RNA BIOLOGY
2019, VOL. 16, NO. 1, 69–81
https://doi.org/10.1080/15476286.2018.1558907

Taylor & Francis
Taylor & Francis Group

RESEARCH PAPER

OPEN ACCESS Check for updates

Transcriptional dynamics of microRNAs and their targets during Drosophila neurogenesis

Peter Menzel [a], Alexandra L. McCorkindale [a], Stefan R. Stefanov [a,b], Robert P. Zinzen [a], and Irmtraud M. Meyer [a,b]

[a]Berlin Institute for Molecular and Systems Biology, Max Delbrück Center for Molecular Medicine, Berlin, Germany; [b]Institute of Biochemistry, Department of Biology, Chemistry, and Pharmacology, Freie Universität Berlin, Berlin, Germany

ABSTRACT

During *Drosophila melanogaster* embryogenesis, tight regulation of gene expression in time and space is required for the orderly emergence of specific cell types. While the general importance of microRNAs in regulating eukaryotic gene expression has been well-established, their role in early neurogenesis remains to be addressed. In this survey, we investigate the transcriptional dynamics of microRNAs and their target transcripts during neurogenesis of *Drosophila melanogaster*. To this end, we use the recently developed *DIV-MARIS* protocol, a method for enriching specific cell types from the *Drosophila* embryo *in vivo*, to sequence cell type-specific transcriptomes. We generate dedicated small and total RNA-seq libraries for neuroblasts, neurons and glia cells at early (6–8 h after egg laying (AEL)) and late (18–22 h AEL) stage. This allows us to directly compare these transcriptomes and investigate the potential functional roles of individual microRNAs with spatiotemporal resolution genome-wide, which is beyond the capabilities of existing *in situ* hybridization methods. Overall, we identify 74 microRNAs that are significantly differentially expressed between the three cell types and the two developmental stages. In all cell types, predicted target genes of down-regulated microRNAs show a significant enrichment of Gene Ontology terms related to neurogenesis. We also investigate how microRNAs regulate the transcriptome by targeting transcription factors and find many candidate microRNAs with putative roles in neurogenesis. Our survey highlights the roles of microRNAs as regulators of differentiation and glioneurognesis in the fruit fly and provides distinct starting points for dedicated functional follow-up studies.

ARTICLE HISTORY
Received 26 July 2018
Revised 3 December 2018
Accepted 4 December 2018

KEYWORDS
Drosophila melanogaster; microRNAs; nervous system; development; spatiotemporal expression; transcriptomics

1. Introduction

MicroRNAs (miRNAs) are a class of small non-coding RNAs involved in post-transcriptional regulation. They are transcribed from precursor microRNA genes, which are processed in the nucleus, exported to the cytoplasm and further processed into typically ~22nt long functional RNA products [1].

These short molecules serve as a guide for binding the RISC complex to target sites located typically in the 3′-UTRs of protein-coding genes, which in turn affects the mRNA translation into protein. MicroRNAs have been shown to play diverse roles in the cell, from differentiation to cell death [2] and are also widely abundant in the nervous system [3].

In the fruit fly *Drosophila melanogaster*, microRNAs reduce mRNA translation mainly by decreasing mRNA levels of their target genes through recruitment of enzymes responsible for mRNA decay and degradation [4–6]. Currently, 238 microRNA genes are annotated in the *Drosophila melanogaster* genome (Ensembl release 85 [7,8]), many of them located in introns of protein-coding genes. MicroRNA target sites in other genes can be computationally predicted and experimentally confirmed [9,10] and individual microRNAs can have hundreds of target sites. Because microRNAs are often evolutionary conserved in metazoans [11], many *D. melanogaster* microRNAs are homologous to human microRNAs [12]. In order to infer the possible functional roles of microRNAs, a range of experimental techniques have been employed in the past, ranging from over-expression and inhibition [13,14] to identifying their expression patterns using *in situ* hybridization or sequencing [15,16].

The development of the *Drosophila melanogaster* embryo is a well studied process on the cellular level. Early neurogenesis is characterized by a series of events, starting with the subdivision of the lateral neurogenic ectoderm at around 2 h after egg laying (AEL), followed by the formation of proneural clusters and phases of neuroblast delamination starting at around 3–4 h AEL. Neuroblasts then undergo several rounds of asymmetric divisions, giving rise to ganglion mother cells, which, starting at around 6 h AEL, then divide further into neurons and/or glia [17,18]. While several transcription factors are known to be key determinants for these cell-fate decisions [19], the role of microRNAs in differentiation is not well characterized so far. Previous studies have shown that microRNAs show diverse spatial and temporal expression patterns during embryonic development [15,20]. Moreover, the expected anti-correlation of microRNA and target gene expression has been experimentally confirmed for several microRNAs [21]. For example, *mir-124* and its target *repo* are often expressed in spatial

CONTACT Irmtraud M. Meyer irmtraud.meyer@cantab.net
Supplementary material for this article can be accessed here.

reciprocity in the central nervous system [21] and *mir-124* is an important regulator for nervous system development in the early embryo [22]. The RNA-binding protein *elav*, which serves as a neural marker gene, is also believed to be regulated post-transcriptionally by microRNAs from the *mir-279/996* family [23]. Other microRNAs have been shown to have particular roles during embryogenesis. For example, mature microRNAs originating from the *mir-309* cluster promote maternal mRNA turnover as part of the zygotic degradation pathway [24]; *mir-1* and *mir-9a* have both been shown to play crucial roles during muscle development [25] and in the specification of sensory organs [26]; and *mir-7* has not only been confirmed to be a regulator in several developmental pathways, but is also expressed in photoreceptor cells [27]. In the Drosophila larvae, *mir-iab4/iab8* targets *Ultrabithorax*, which regulates the self-righting ability [28]. The paralogous microRNAs *mir-263a/b* down-regulate the pro-apoptotic gene *head involution defective* in the developing eye [29]. Another important microRNA, *mir-184*, is crucial for the development of the female germline during early embryogenesis [30].

In situ hybridization probes to nascent microRNA transcripts in the Drosophila embryo showed that, for example, *mir-124, mir-315*, the cluster comprising *mir-13b* and *mir-2c*, and others are specifically expressed in the embryonic nervous system [15]. In the developed fly, several microRNAs have been shown to be crucial in nervous system function. For example *mir-9c, mir-31a*, and *mir-980* are involved in Drosophila memory formation [31,32]. Another example is *mir-276a*, which regulates expression of the dopamine receptor *DopaR*, a central actor in olfactory memory formation [33].

To summarize, while it has already been possible to experimentally confirm the functional roles of select Drosophila microRNAs in dedicated experiments [34,35], the functional importance of the majority of microRNAs remains elusive.

Most of the existing quantitative expression studies either focus on specific microRNAs and their expression levels or on whole-embryo microRNA expression levels during embryonic development. Ideally, however, one would like to perform transcriptome-wide studies of microRNAs and their target genes in a tissue- or cell type-specific manner, in order to get an unbiased and more accurate view of their potential function during embryonic development. As there is little known so far about the cell type-specific expression and regulatory function of microRNAs during early neurogenesis, we analyze the spatiotemporal expression of microRNAs and their target genes during *Drosophila melanogaster* neurogenesis at two time points using the *DIV-MARIS* protocol introduced in [36], which is adapted from *MARIS* [37], and uses marker-based fluorescence sorting of individual embryonic cells, followed by RNA extraction and sequencing. For this purpose, we create dedicated sequencing libraries for small RNA transcripts at two developmental stages and from three different cell types. The combination of these smallRNA-Seq data sets and the standard RNA-Seq data sets from [36] allows us to simultaneously study the expression patterns of both microRNAs and their target genes. Subsequent *in silico* analysis allows us to identify the major cell type-specific microRNAs and their putative regulatory roles in Drosophila neurogenesis in a quantitative manner and with unprecedented spatiotemporal resolution.

2. Results

2.1. Small RNA sequencing of neural cell types during embryogenesis

In this study, we create smallRNA-Seq libraries for neuroblasts, neurons and glia cells at 6–8 h after egg laying (AEL) and 18–22 h AEL using the *DIV-MARIS* protocol, which is described in detail in [36]. In brief, *D. melanogaster* embryos were collected at the specified time points and the cell populations comprising neuroblasts, neurons, and glia were sorted after staining by FACS using primary directed and fluorescent secondary antibodies against the endogenous markers *prospero* (*pros), elav*, and *repo*, respectively. The specificity of the antibodies for the three cell types has been confirmed in [36]. The early collection bin at 6–8 h AEL comprises neuroblast proliferation and diversification into neurons and glia, whereas the late collection bin at 18–22 h AEL marks the end of embryogenesis with fully differentiated neurons and glia. In total, 20 samples are obtained from the three neural cell types at two time points, with marker-positive and marker-negative FACS selection in biological duplicates (Figure 1). From each sample, the small RNA fraction was sequenced using Illumina high-throughput sequencing (Supplementary Table 1). After adapter trimming, 584 million reads remained, of which 343 million reads were mapped to the Drosophila genome. Of these, 146 million reads were counted towards the 238 annotated microRNA genes.

