organism. In this study, a *pasha*-mutant *D. melanogaster* strain was found to express only 25% of the *Gpdh* levels typical of their wild type counterparts. Such mutants lack the ability to produce Pasha, a protein integral during miRNA-processing, and as a consequence do not produce mature miRNAs. As miRNA-centric regulation often culminates in the depletion of their targets, the concurrent downregulation of *Gpdh* observed in their absence here therefore alludes to two possibilities: one, that rather than being explicitly bound and repressed by miRNAs, *Gpdh* expression relies on their action upon an upstream *Gpdh*-antagonist; or two, that *Gpdh* may come under the regulation of another class of miRNA-like elements called mirtrons, which do not require Pasha to be processed into their functional form. The preliminary findings in this study further highlights the imperative nature of miRNAs in regulating metabolic processes and subsequently, ensuring proper organismal development and its continued survival.

Keywords: Gpdh, miRNA, Drosophila, Pasha mutant

INTRODUCTION

Metabolism drives life: no biotic organism is capable of survival without it. Often, multiple metabolic pathways converge together to enable growth and progression, maintenance of homeostatic balance, and many other vital processes. Glycerol-3-phosphate dehydrogenase (Gpdh) is a crucial protein involved in both the glycerol metabolic pathway and the alpha-glycerophosphate cycle. These are components of a larger network forming the phospholipid metabolism pathway (Carmon and MacIntyre 2010; Liu 2010; Azzam and Liu 2013). As an enzyme, Gpdh primarily functions to reversibly catalyze the conversion of dihydroxyacetone phosphate (DHAP) to sn-glycerol 3-phosphate in the cytosol. This action eventually leads to the production of glycerol, utilized for osmoregulation purposes within the cell. In humans, a second isoform of Gpdh is differentially localized to the mitochondria and helps in the maintenance of redox potential across its inner membrane (Harding et al. 1975).

Three isoforms of *Gpdh* are found in *Drosophila* (Grell, 1967). Two of these are detected during larval development, whilst the third is exclusively adult (von Kalm et al. 1989). Changing levels of Gpdh activity are shown to coincide with certain points of larval growth, pupal histolysis, and adult tissue differentiation (Wright and Shaw, 1969). The interdependent localization patterns of the Gpdh-1 isoform of the enzyme along with GAPDH and aldolase in *Drosophila* has been proven to be crucial in controlling flight ability and wing structure integrity (Sullivan et al. 2003; Wightman et al. 2013). Antibody staining further characterizes the relationship of these three proteins, and how they are associated to each other within the Z-discs and M-lines of the thoracic region (Wojtas et al. 1997; Sullivan et al. 2003). Such discovery not only highlights Gpdh's importance during development, but also displays that correlated enzymes within a pathway are purposely co-localized together. Gpdh is implicated in ethanol tolerance and processing in *Drosophila* as well (Geer et al. 1983; Geer et al. 1993). An overexpression of Gpdh-1 is known to reduce ethanol production (Nevoight and Stahl, 1996).

Despite the extensive knowledge we now know about Gpdh, very little information regarding its regulation is available. In recent years, an emerging power player in metabolic pathway control within *D. melanogaster* is the small, non-coding RNA (ncRNA). MicroRNAs (miRNAs) is one such species of ncRNAs most implicated in a wide range of developmental processes within the fly. Approximately 204 loci are identified as canonical miRNA genes, with another 34 identified as mirtron genes (Lyu et al. 2014). Their biogenesis occurs in a sequential manner (Azlan et al. 2016), and is dependent upon the initial cleavage of long pri-miRNAs by a pair of proteins called Pasha and Drosha (Denli et al. 2004; Gregory et al. 2004; Martin et al. 2009). In its effective mature state, the miRNA is only ~22 nucleotides in length. Nonetheless, its potency as a part of the RNA-induced silencing complex (RISC) (Lund et al. 2004) has been proven time and time again (Mallory and Vaucheret 2010; Tomari et al. 2007; Ambros 2004).

