A Computational Approach to Identifying miRNAs Implicated in *Drosophila* Neurodevelopment

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Abstract: *miRNAs are known to regulate many aspects of neurodevelopment. They participate at different stages of this process from early embryogenesis to adult stage. Their various and specific functions begin to be unraveled in many model systems.*

Important part of neurogenesis, which generates mature neurons from progenitor cells, is the nerve growth and the formation of synapses. As they underlie the neuronal network formation, perturbation of their proper regulation causes different neuro-developmental diseases in human.

In our study we used the model organism Drosophila to identify by a computational approach miRNAs, targeting genes, which control axonal growth and synaptogenesis. We screened preselected groups of genes, known to regulate these processes and identified several micro-RNAs as likely candidates for their expression control. We found five miRNAs, which have been reported earlier to associate with dFMRP (Drosophila Fragile X mental Retardation Protein 1) and which target only a small number of specific genes. We also identified several new miRNA candidates likely implicated in synaptogenesis.

Keywords: *miRNA, Computational approach, Drosophila, Nerogenesis, Synaptogenesis, dFMRP.*

Introduction

MicroRNAs (miRNAs) are about 18-25 nt long small RNAs which function to regulate the activity and stability of specific messenger RNA targets. miRNAs are transcribed from sequences which reside within coding regions (intronic or exonic) or between annotated genes [4, 44]. Intergenic miRNAs are located in clusters. They have their own promoters and are transcribed as long policistronic RNA-molecules [29]. Intronic miRNAs are co-ordinately expressed with the corresponding host genes [11].

Most miRNAS are transcribed as over 1 kb long primary miRNAs (pri-miRNAs) which are recognized and cleaved by the nuclear RNAse III Drosha to generate precursors miRNAs (pre-miRNAs) of about 70 nt which are exported into the cytoplasm (see the reviews in [18, 33, 36]). They are cleaved by the RNAse III Dicer into double stranded RNAs of about

22 nt. These small molecules are loaded onto the RISC-complex (RNA-induced silencing complex). This complex directs miRNAs to their target mRNAs where they bind complementary usually to the 3'untranslated regions. In some cases they bind to the 5' untranslated regions or to other parts of the target mRNA-transcripts [8, 37].

Binding to target mRNAs leads to repression of gene expression which may be due to different mechanisms at the level of translation or mRNA-degradation [16].

Predictions, obtained through different methods, indicate that one miRNA can target tens of transcripts and conversely a single transcript can be targeted by many different miRNAs [2, 32, 47].

There is a great amount of data showing that miRNAs are enriched in human and mammalian brain. Their expression there is higher than in any other animal organ [1, 5-6, 48].

Over 500 miRNAs have been annotated and are expected to function in the human brain in diverse biological pathways controlling neurodevelopment [36, 40, 51].

miRNAs play important roles in neurite growth, synaptic development, neuronal plasticity, learning and memory [3, 10, 17, 30, 33, 46, 50].

Some miRNAs (such as miR-124, miR-128) are preferentially expressed in neurons, others (such as miR-23, miR-26, and miR-29) are more strongly expressed in glia, and some (miR-9 and miR-125) are evenly represented in both cell types [52]. Even within a single cell a compartmentalization effect exists with respect to the miRNA content [27]. All these findings imply that miRNAs must have diverse and specific functions in the human brain. Most of them remain so far unknown.

The fruit fly *Drosophila melanogaster* is a very useful genetic model system for studying different stages of neurogenesis, including nerve growth, axonal path-finding and synapse formation as the ultimate step in the nervous system wiring. Many molecular signaling pathways, involved in these processes have been described. They are tightly and dynamically regulated and numerous participating proteins, protein complexes and their corresponding genes have been established.

In our work we focused on nine preselected groups of genes, controlling axonal growth in *Drosophila* [45] and another group of genes, which are involved in synapse formation and for which the available information was taken from Flybase (http://www.flybase.org/).

We identified miRNAs, targeting all these genes. Half of these miRNAs are specific for particular gene/set of genes, others do not show a similar specificity.

Five of the 60 predicted miRNAs have been shown previously to physically interact with *Drosophila* FMRP (Fragile X Mental Retardation Protein 1). Many of the miRNA/gene matches have not been reported before with a role in synaptic formation.