2.2. Cell type-specific microRNA profiles and differential expression

The first key question to address is which microRNAs are significantly differentially expressed between the marker-positive and the respective marker-negative FACS fractions. After read mapping, 93 out of the 238 annotated fly microRNAs have at least 1000 mapped reads across all samples, which we use as a minimum threshold for a microRNA to be considered in our *in silico* analyses. Of those 93, 29 microRNAs are among the top 20 expressed microRNAs across all marker-positive fractions (Figure 3(a)). The five most abundant microRNAs across all samples are *mir-184, mir-7, mir-276a, mir-10*, and *mir-286* and we find in total 13 microRNAs that are among the top 20 in each of the marker-positive fractions. This shows that microRNAs that are highly expressed in the marker-positive fractions in terms of absolute abundance are not necessarily unique to the cell type (compare e.g. *mir-184* in neuroblasts and glia at 6–8 h AEL) or developmental stage (compare e.g. *mir-10* for neurons at both time points).

In fact, only the microRNAs highlighted by a black arrow in Figure 3(a) show significant changes in expression between the marker-positive and marker-negative FACS fractions. Of all expressed microRNAs, we find 74 microRNAs that are significantly differentially expressed in at least one sample (Figure 4), with 39 microRNAs up-regulated and 43 microRNAs down-regulated in the

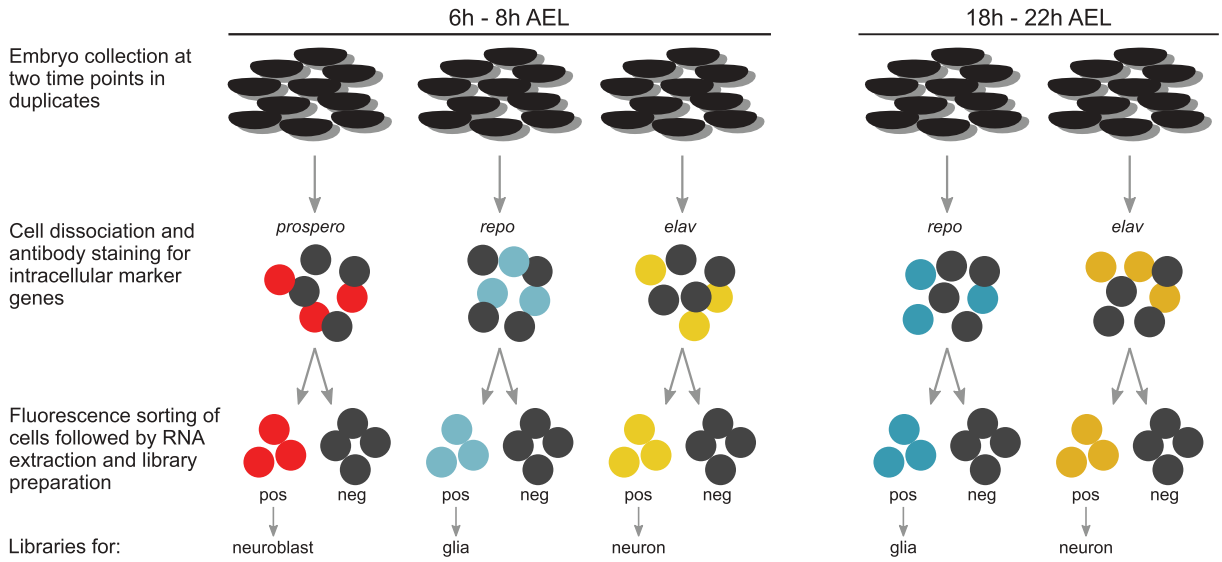


Figure 1. Overview of sample collection, cell sorting and creation of FACS-sorted marker-positive and marker-negative libraries for RNA-sequencing.

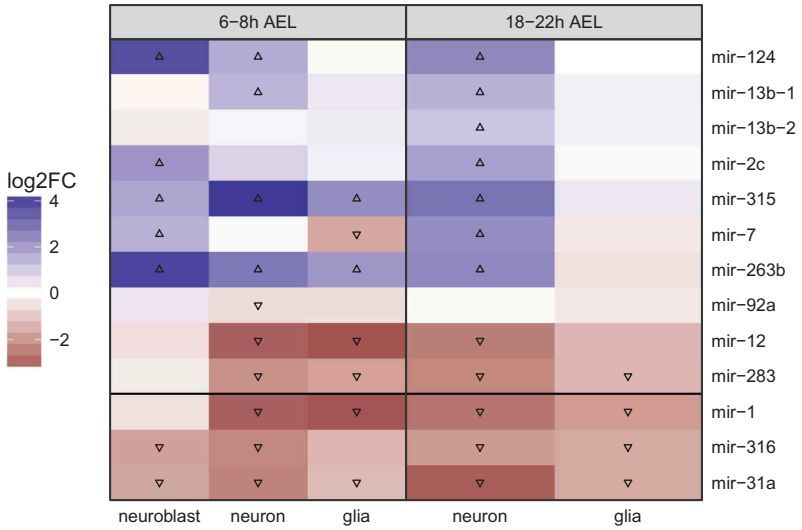


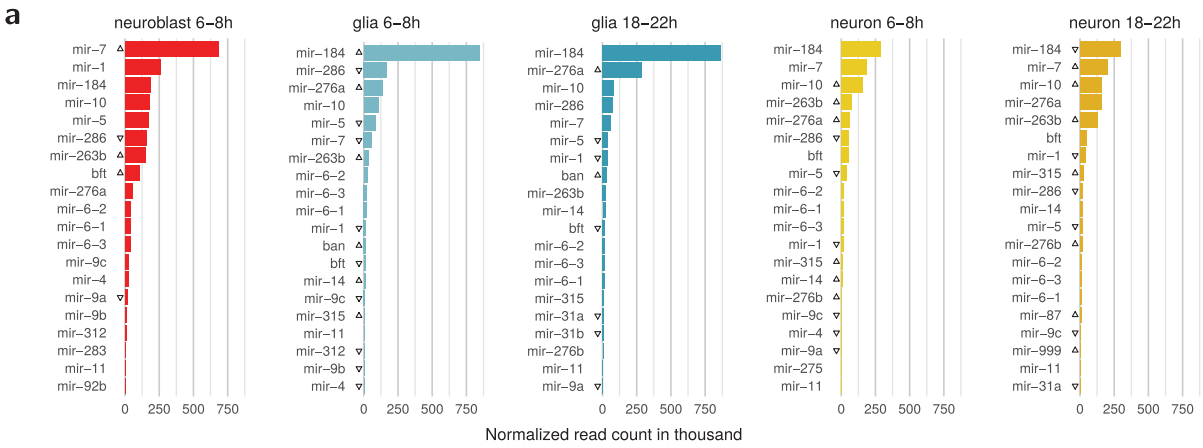
Figure 2. Differential expression of microRNAs that were associated with the nervous system using *in situ* hybridization probes [15] (top) and three non-neural microRNAs (bottom). Colors denote DESeq2 log2FC between marker-positive and marker-negative FACS fractions for each sample and arrows denote significant up- or down-regulation.

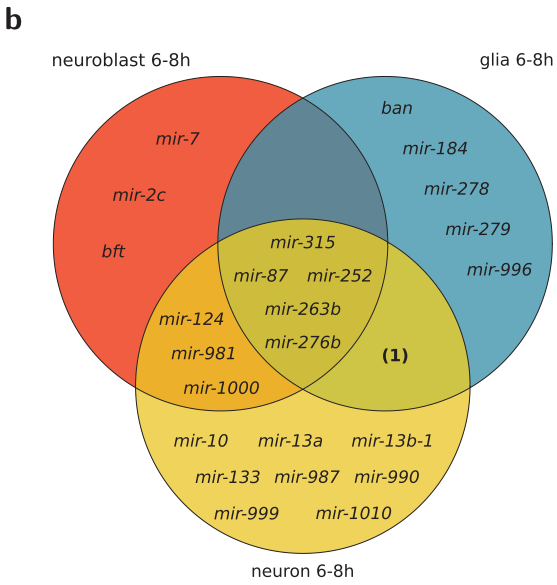
specific cell type compared with the marker-negative fraction (Supplementary Figures 1/2).