Specific miRNAs have been implicated in the regulation of metabolic components within *Drosophila*. MiR-8 response to steroids such as ecdysone positively affects body growth (Jin et al. 2012). The co-operative nature of certain miRNAs with one another during metabolism regulation is demonstrated here, as the steroid cycle is known to be modulated by the activities of miR-14 (Varghese et al. 2007). The relationship between these two miRNA species became even more evident when miR-14 was found to additionally affect insulin production by secretory cells within the brain (Varghese et al. 2010). MiR-8 had previously been shown to target the mRNA of the *u-shaped* (*USH*) gene, a repressive interaction ultimately influencing the insulin response pathway and fat accumulation (Hyun et al. 2009).

Here, we show how the disabling of miRNA-machinery leads to implicitly lower levels of *Gpdh* expression. This finding provides an early insight into the relationship between miRNAs and the gene's enzymatic activities, strengthening the stance that metabolic control within *D. melanogaster* heavily involve these small regulatory elements. Future research aimed towards understanding how this came to be shall monumentally improve our understanding of the ways in which miRNAs help shape and influence crucial processes within the cell.

MATERIALS AND METHODS

Fly Stock Maintenance and Sample Collection

The fly lines utilized in this study were available stocks obtained from Bloomington Drosophila Stock Centre (BDSC). Wild-type is *y w*. A line heterozygous for *pasha*^{LL03360} (FBgn0039861) mutation (Berdnik et al. 2008) was chosen. Homozygous mutants were generated through backcrossing. All stocks were

grown and maintained at 25°C on standard cornmeal fly feed. Wild-type and experimental samples were collected at the end of the 3rd instar larvae stage.

RNA Extraction and CDNA Production

Larval samples were subjected to homogenization using QIAshredder (Qiagen), followed immediately with RNA extraction with the RNeasy Plus Mini Kit (Qiagen) as described by the included protocol. Stock RNA samples were kept at -80°C. Reverse transcription was carried out using QuantiTect Reverse Transcription Kit (Qiagen) with a prior genomic DNA removal step added, as per manufacturer's suggestions. Freshly synthesized cDNA were diluted to a factor of 1:10, and immediately stored at -20°C until further required.

Primer Design and Quantitative PCR

The primers for Gpdh were: Gpdh-F 5'- AATCGCGGAGCCAAGTAGTACT-3' and Gpdh-R 5'-TCGATGGACTCGCTGATGTG-3. The ribosomal protein component *Rp49* was chosen as the normalization control. The primers designed for the gene were RP49-F 5'-CCGCTTCAAGGGACAGTATCTG-3' and RP49-R 5'- ATCTCGCCGCAGTAAACGC-3'. For each 10µl qPCR reaction, 1µl of previously prepared diluted cDNA was utilized alongside 1uM of primers and Fast SYBR Green Master Mix (Applied Biosystem). For each sample, three technical replicates were prepared. qPCR were carried out using 7500 Fast Real-Time PCR System (Applied Biosystems) using the *Fast* setting: initial denaturation at 95°C for 20 seconds, followed by 40 repeats of denaturation at 95°C for 3 seconds and simultaneous primer annealing and elongation at 60°C for 30 seconds. A final round of denaturation and annealing-extension was included at 95°C for 15 seconds and 60°C for 1 minute, respectively. qpCR runs were repeated with an additional biological replicate for the purpose of validation and consolidation observed results.

***In silico* Prediction of Binding miRNAs**

A 3'UTR sequence redundant for all isoforms of *Gpdh* (FlyBase ID#CG9042) was identified for TargetScanFly, Version 6.2, last updated in 2015 (Kheradpour et al., 2007). This online prediction tool (http://www.targetscan.org/cgi-bin/targetscan/fly_12/) identifies mRNA-targets of miRNA based on complementary-seed-pairing (Lewis et al., 2003). Those displaying agreeable seed sequences to the 3'UTR are therefore shortlisted as potential *Gpdh*-targeting miRNAs, ranked in order of predicted strength in their binding. An additional weightage in the form of branch length scores is used as a measure of conservation of miRNA binding sites. Scores lower than 3.16 (i.e. 60% of branch lengths) is deemed poorly conserved. The lower the score, the more preliminary the prediction, the less likely is the miRNA-mRNA interaction to actually take place *in vivo* (Kheradpour et al., 2007).