Materials and methods

We compiled a list of *Drosophila* genes and grouped them according to their functions in nerve growth and in synaptogenesis (see Table 1, Results and discussion). Sets 1-9 are regulators of axonal growth, led by the growth cones, navigating axons by rearrangements of the cytoskeleton [45]. Set 10 contains genes, which encode molecular scaffolds, cell adhesion proteins and proteins, required in epithelial cell polarity. They all participate in the late stages of neurogenesis-synaptogenesis.

The list of genes grouped into sets (see Table 1) were recorded as a CSV (Comma Separated Values) file. Pre-computed data for miRNA matching transcripts for the whole *D. melanogaster* genome was obtained from the MicroCosm Targets database: http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/. The algorithm used to compute the data is discussed in more detail in [15].

Both the file containing our genes of interest and the pre-computed data were fed into a Perl program, which extracted from the pre-computed data all matches involving transcripts of genes which were on the list (see Table 1). Transcripts were identified by their annotation ID, as it was found out that the names were sometimes missing. The output from the program was used to generate Table 2 in the Appendix.

Results and discussion

In our work we analyzed the genes, overviewed in Table 1. They were distributed in groups, according to their functions in particular pathways for particular steps of actin and microtubule cytoskeleton remodeling, which underlie the elongation of axons, their guidance towards the target cells and the formation of the synaptic terminals [21, 25, 43, 49, 61].

An attempt to identify animal miRNA/target pairs based solely on sequence complementarity tends to give a high number of false positive results [13]. One way to reduce the number of false positives is to take into account the free energy (ΔG) of putative target sites together with their immediate context, where low values of ΔG translate into greater likelyhood for a linear secondary structure allowing greater accessibility for miRNA repression [60].

Another way is based on the observation that experimentally confirmed mirna-binding sites tend to be highly conserved between related species [7], therefore multiple sequence alignment could be used to filter out less conserved regions, thus reducing the likelihood of false positive results.

Data describing predicted miRNA/target pairs for the whole *D. melanogaster* genome, which employs both of the above techniques was taken from the "MicroCosm Targets" database. It is generated using the "Miranda" algorithm which first identifies the complementary sequences then filters the results by assessing their thermodynamic stability using the Vienna RNA folding library routines and finally tests their conservation status through multiple sequence alignment.

The resulting data is available for download as text files from http://www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets/v5/download.pl.

Table 1. Groups of genes analyzed according to their function

Set No	Function	Genes	References
1	Actin filament nucleation and elongation	*Sop2, Arp66B, Arp14D, Arc-p34, Arpc3A, Arc-p20, DAAM, p16-ARC, Arpc3B, ena*	Reviewed in: [45]
2	Actin monomer binding	*capt, cib, chic, twf*	Reviewed in: [45]
3	Barbed-end capping	*Cpa, cpb*	Reviewed in: [45]
4	Pointed-end depolymerization/severing	*tsr, fliI, qua*	Reviewed in: [45]
5	Actin filament bundling	*sn, Actn, Fim, cher*	Reviewed in: [45]
6	Retrograde flow of filamentous atin	*zip, sqh*	Reviewed in: [45]
7	Microtubule plus-end binding	*CLIP-190, chb, Eb1, Apc, Apc2, CG18190*	Reviewed in: [45]
8	Microtubule stabilising	*futsch, tau*	Reviewed in: [45]
9	Microtubule-actin linkage	*shot, pod1*	Reviewed in: [45]
10*	Synaptogenesis: - *Molecular scaffolds* - *Cell adhesion proteins* - *Epithelial cell polarity*	*dlg1, scrib, lgl, Fas2, baz(par-3), par-6, par-1, arm, ed*	Reviewed in: [19, 38, 53, 57, 58]

**Only genes for which there was a record in the "MicroCosm Targets" database (http://ebi.ac.uk/enright-srv/microcosm/htdocs/targets/ are included.*

We used custom software to extract only the information relevant to our study from the raw data and present the results in tabular form (see Appendix, Table 2).