Venn diagrams in Figure 3(b,c) show the shared up- and down-regulated microRNAs between the different cell types and time points. The five microRNAs *mir-252, mir-263b, mir-276b, mir-87*, and *mir-315* are up-regulated, whereas the 6 microRNAs *mir-3, mir-8, mir-9a, mir-31a, mir-31b*, and *mir-286* are down-regulated in all three neural cell types at 6–8 h AEL. Additionally, *mir-375, mir-5, mir-283, mir-312*, and *mir-1* are down-regulated in both glia and neurons at both time points. We find 11 shared up-regulated microRNAs in neurons with additional 7 microRNAs exclusively up-regulated in late fully differentiated neurons. In glia, we find only *mir-279* and *ban* to be up-regulated at both time points. Similarly, another 19 microRNAs are down-regulated exclusively in neurons, whilst there are only 2 microRNAs (*bft* and *mir-7*) that are down-regulated exclusively in glia cells. Only *mir-2a-2* and *mir-1017* are down-regulated exclusively in neuroblasts. This indicates a higher regulatory complexity of neurons compared with the other two cell types.

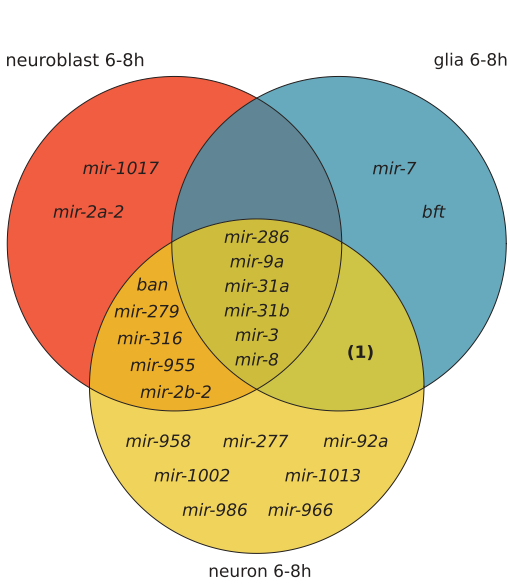
2.3. Specificity of smallRNA-seq compared with in situ hybridization

The localization of *Drosophila melanogaster* microRNAs across the embryo has previously been characterized using *in situ* hybridization probes to nascent microRNA precursor transcripts [15]. We can therefore assess the microRNA tissue specificity of *DIV-MARIS* by comparing the differential microRNA expression between marker-positive and marker-negative fractions of our samples with those microRNAs (or microRNA clusters) that showed expression in the nervous system using

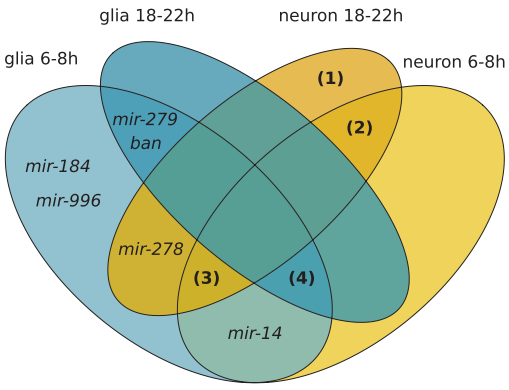




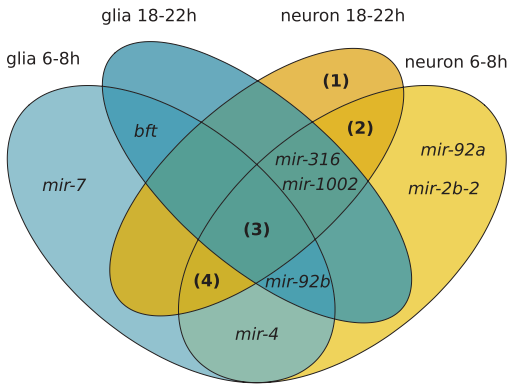
(1) *mir-14, mir-137, mir-190, mir-219, mir-276a, mir-284, mir-927, mir-932, mir-993, mir-1017*



(1) *mir-1, mir-4, mir-5, mir-9b, mir-9c, mir-12, mir-310, mir-311, mir-312, mir-92b, mir-283, mir-375*



(1) *mir-2c, mir-7, mir-13b-2, mir-998, mir-1012, mir-4969, mir-iab-4*
(2) *mir-10, mir-13a, mir-13b-1, mir-124, mir-133, mir-981 mir-987, mir-990, mir-999, mir-1000, mir-1010*
(3) *mir-87, mir-137, mir-190, mir-219, mir-263b, mir-276b, mir-284, mir-315, mir-927, mir-932, mir-993, mir-1017*
(4) *mir-252, mir-276a*



(1) *mir-184, mir-252, mir-281-1, mir-281-2, mir-305, mir-314, mir-956, mir-980, mir-996*
(2) *mir-277, mir-279, mir-955, mir-958, mir-966, mir-986, mir-1013, ban*
(3) *mir-1, mir-3, mir-5, mir-8, mir-9a, mir-986, mir-31a, mir-31b, mir-283, mir-312, mir-375*
(4) *mir-9b, mir-9c, mir-12, mir-286, mir-310, mir-311*

Figure 3. (a) The 20 most abundant microRNAs in marker-positive fractions for each sample. The arrows indicate significant up- or down-regulation in the marker-positive vs. marker-negative fractions. (b,c) Overlap of up-regulated (b) and down-regulated (c) microRNAs in marker-positive vs. marker-negative fractions in the 6–8 h AEL samples (top) and glia/neuron samples at both time points (bottom).

in situ hybridization. In the comparison, we also include 3 microRNAs that are known to be primarily expressed in non-neural tissues, such as *mir-1*, which is highly abundant in the mesoderm [25]. As the comparison in Figure 2 shows, our observations are mostly in very good agreement with the previous observations in [15]. The microRNAs *mir-124, mir-13b-1/2, mir-2c, mir-315, mir-7*, and *mir-263b* that Aboobaker *et al.* find to be associated with the nervous system, see top seven rows in Figure 2, show indeed a significant over-expression among neurons and neuroblasts at the early and in neurons at