RESULTS AND DISCUSSION

Pasha* Mutants Display Significantly Reduced Levels of *Gpdh

Homozygous mutants of *pasha* do not survive to adulthood. Development instead halted at 3rd instar larvae prior to pupation. This in itself demonstrated the importance of small RNA activity in ensuring survival. The protein Pasha which mutants lacked is one-half of the first processing complex of pri-miRNA alongside Drosha. Its elimination meant impairment of the small RNA machinery as a whole, rendering

lethality beyond the larval stages of *Drosophila* development. The changes in Ct values of *Gpdh* and *RP49* between wild-type and *pasha* mutant 3rd instar larvae were first analyzed. As shown in Table 1, the mean Ct values for *RP49* in wild type 3rd instar larvae are 17.736 and 17.582 with standard deviations of 0.054 and 0.094, whereas the mean Ct values in *pasha* mutant 3rd instar larvae are 16.912 and 17.765 with a standard deviation of 0.105 and 0.029, respectively. Comparatively, *Gpdh* is less highly-abundant to *RP49*, with average Ct values in wild type 3rd instar larvae of 22.307 and 21.924 with standard deviation of 0.048 and 0.012, and averages of 24.253 and 25.144 with a standard deviation of 0.059 and 0.022 in *pasha* mutant 3rd instar larvae, respectively. It is already clear from these initial observations that whilst *RP49* abundance remain stable regardless of *pasha* mutation, *Gpdh* levels appear to be signficantly reduced in the *pasha* mutant when compared to wild-type 3rd instar larvae.

To further reiterate this finding, the ΔΔCt method of calculating fold-change was applied. The 'Δ' or delta values were obtained as (a) differences in *Gpdh* Ct values when normalized against an *RP49* standard curve, and (b) differences in *Gpdh* Ct values against *RP49* in the bivariate data. The results are shown as percentages in Figure 1. Levels of Gpdh in *pasha* mutant 3rd instar larvae is only about 25% of that of wild-type 3rd instar larvae. These results show that in the absence of most miRNAs, the level of *Gpdh* is reduced.

This observation is peculiar as miRNA are known to act through suppression and degradation. Consequently for many well-studied miRNAs, relationships with their targets are often antagonistic. The elimination of the regulating miRNA typically results in heightened expression levels of its target mRNA(s). Nonetheless, some miRNAs have previously been shown to instead upregulate genes (Vasudevan & Tong 2007; Place et al. 2008), although such events are rare. In the case of *Gpdh* in *Drosophila*, and knowing that most miRNAs repress genes, it is most likely that the concurrent reduction of miRNA and *Gpdh* is the result of miRNAs targeting an upstream component of the Gpdh pathway, rather than directly eliciting *Gpdh* mRNA itself. It is however unclear at the moment, which miRNAs are responsible. The mutation in *pasha* is expected to disrupt most miRNA. The answer could thus lie with mirtrons, which bypass Drosha and Pasha cleavage on its way to becoming mature miRNA (Okamura et al. 2007; Ruby et al. 2007).

In humans, *Gpdh* was shown to be regulated by hsa-miR-30 (Zaragosi et al. 2011). To see whether this is also true in flies, *in silico* target prediction analysis of the *Gpdh* 3' UTR using TargetScan Fly was performed. Results show that the gene does not have conserved miRNA binding sites. With less stringent scoring parameters applied, only 12 poorly conserved target sites were identified, summarized in Table 2 below. These are binding regions for dme-miR-985, dme-miR-999, dme-miR-124, dme-miR-9 and dme-miR-4, amongst others. Noticeably, *Drosophila* does not appear to have an equivalent of hsa-miR-30. This could explain the reason why the antagonistic-rule of miRNA-target relationships was not adhered to, as *Gpdh* might not be directly regulated by a miRNA in the drosophilid cell. Regardless, future studies addressing the peculiarities observed in this study are necessary.