In our work we were interested in the miRNA expression control of the genes, which are involved in different stages of neurogenesis and synaptogenesis. For some of these genes our previous experiments showed that they interact genetically with *dfmr1* – the *Drosophila* ortholog of the *Fragile X mental retardation 1*(*FMR1*) in human [20]. It is established that dFMRP – *Drosophila* Fragile X Mental Retardation Protein, which is an RNA-binding protein, encoded by *dfmr1*, negatively regulates translation of specific neuronal mRNAs [28, 31, 42, 54, 59].

One possible mechanism by which dFMR1 could exert this regulatory function is based on its physical association with components of the RNA interference (RNAi) pathway – Dicer, Ago2 and Ago1 [9, 23]. Further data demonstrate that mammalian and *Drosophila* FMRP associates with endogenous miRNAs, some of which have been identified [14, 30].

A model has been created, suggesting that FMRP facilitates assembly of miRNAs at specific mRNA target sequences [39].

We found 60 miRNAs, targeting the preselected sets of 45 genes. Among them we found 3 miRNAs for genes which are CREB-inducible and involved in learning and memory (mir-9, mir-124, mir-219) and which were found by other authors to co-immunoprecipitate with FMRP from mouse brains [34] (see also Appendix, Table 2). These miRNAs regulate mainly targets from our set 1 (see Table 1), containing genes for actin filament nucleation and elongation. Two other predicted miRNAs – mir-100 and let-7 also bind FMRP [34]. Two other miRNAs – mir-100 and let-7 target *sqh* (spaghetti *squash*) – a gene, which encodes a non-muscle myosin light chain, involved in many biological processes, including actin cytoskeleton regulation [26]. *sqh* is also targeted by miRNA 219. mir-100 targets par-6 – a gene, involved in epithelial cell polarity and synaptogenesis (see Table 1).

Among the miRNAs from our results there are some, which have been reported earlier as potential regulators of the axon guidance pathway – mir-275, mir-318, mir-288, mir-282, mir-304, mir-263b, mir-306, mir-133, mir-274 [15].

Several miRNAs have been identified and validated earlier as important factors in synaptic development – mir-1, let-7, mir-125b, mir-134, cluster mir-310 [10, 14, 24, 56]. In our study we found that let-7 targets *sqh* and 312 miRNA (as part ot 310 cluster) targets CLIP-190 – a gene, involved in microtubule growth (Table 1).

On the other hand, we found many new miRNAs, potentially targeting genes for synaptic formation (set 10, encoding molecular scaffolds, cell adhesion proteins and proteins/epithelial cell polarity proteins), like: mir-1003, mir-316, mir-210, mir-278, mir-308, mir-13a, mir-4, mir-2c, mir-1016, mir-6, mir-79, mir-1012, mir-2b, mir-1017, mir-5, mir-9a, mir-13b, mir-287, mir-100, mir-2a. It is worth mentioning, that this particular set of genes appears to be most tightly regulated by miRNAs. Within this group is the gene *scrib,* which is a scaffolding protein with important synaptic functions (and which is controlled by the highest number of miRNAs among the sets analyzed – 12 miRNAs).

The list of genes in each functional group is by no means exhaustive, therefore we cannot make definite conclusions about the level of miRNA control in each case, and however, inferences can be made about the role of specific miRNA species. The data obtained by means of computational approaches opens possibilities for the physiological validation of the miRNAs predicted.

Alltogether, our analysis of the data showed that:

- A total of 60 miRNAs were implicated in the regulation of the genes of interest.
- The mean number of regulated genes per miRNA was 1.6, with highest number 4.
- The mean number of regulating miRNAs per gene was 2.16.
- Among the 45 genes of interest 11 didn't have any regulating miRNAs and one was unannotated.
- About half of all predicted miRNAs seem to preferentially target genes within a single functional group, while the rest are not specific.
- The only group for which lacked predicted miRNA targets is group 9 - microtubule-actin linkage
- The groups with the highest apparent score for mirRNA control (defined as number of miRNA/number of protein coding genes) were groups 7 with 4.