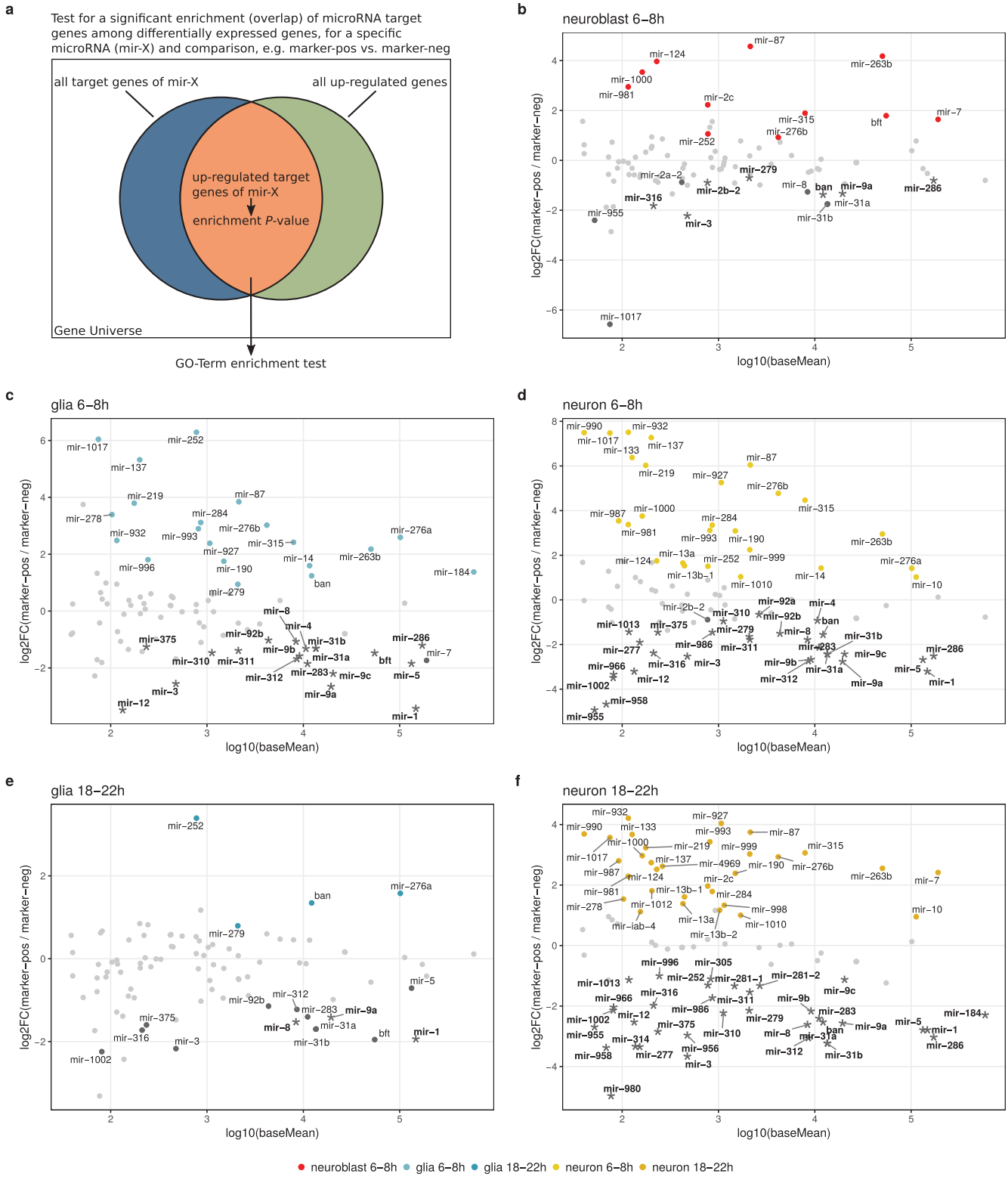


Figure 4. (a) Illustration of target gene enrichment test (b–f) Differential microRNA expression between marker-positive and marker-negative FACS fractions. Colored points denote significantly differentially expressed microRNAs and asterisks denote microRNAs with significantly enriched target genes among genes that are regulated in the opposite direction (see (a)).

the late stage. This comparison highlights the superior cell type-specific resolution of *DIV-MARIS* compared with the *in situ* hybridization technique as *DIV-MARIS* can differentiate between the three neural cell types. For example, *DIV-MARIS* is able to verify that, at the early time point, only *mir-315* and *mir-263b* are significantly up-regulated in glia cells. Two microRNAs, the clustered microRNAs *mir-12* and *mir-283,* which Aboobaker *et al.* find to be associated with only a subset of the peripheral nervous system, are indeed only lowly expressed in all neural cell types. Moreover, our data confirm that these two microRNAs are both down-regulated in neurons and glia at both stages. For microRNA *mir-92a*, which Aboobaker *et al.* find to be expressed in brain primordium and ventral nerve cord, we only see a slight down-regulation in early neurons and no difference in the other samples. At the same time, *mir-1, mir-316*, and *mir-31a* are down-regulated as expected in all neural cell types.

2.4. Comparison between neural cell types and time points

One advantage of using FACS sorted neural cell population is the ability to directly compare the marker-positive fractions of different cell types and time points with each other. This allows for a more sensitive analysis between cell types without interference of the diverse cell population in the marker-negative fractions. Across all samples, 69 microRNAs are differentially expressed in at least one sample pair (Figure 5). We find 11 microRNAs that are down-regulated in glia and neurons compared with neuroblasts, where *mir-1* shows the highest down-regulation from neuroblasts to neurons and glia at 6–8 h AEL (Figure 5(a,b)). In the comparison between neurons and glia, *mir-5, mir-9b, mir-184, mir-252, mir-276a, mir-279, mir-286, mir-312*, and *ban* are up-regulated in glia and *mir-7, mir-124, mir-190, mir-219, mir-927, mir-999*, and *mir-1000* are up-regulated in neurons at both time points (Figure 5(e,f)). Similarly, the comparisons between neuroblasts and glia as well as neurons and glia at 6–8 h AEL show that *mir-184, mir-252, mir-276a, mir-279, mir-996*, and *ban* are up-regulated, whereas *mir-7, mir-124, mir-999*, and *bft* are down-regulated in glia compared with both neurons and neuroblasts.

2.5. Effect of differential microRNA expression on their target genes

MicroRNAs serve as modulators of gene expression by regulating the translation of mRNAs of their target genes via direct *trans* RNA-RNA interactions. It is thus of key interest to investigate how the differential expression of microRNAs affects the mRNA levels of their target genes. To this end, we combine the *in silico* assessment of our small transcriptome data with the standard transcriptome sequencing data from [36], which were obtained using the same *DIV-MARIS* protocol. As before, we measure the differential expression of protein-coding genes between the marker-positive and marker-negative FACS fractions, between the three neural cell types as well as the early and late developmental stage (Supplementary Figures 3 and 5). In order to assess the potential impact of microRNA-mediated regulation of target genes on nervous system development, the figures only show genes that have predicted microRNA target sites and are associated with Gene Ontology (GO) terms related to the development of the nervous system, *i.e.* all GO *Biological Process* terms in the sub-tree of the ontology under the term *nervous system development, GO:0007399* (*GO-nsd* terms). In the comparison between marker-positive and marker-negative fractions, we find the highest number of differentially expressed genes associated with GO-nsd terms in neurons followed by glia and neuroblasts, with a much higher number of genes that are up-regulated in the marker-positive fractions than down-regulated (Supplementary Figure 3). In the comparison between the three cell types, we see that the changes in expression follow the developmental stages, where the highest number of differentially expressed genes is found in the comparison between glia and neurons at 18–22 h AEL, followed by glia and neurons at 6–8 h AEL (Supplementary Figure 5). The smallest differences are observed in the comparison within glia and within neurons at early vs. late time points. We also count the number of differentially expressed microRNA target genes that are up- or down-regulated upon microRNA down- or up-regulation (Supplementary Figure 4). Similar to the microRNAs, the highest number of differentially expressed genes in only one cell type is found in neurons and the highest overlap is observed between neurons and glia, and only 51 and 10 genes are commonly up- or down-regulated, respectively, in all three cell types.

While this overview gives a general sense of the change of expression of microRNAs and their target genes between the three neural cell types, the regulatory impact of each microRNA on the protein-coding transcriptome can be tested further by gene set enrichment analysis. To this end, we used the sets of all differentially expressed genes in each comparison and the sets of predicted microRNA target genes to test each microRNA for a significant enrichment of its target genes among all genes that are regulated in the opposite direction of the microRNA (Figure 4(a)). This enrichment test allows us to quantitatively link the differentially expressed microRNAs to distinct subsets of their target genes and measure statistical significance of the putative regulatory contribution of each of the differentially expressed microRNAs on the protein-coding transcripts. Note that this test includes all target genes and is not restricted by GO term annotations. Across all comparisons, 48 of 82 differentially expressed microRNAs show a significant enrichment in at least one sample pair (marked by asterisks in Figures 4(b–f) and Figure 5). Remarkably, we find a significant enrichment only for microRNAs that are down-regulated in the marker-positive fractions in every comparison between marker-positive and marker-negative fractions (Figures 4(b–f). In the comparisons between the cell types (Figure 5), all of the microRNAs that are up-regulated in glia compared with neurons show a significant enrichment, whereas *mir-124* is the only microRNA with enriched target genes that is up-regulated in neurons compared with glia at both time points (Figure 5(e–f)). When comparing neuroblasts and glia, four microRNAs, *mir-1, mir-9a, mir-9c*, and *mir-124*, are up-regulated in neuroblasts and show an enrichment of their target genes, whereas *mir-190, mir-252, mir-279*, and *mir-996* are up-regulated in glia and have enriched target genes (Figure 5(a)). In the neuron vs. neuroblast comparison, the enrichment of target genes is significant for all of