CONCLUSIONS

Not only is the miRNA-machinery in *D. melanogaster pasha*-mutants impaired, their survival is also compromised, as they do not develop into adults. This study shows that *Gpdh* levels were considerably reduced in larval tissue of *D. melanogaster* lacking *pasha* expression. As an important enzyme in various metabolic pathways within the fly, this paralleled relationship between *Gpdh* to miRNA availability emphasizes the importance of metabolism to organismal survival. The unexpected downturn of *Gpdh* in the absence of miRNAs also implies that its mRNA may not in itself be a direct target; rather, an upstream antagonist of Gpdh expression is the component of *Gpdh*-involving pathways heavily guarded by such

ncRNAs. Another possibility is that rather than canonical miRNAs, *Gpdh* is regulated by a novel group of Pasha-independent ncRNAs called mirtrons. Regardless, these findings all point towards the dependency of *Gpdh* upon the miRNA-machinery in maintaining normal levels of its expression within *D. melanogaster*, inevitably dictating whether events requiring its dehydrogenase functions run smoothly or otherwise. An improvement to the methods seen here is to assess *Gpdh* expression level in regards to more than just *RP49*. The inclusion of two or more genes for normalization is encouraged to compensate for each other's intrinsic fluctuations, and in this case will provide a less-biased estimation of *Gpdh* level changes. If the same observations do indeed persist after these more stringent parameters have been introduced, proteomics and co-precipitation methods could be applied to truly decipher how small RNAs may influence *Gpdh* and simultaneously, the many metabolic processes the enzyme is involved in.

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Table 1: Mean Ct and standard deviation of wild-type and *pasha* mutant samples

Sample	Target	Mean Ct	Standard Deviation
Wild type 3rd instar larvae - 1	*RP49*	17.736	0.054
Wild type 3rd instar larvae - 2	*RP49*	17.582	0.094
pasha mutant 3rd instar larvae - 1	*RP49*	16.912	0.105
pasha mutant 3rd instar larvae - 2	*RP49*	17.765	0.029
Wild type 3rd instar larvae - 1	*Gpdh*	22.307	0.048
Wild type 3rd instar larvae - 2	*Gpdh*	21.924	0.012
pasha mutant 3rd instar larvae - 1	*Gpdh*	24.253	0.059
pasha mutant 3rd instar larvae - 2	*Gpdh*	25.144	0.022

Table 2. *In silico* prediction of miRNAs which may bind to the 3' UTR region of *Gpdh* mRNA (CG9042). Outcomes listed were obtained from TargetScanFly, Version 6.2.

dme-MiRNA	Target on 3'UTR of *Gpdh*	Branch length score
miR-985	Position 28-34	2.51
miR-999	Position 112-118	0.41
miR-124	Position 140-146	0.41
miR-976	Position 163-170	1.82
miR-981	Position 184-190	0.41
miR-979	Position 195-201	0.11
miR-9b	Position 210-216	1.43
miR-9c	Position 210-216	1.43
miR-9a	Position 210-216	1.43
miR-4	Position 220-226	2.51
miR-1001	Position 386-392	0.30
miR-927	Position 476-482	0.13

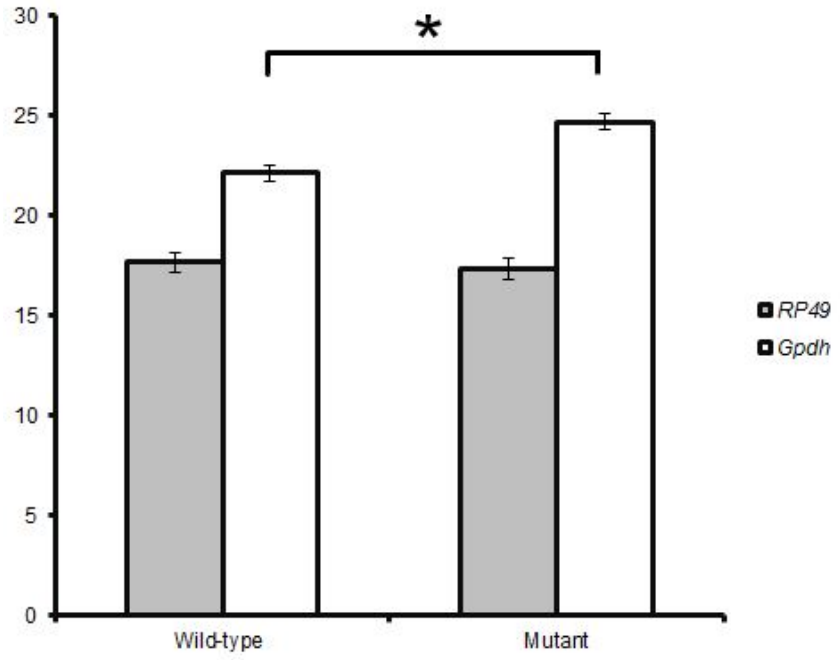

Figure 1: Expression levels, as expressed in Cq terms, of *Gpdh* normalized to *RP49* in wild type versus *pasha* mutant 3rd instar larvae. Statistical analysis by Student T-Test affirms the significant decrease in *Gpdh* levels in mutants compared to wild-type (**P<0.001*)

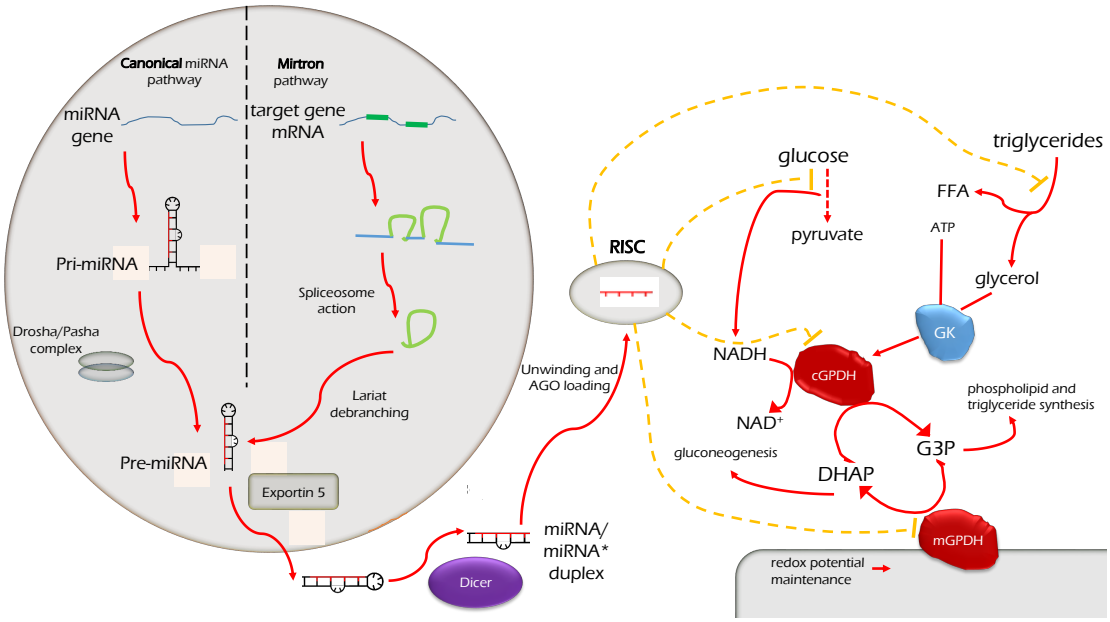

Figure 2: Biogenesis of MicroRNAs and the predicted points in *Gpdh*-involved pathways at which they might assert a regulatory role