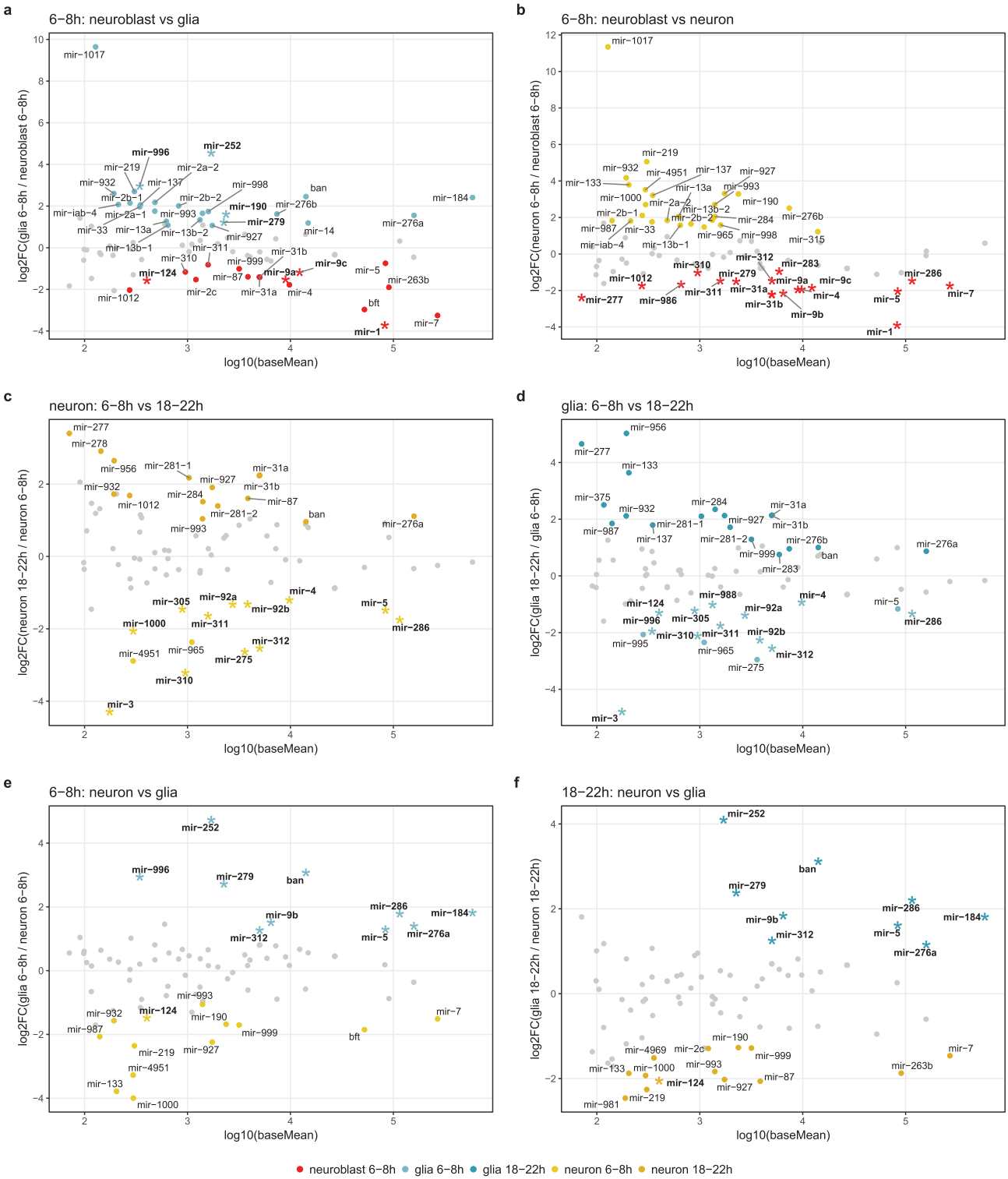


Figure 5. Differential microRNA expression between cell types and time points. Colored points denote significantly differentially expressed microRNAs. Asterisks denote microRNAs with significantly enriched target genes among genes that are up-regulated in the opposite direction.

the microRNAs that are up-regulated in neuroblasts, but for none of the microRNAs that are up-regulated in neurons (Figure 5(b)). Further, in the comparison between the 6–8 h and 18–22 h AEL time points within glia and neurons, we only find microRNAs with enriched target genes among those, that are down-regulated in the late time points, and many of those are shared between glia and neurons, such as *mir-3, mir-4, mir-5, mir-92a/b, mir-310/311/312* and *mir-286* (Figures 5(c,d)). Note that some microRNAs,

especially those with high sequence identity, share the same or similar sets of target genes (Supplementary Figure 6) and therefore behave similarly in this analysis. For example, *mir-31a/b* are exactly overlapping in the plots.

2.6. Gene ontology enrichment analysis

The gene set enrichment tests give an unfiltered view on the regulatory effect of the differentially expressed microRNAs by considering all differentially expressed target genes. In order to cross-check the tissue-specificity of the microRNAs and further detailize putative microRNA functions, we performed Gene Ontology (GO) enrichment tests on the sets of differentially expressed microRNA target genes (middle section in Figure 4(a)) to find over-represented GO-nsd terms that are associated with that particular gene set. The combination of the target gene enrichment and the GO term enrichment will give additional evidence for the specific regulatory roles of each microRNA via the more fine-grained functions exerted by their respective target genes.

Our comparisons between marker-positive and marker-negative fractions identifies in total 40 microRNAs with enriched target genes (those marked by asterisks in Figure 4) that have at least one significantly enriched GO-nsd term among their target genes (Supplementary Figure 7). Figure 6 summarizes the top 10 enriched GO-nsd terms for microRNA target genes in neuroblasts as well as neurons and glia at 18–22 h AEL. Of the 7 microRNAs in neuroblasts, only *mir-2b-2* has target genes associated with neuroblast development and differentiation, whereas the other 6 microRNAs *mir-3, mir-9a, mir-279, mir-286, mir-316*, and *ban* have enriched terms related to neuron development and differentiation among their target genes. In glia we find that *mir-1, mir-8, mir-9a* have target genes enriched for terms related to gliogenesis, but only few terms related to neuron development. Additionally, we observe that the target genes of *mir-5, mir-316*, and *mir-31a/b* are significantly enriched for glial

Figure 6. Top 10 GO *Biological Process* terms in the GO sub-tree *nervous system development, GO:0007399*, that are significantly enriched in the set of up-regulated protein-coding genes that are targets of down-regulated microRNAs in the comparison between positive and negative FACS fractions for neuroblasts at 6–8 h AEL and neuron/glia at 18–22 h AEL. Shown are only microRNAs having a significant overlap of their target genes with differentially expressed genes.

cell development and differentiation (Supplementary Figure 7 (c)), however, the hypergeometric test for target enrichment among up-regulated genes was not significant for these three microRNAs. All of the 35 microRNAs that are down-regulated in neurons at 18–22 h AEL have a significant enrichment of their target genes among genes that are up-regulated in neurons and all but two have enriched terms related to neuron development and differentiation among their up-regulated target genes. We also see a similar situation in neurons at 6–8 h AEL (Supplementary Figure 7(d)).

Figure 7 and Supplementary Figure 8 show the corresponding GO-nsd term enrichment for microRNA target genes in the comparisons between the three cell types. Again, we find the highest number of down-regulated microRNAs with enriched GO-nsd terms among their up-regulated target genes in neurons. In glia cells, *mir-1, mir-9a, mir-9c*, and *mir-124* are down-regulated in glia compared with neuroblasts and their up-regulated target genes are enriched for terms related to gliogenesis. In the comparison between glia and neurons only *mir-124* is down-regulated in glia and has enriched gliogenesis terms among its up-regulated target genes. However, also *mir-190, mir-263b, mir-927, mir-932, mir-987*, and *mir-1000* show enrichment of terms related to gliogenesis (Supplementary Figure 8(c/d)), but their target genes are not significantly enriched among the genes

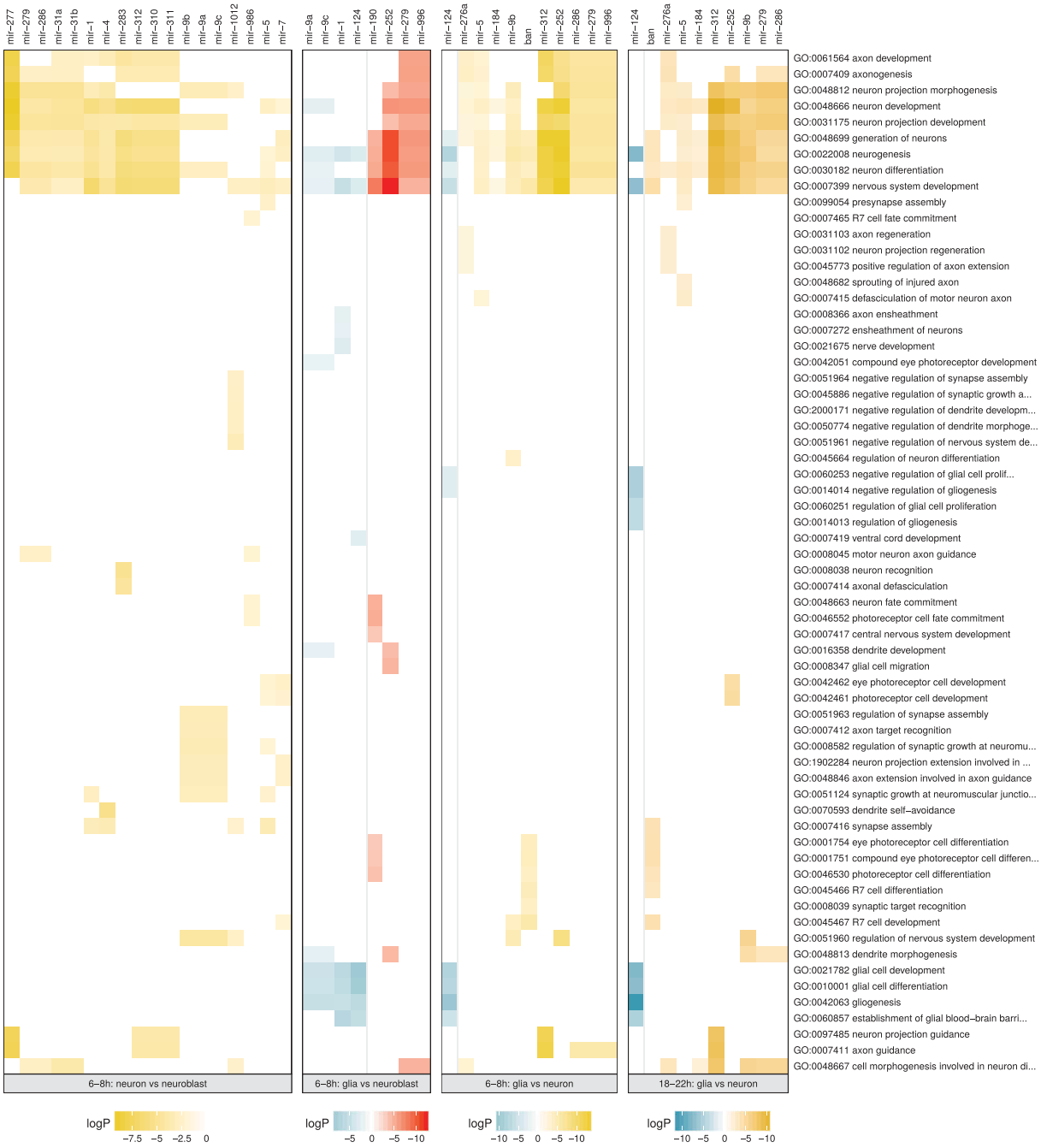


Figure 7. Top 10 GO *Biological Process* terms in the sub-tree *nervous system development, GO:0007399*, that are significantly enriched in the sets of differentially expressed protein-coding genes that are targets of differentially expressed microRNAs in the comparisons between cell types. Shown are only those microRNAs having a significant overlap of their target genes with differentially expressed genes in the opposite direction.

up-regulated in glia. The three microRNAs *mir-252, mir-996*, and *mir-279* are up-regulated in glia compared with neurons and neuroblasts and their targets show enriched GO terms related to neuron development, axonogenesis and related terms. Further, *mir-5, mir-276a, mir-184*, and *mir-312* show the same effect in the neuron vs. glia comparison at both time points. In the comparison between neuroblasts and neurons, we only find microRNAs with enriched target genes that are down-regulated in neurons, but not in neuroblasts. However, *mir-33, mir-315, mir-932, mir-965*, and *mir-987* are down-regulated in neuroblasts and their target genes have enriched terms related to neuroblast differentiation and neuroblast fate commitment (Supplementary Figure 8(b)). In neurons, we find especially the down-regulated microRNAs *mir-277, mir-279, mir-286, mir-31a/b, mir-1, mir-4, mir-283, mir-310/311/312* to have enriched terms related to neuron development among their target genes.

2.7. Regulation of transcription factors through microRNAs

While protein expression can be directly regulated by microRNAs, it can also be controlled indirectly through the regulation of transcription factors (TFs) that are targets of microRNAs. As transcription factors typically control more than one gene, they can potentially multiply the regulatory effect of microRNAs. For example, down-regulation of a single microRNA would result in up-regulation of a transcription factor, which in turn alters the expression of multiple genes. In order to discover this type of regulatory chain, we connect the differential expression of microRNAs, transcription factors, and protein coding genes by their pair-wise target sites, again followed by GO enrichment analysis as above.

Similar to microRNAs, we find many more differentially expressed transcription factors between marker-positive and marker-negative fractions in neuroblasts and neurons compared with glia cells (Supplementary Figure 9). Likewise, the comparisons between the three neural cell types show more up-regulated transcription factors in neurons compared with glia as well as in neuroblasts compared with both neurons and glia (Supplementary Figure 10). We used the same hypergeometric test for finding TFs that show a significant overlap of their target genes with the set of genes that are regulated in the same direction (those marked by asterisks in Supplemenatry Figures 9 and 10) and perform GO term enrichment tests to analyze the functions of the up-regulated TF target genes. In the comparison between marker-positive and marker-negative fractions in neuroblasts and neurons, we find several TFs with enriched GO-nsd terms related to neuroblast and neuron development among their target genes, but not in glia (Supplementary Figure 11). However, in the comparison between the cell types, the transcription factors *Dll, slp1, dl*, and *twi* have significantly enriched target genes associated with terms related to gliogenesis (Supplementary Figure 12).

When using the transcription factors as an intermediate step between each differentially expressed microRNA and protein-coding genes, we can perform Gene Ontology enrichment analysis on the sets of up-regulated genes that are targeted by up-regulated transcription factors that in turn are targeted by a down-regulated microRNA and *vice versa*. This reveals the impact of each microRNA on the protein-coding transcriptome through the up-regulation of TFs. As there are comparatively few transcription factors among all genes, the hypergeometric test for finding significant overlaps between microRNA target genes and differentially expressed TFs cannot be applied. We therefore consider all differentially expressed microRNAs targeting all differentially expressed TFs in the individual comparisons. In the comparisons between marker-positive and marker-negative fractions (Supplementary Figure 13), we again find that most of the down-regulated microRNAs in neurons show enriched terms related to neuron development in the regulated protein-coding genes. Especially the *mir-310/311/312* family targets 12 transcription factors, which in turn target up-regulated genes in neurons with enriched terms related to neuron development and differentiation. In neuroblasts, especially *ban, mir-8, mir-31a/b, mir-315*, and *mir-1017* are significantly enriched for neuroblast differentiation. When comparing the different cell types directly, we find that genes regulated by *mir-87, mir-190, mir-927*, and *mir-932* are enriched for terms related to gliogenesis and *ban, mir-5, mir-9b, mir-252*, and *mir-312* show enriched terms related to neuron development (Supplementary Figure 14). We also find many microRNAs in the comparison between neuroblasts and neurons/glia, that act through TFs on genes that are associated with neuroblast commitment and differentiation. This is in contrast to the purely microRNA-based analysis, where there were almost no microRNAs with significant enrichment of these terms.

3. Discussion

Our survey investigates the expression of microRNAs based on dedicated transcriptome data sets for neuroblasts, neurons and glia at two distinct developmental stages during Drosophila neurogenesis. The superior cell type-specific resolution of *DIV-MARIS* combined with whole transcriptome sequencing allows for an in-depth *in silico* analysis to identify microRNAs that may play key functional roles during neurogenesis. Besides comparing the microRNA expression profiles between those three neural cell types, our joint analysis of small and total RNA-seq data sets can assess the effects that these microRNAs may exert on the protein-coding transcriptome, either via direct *trans* RNA-RNA interactions between microRNA and the mRNAs of their target genes or, indirectly, via transcription factors that are targeted by these microRNAs.

We compared the microRNA expression profiles in our samples to several microRNAs that have been previously been shown to be expressed in the nervous system by *in situ* hybridization probes against pre-microRNA transcripts [15], and found that smallRNA-Seq recapitulates and refines the observations therein. The *DIV-MARIS* approach allows for the cell type-specific analysis of individual microRNAs and we find significant differences between neurons and glia. SmallRNA-sequencing can also resolve the high divergence of expression between the individual microRNAs in a cluster, such as *mir-309/3/286/4/5/6-1,2,3*, and we see that *mir-5* and *mir-286* are differentially expressed between neurons and glia, for example. Additionally, our protocol can also detect low-

abundance microRNAs, such as the *mir-310 - mir-313* cluster, and their differential expression between the neural cell types.

Gene set enrichment tests for microRNA targets and GO terms among the target genes strongly support the role of microRNAs as inhibitors of genes and transcription factors in all three cell types. It is therefore mandatory to investigate both up-regulated as well as down-regulated microRNAs for characterizing a possible regulatory effect of differential microRNA expression on each cell type. We find significant evidence that it is the significantly *down-regulated* microRNAs that are steering the development of the neural cell types via down-regulation of their target genes, and that high absolute expression levels of microRNAs in the marker-positive fractions do not imply functional relevance. In the following we discuss microRNAs that were previously described to be associated with Drosophila embryogenesis and the nervous system.

The two most abundant microRNAs in neuroblasts are *mir-7* and *mir-1*, which are also among the top 20 most abundant microRNAs in glia and neurons. However, they are highly down-regulated in these two cell types compared with neuroblasts. The muscle-specific *mir-1* is down-regulated in both glia and neurons in the marker-positive vs. marker-negative fractions and its target genes are significantly enriched among up-regulated genes, which in turn are enriched for GO terms related to neuron differentiation and gliogenesis, respectively. This observation supports a view that *mir-1* possibly acts by repressing the differentiation into neuroglial cell types. On the other hand, *mir-7* is expressed highest in neuroblasts and lowest in glia cells.

The highly conserved *mir-184* is the most abundant microRNA in neurons and glia and the third most abundant in neuroblasts. It has previously been found to be highly expressed in the early embryo and the following stages [30] and is part of a regulatory network controlling adult neural stem/progenitor cell differentiation [38], but is also involved in inhibiting the overexpression of *Glioactin* [39]. Our comparison between the three embryonic neural cell types reveals that *mir-184* is up-regulated in glia compared with neuroblasts and neurons at both time points. Its up-regulated target genes in neurons are enriched for GO terms related to neuron development. Interestingly, its up-regulated target genes in neuroblasts are exclusively enriched for five terms related to neuroblast differentiation, but not for neuron development. This suggests that *mir-184* acts as an inhibitor of neuronal cell fate. Similarly, both *mir-5* and *mir-286* are among the top 15 most abundant microRNAs in all samples and the comparison between marker-positive and marker-negative fractions shows indeed a down-regulation of both in most marker-positive samples. However, the comparison between the cell types reveals a down-regulation of both microRNAs in neurons compared with neuroblasts and glia and their target genes are associated with GO terms related to neuron development.

One of the most studied microRNAs in the nervous system is *mir-124*. In our experiment, we find that *mir-124* increases in abundance from neuroblasts over neuron 6–8 h to neuron 18–22 h AEL, however its relative expression among all microRNAs is rather low. It is up-regulated in neuroblasts as well as in differentiating (6–8 h AEL) and differentiated (18–22 h AEL) neurons, which was is also observed in mouse [40,41], where *mir-124* and the *mir-9* family play distinct roles in post-mitotic neurons [42]. When comparing the neural cell types, *mir-124* is significantly down-regulated in glia compared with the other two and it is the only microRNA that has a significant overlap of its targets with up-regulated genes in neurons at both time points, which is also seen in neuroblasts. GO enrichment analysis shows that those genes have significantly enriched terms related to gliogenesis. These observations support the theory that *mir-124* is an important regulator distinguishing neuron and glia cell development, which is also supported by the previous observation that *mir-124* and its target *repo*, the marker for glia cells, are expressed in spatial reciprocity [21].

The microRNA pair *mir-31a/b*, which are also conserved in human, are down-regulated in all three neural cell types and both time points in the marker-positive vs. marker-negative fractions. Previously, the expression of *mir-31a* was found to be required for memory formation [31] and is also a regulator of glia homeostasis [43] in adult flies. In this study, we find no expression difference between neurons and glia, but a down-regulation in glia and neurons compared with neuroblasts. Further, they are significantly higher expressed at 18–22 h compared with 6–8 h AEL. Both microRNAs have also significantly enriched GO terms related to glia and neuron development as well as terms related to axonogenesis and axon guidance among their target genes. This indicates that they also act by inhibiting gene expression in the developing nervous system.

The three microRNAs *mir-9-a/b/c* are down-regulated across neurons and glia, and their up-regulated target genes are enriched for terms related to neuron differentiation and related terms. The down-regulation is also stronger in glia/neurons compared with neuroblasts, supporting the hypothesis that they inhibit neural cell fate, which has previously been observed in sensory organ precursors in the peripheral nervous system [26]. Their target genes show enrichment for terms relating to gliogenesis but also neurogenesis. In the comparison between glia and neurons, *mir-9b* is down-regulated in neurons at both time points and its target genes are enriched for several GO terms related to neuron differentiation. Similarly, the two microRNAs *mir-279* and *mir-996* were previously described as post-transcriptional inhibitors of *elav* [23]. Accordingly, here we found *mir-279* to be up-regulated in glia compared with neuroblasts as well as neurons at both time points. It has also enriched target genes that are up-regulated in neurons, which are associated with GO terms related to neuron development. Similarly, *mir-996* up-regulation was detected in glia compared with neurons and neuroblasts at 6–8 h AEL, but not at 18–22 h AEL, with identical effects on target gene regulation as *mir-279*.

Besides those, we also detect several other microRNAs that are differentially expressed between the marker-positive and marker-negative fractions or between the neural cell types and have differentially expressed target genes with enriched Gene Ontology terms related to nervous system development. In summary, the sequencing of the smallRNA fraction of specific cell types enables a more detailed

understanding of spatiotemporal microRNA expression during Drosophila neurogenesis. The combination with sequencing of the protein-coding transcriptome and analysis of target genes and associated Gene Ontology terms supports the characterization of individual microRNAs and their putative roles in the different cell types.

4. Materials and methods

The *DIV-MARIS* protocol is fully described in [36]. Briefly, Drosophila embryos were collected at time points 6–8 h and 18–22 h after egg laying (AEL) (Figure 1). Embryos were dissociated into single-cell suspensions, cells were fixed in 4% formaldehyde. Fixed cell suspensions were immunostained under RNase-free conditions and FACS-purified using a FACSAria II cell sorter (BD Biosciences). Marker-positive and marker-negative cell populations were collected in biological duplicates. FACS-purified cells were subjected to cross-linking reversal and Proteinase K digestion prior to RNA isolation. From both FACS fractions, RNA was extracted and prepared for strand-specific Illumina sequencing using the NEBNext Small RNA protocol (New England BioLabs). Libraries were then sequenced on the Illumina HiSeq 2500 using single-end reads of length 51bp.

After sequencing, adapter sequences were removed from the reads using AdapterRemoval ver 2.1.7 [44] with options –– trimns –– trimqualities –– minlength 15 –– minadapteroverlap 3 –– adapter1 AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC. The trimmed reads were mapped to the Drosophila melanogaster genome BDGP 6 using bowtie ver 1.1.2 [45] with the options -m 10 -a –– strata –– best -S -l 10. Using the ENSEMBL85 gene annotation, the number of reads per genomic feature were counted by featureCounts 1.5.1 using fractional counting of multi-mapping reads with the options -M –– fraction -O -s 1. From the per-gene counts, differential expression analysis was done using DESeq2 ver 1.16.1 [46] in R v3.4.3 [47]. Only microRNAs with more than 1000 reads across all conditions were included in the analysis and microRNAs were called as significantly differentially expressed if $|\log2FC| > 0.5$ and FDR-adjusted P-value < 0.05.

The RNA-Seq data from [36] are accessible at the GEO database with accession GSE106095. We used STAR [48] to map the paired-end 2x75bp reads to the same genome and to count the number of reads per genomic feature. Genes were called differentially expressed if $|\log2FC| > 1.0$ and FDR-adjusted P-value < 0.05. Mappings between microRNAs and transcription factors with their target genes were downloaded from the DroID database ver. 2015_12 [49].

Using the DroID database, predicted microRNA targets were pooled into sets of target genes for every microRNA. The generated gene sets were used to test the differentially expressed genes in each comparison for enrichment in targets for each differentially microRNA using the hypergeometric test ($P < 0.01$). Gene Set Enrichment for Gene Ontology terms was done using the topGO package in R [50] and for each set of microRNA/TF target genes, we selected the top 10 significant ($P < 0.01$) *Biological Process* terms in the sub-tree *nervous system development, GO:0007399*.

The smallRNA-Seq sequencing reads are available in the GEO database with accession number GSE117245.

Disclosure statement

No potential conflict of interest was reported by the authors.

ORCID

Peter Menzel http://orcid.org/0000-0003-4964-0685
Alexandra L. McCorkindale http://orcid.org/0000-0002-0840-0784
Stefan R. Stefanov http://orcid.org/0000-0003-4341-8725
Robert P. Zinzen http://orcid.org/0000-0002-8638-5102
Irmtraud M. Meyer http://orcid.org/0000-0002-4048-3479

References

[1] Bartel DP, Chen CZ. Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. Nat Rev Genet. 2004 May;5(5):396–400.
[2] Ivey KN, Srivastava D. MicroRNAs as regulators of differentiation and cell fate decisions. Cell Stem Cell. 2010 Jul;7(1):36–41.
[3] McNeill E, Van Vactor D. MicroRNAs shape the neuronal landscape. Neuron. 2012 Aug;75(3):363–379.
[4] Antic S, Wolfinger MT, Skucha A, et al. General and MicroRNA-Mediated mRNA degradation occurs on ribosome complexes in Drosophila cells. Mol Cell Biol. 2015 Jul;35(13):2309–2320.
[5] Guo H, Ingolia NT, Weissman JS, et al. Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature. 2010 Aug;466(7306):835–840.
[6] Tat TT, Maroney PA, Chamnongpol S, et al. Cotranslational microRNA mediated messenger RNA destabilization. Elife. 2016 Apr;5. DOI:10.7554/eLife.12880.
[7] Gramates LS, Marygold SJ, Santos GD, et al.; the FlyBase Consortium. FlyBase at 25: looking to the future. Nucleic Acids Res. 2017 Jan;45(D1):D663–D671.
[8] Yates A, Akanni W, Amode MR, et al. Ensembl 2016. Nucleic Acids Res. 2016 Jan;44(D1):D710–6.
[9] Berezikov E, Robine N, Samsonova A, et al. Deep annotation of Drosophila melanogaster microRNAs yields insights into their processing, modification, and emergence. Genome Res. 2011 Feb;21(2):203–215.
[10] Perry N, Volin M, Toledano H. microRNAs in Drosophila regulate cell fate by repressing single mRNA targets. Int J Dev Biol. 2017;61(3–5):165–170.
[11] Wheeler BM, Heimberg AM, Moy VN, et al. The deep evolution of metazoan microRNAs. Evol Dev. 2009;11(1):50–68.
[12] Ibanez-Ventoso C, Vora M, Driscoll M. Sequence relationships among C. elegans, D. melanogaster and human microRNAs highlight the extensive conservation of microRNAs in biology. PLoS One. 2008 Jul;3(7):e2818.
[13] Leaman D, Chen PY, Fak J, et al. Antisense-mediated depletion reveals essential and specific functions of microRNAs in Drosophila development. Cell. 2005 Jul;121(7):1097–1108.
[14] Loya CM, Lu CS, Van Vactor D, et al. Transgenic microRNA inhibition with spatiotemporal specificity in intact organisms. Nat Methods. 2009 Dec;6(12):897–903.
[15] Aboobaker AA, Tomancak P, Patel N, et al. Drosophila microRNAs exhibit diverse spatial expression patterns during embryonic development. Proc Natl Acad Sci USA. 2005 Dec;102(50):18017–18022.
[16] Bushati N, Cohen SM. microRNA functions. Annu Rev Cell Dev Biol. 2007;23(1):175–205.
[17] Homem CC, Knoblich JA. Drosophila neuroblasts: a model for stem cell biology. Development. 2012 Dec;139(9):4297–4310.
[18] Sousa-Nunes R, Cheng LY, Gould AP. Regulating neural proliferation in the Drosophila CNS. Curr Opin Neurobiol. 2010 Feb;20:50–57.
[19] Doe CQ. Temporal patterning in the Drosophila CNS. Annu Rev Cell Dev Biol. 2017 Oct;33(1):219–240.

[20] Aravin AA, Lagos-Quintana M, Yalcin A, et al. The small RNA profile during Drosophila melanogaster development. Dev Cell. 2003 Aug;5(2):337–350.
[21] Stark A, Brennecke J, Bushati N, et al. Animal MicroRNAs confer robustness to gene expression and have a significant impact on 3′UTR evolution. Cell. 2005 Dec;123(4):1133–1146.
[22] Sun K, Westholm JO, Tsurudome K, et al. Neurophysiological defects and neuronal gene deregulation in Drosophila mir-124 mutants. PLoS Genet. 2012 Feb;8(2):e1002515.
[23] Sanfilippo P, Smibert P, Duan H, et al. Neural specificity of the RNA-binding protein elav is achieved by post-transcriptional repression in non-neural tissues. Development. 2016 Dec;143 (23):4474–4485.
[24] Bushati N, Stark A, Brennecke J, et al. Temporal reciprocity of miRNAs and their targets during the maternal-to-zygotic transition in Drosophila. Curr Biol. 2008 Apr;18(7):501–506.
[25] Sokol NS, Ambros V. Mesodermally expressed Drosophila microRNA-1 is regulated by twist and is required in muscles during larval growth. Genes Dev. 2005 Oct;19(19):2343–2354.
[26] Li Y, Wang F, Lee JA, et al. MicroRNA-9a ensures the precise specification of sensory organ precursors in Drosophila. Genes Dev. 2006 Oct;20(20):2793–2805.
[27] Li X, Carthew RW. A microRNA mediates EGF receptor signaling and promotes photoreceptor differentiation in the Drosophila eye. Cell. 2005 Dec;123(4):1267–1277.
[28] Picao-Osorio J, Johnston J, Landgraf M, et al. MicroRNA-encoded behavior in Drosophila. Science. 2015 Nov;350(6258):815–820.
[29] Hilgers V, Bushati N, Cohen SM. Drosophila microRNAs 263a/b confer robustness during development by protecting nascent sense organs from apoptosis. PLoS Biol. 2010 Jun;8(7):e1000396.
[30] Iovino N, Pane A, Gaul U. mir-184 has multiple roles in Drosophila female germline development. Dev Cell. 2009 Jul;17 (1):123–133.
[31] Busto GU, Guven-Ozkan T, Fulga TA, et al. microRNAs that promote or inhibit memory formation in Drosophila melanogaster. Genetics. 2015 Jun;200(3):569–580.
[32] Busto GU, Guven-Ozkan T, Davis RL. MicroRNA function in Drosophila memory formation. Curr Opin Neurobiol. 2017 Apr;43:15–24.
[33] Li W, Cressy M, Qin H, et al. MicroRNA-276a functions in ellipsoid body and mushroom body neurons for naive and conditioned olfactory avoidance in Drosophila. J Neurosci. 2013 Mar;33(49):5821–5833.
[34] Chawla G, Sokol NS. MicroRNAs in Drosophila development. Int Rev Cell Mol Biol. 2011;286:1–65.
[35] Smibert P, Lai EC. A view from Drosophila: multiple biological functions for individual microRNAs. Semin Cell Dev Biol. 2010 Sep;21(7):745–753.
[36] McCorkindale AL, Wahle P, Jungreis I, et al. A gene expression atlas of embryonic neurogenesis in Drosophila reveals complex spatiotemporal regulation of lncRNAs. https://www.biorxiv.org/content/early/2018/12/27/483461.
[37] Hrvatin S, Deng F, O'Donnell CW, et al. MARIS: method for analyzing RNA following intracellular sorting. PLoS One. 2014;9(3):e89459.
[38] Liu C, Teng ZQ, Santistevan NJ, et al. Epigenetic regulation of mir-184 by MBD1 governs neural stem cell proliferation and differentiation. Cell Stem Cell. 2010 May;6(5):433–444.
[39] Sharifkhodaei Z, Padash-Barmchi M, Gilbert MM, et al. The Drosophila tricellular junction protein gliotactin regulates its own mRNA levels through BMP-mediated induction of mir-184. J Cell Sci. 2016 Apr;129(7):1477–1489.
[40] Bak M, Silahtaroglu A, Møller M, et al. MicroRNA expression in the adult mouse central nervous system. Rna. 2008 Mar;14(3):432–444.
[41] Yu JY, Chung KH, Deo M, et al. MicroRNA mir-124 regulates neurite outgrowth during neuronal differentiation. Exp Cell Res. 2008 Aug;314(14):2618–2633.
[42] Yoo AS, Staahl BT, Chen L, et al. MicroRNA-mediated switching of chromatin-remodelling complexes in neural development. Nature. 2009 Jul;460(7255):642–646.
[43] Foo LC, Song S, Cohen SM. mir-31 mutants reveal continuous glial homeostasis in the adult Drosophila brain. Embo J. 2017 May;36(9):1215–1226.
[44] Lindgreen S. AdapterRemoval: easy cleaning of next generation sequencing reads. BMC Res Notes. 2012 Jul;5(1):337.
[45] Langmead B, Trapnell C, Pop M, et al. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 2009;10(3):R25.
[46] Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15(12):550.
[47] R Core Team. R: A language and environment for statistical computing. Vienna (Austria): R Foundation for Statistical Computing; 2015.
[48] Dobin A, Davis CA, Schlesinger F, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics. 2013 Jan;29(1):15–21.
[49] Murali T, Pacifico S, Yu J, et al. DroID 2011: a comprehensive, integrated resource for protein, transcription factor, RNA and gene interactions for Drosophila. Nucleic Acids Res. 2011 Jan;39 (suppl_1):D736–43.
[50] Alexa A, Rahnenfuhrer J. topGO: enrichment analysis for gene ontology. 2016.