- 60 unique miRNAs are implicated in the regulation of our set of 45 genes of interest – out of a total of about 13 909 protein coding genes. This result is close to the median for a random set of 45 genes, which means that as a whole our genes of interest are not any more tightly regulated than expected. Specific sub-sets (ex. group 7) however display a higher level of apparent interactions with miRNA. This hints at a correlation between miRNA regulation and specific biological functions.

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Appendix

Table 2, sets 1 to 5

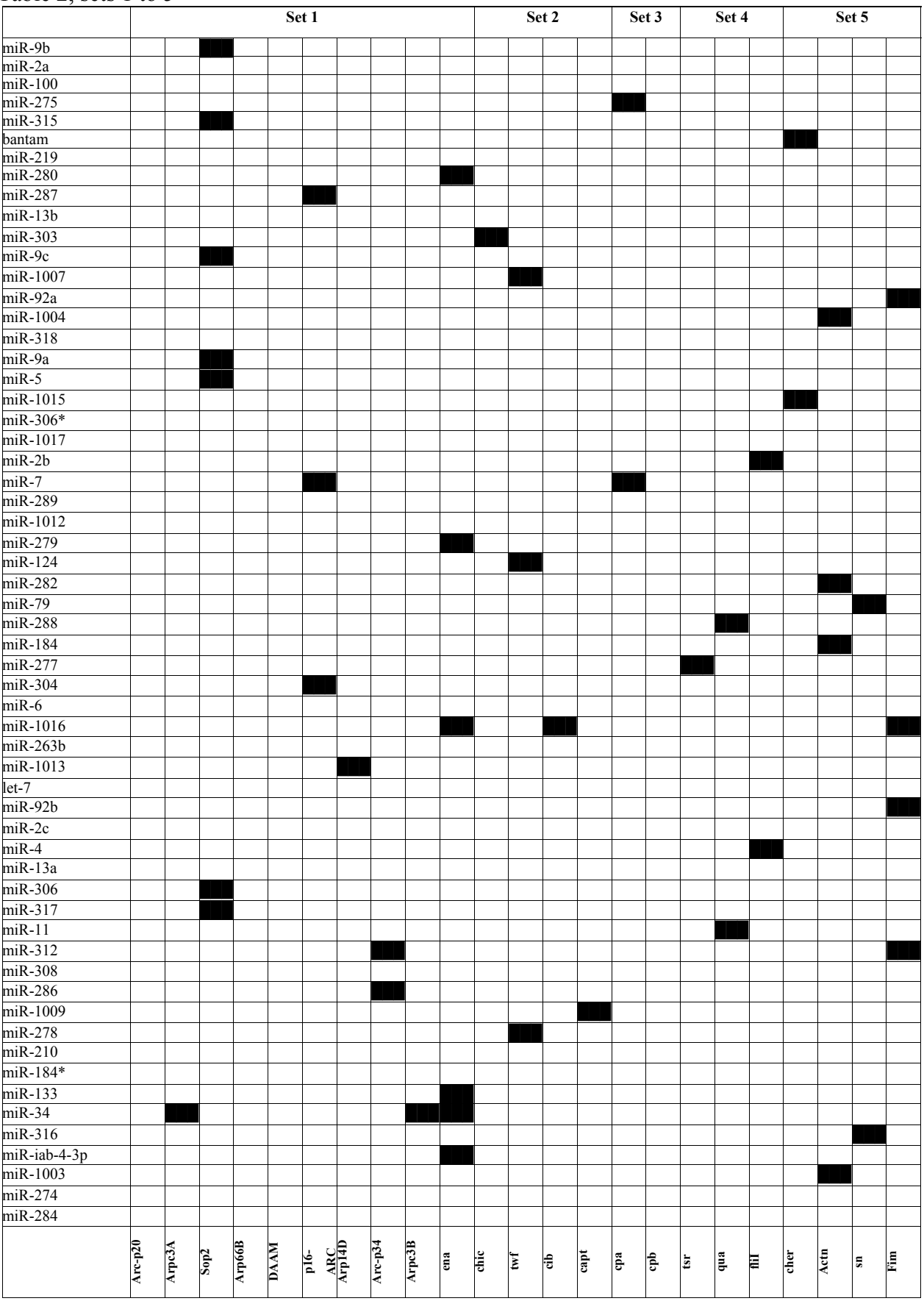
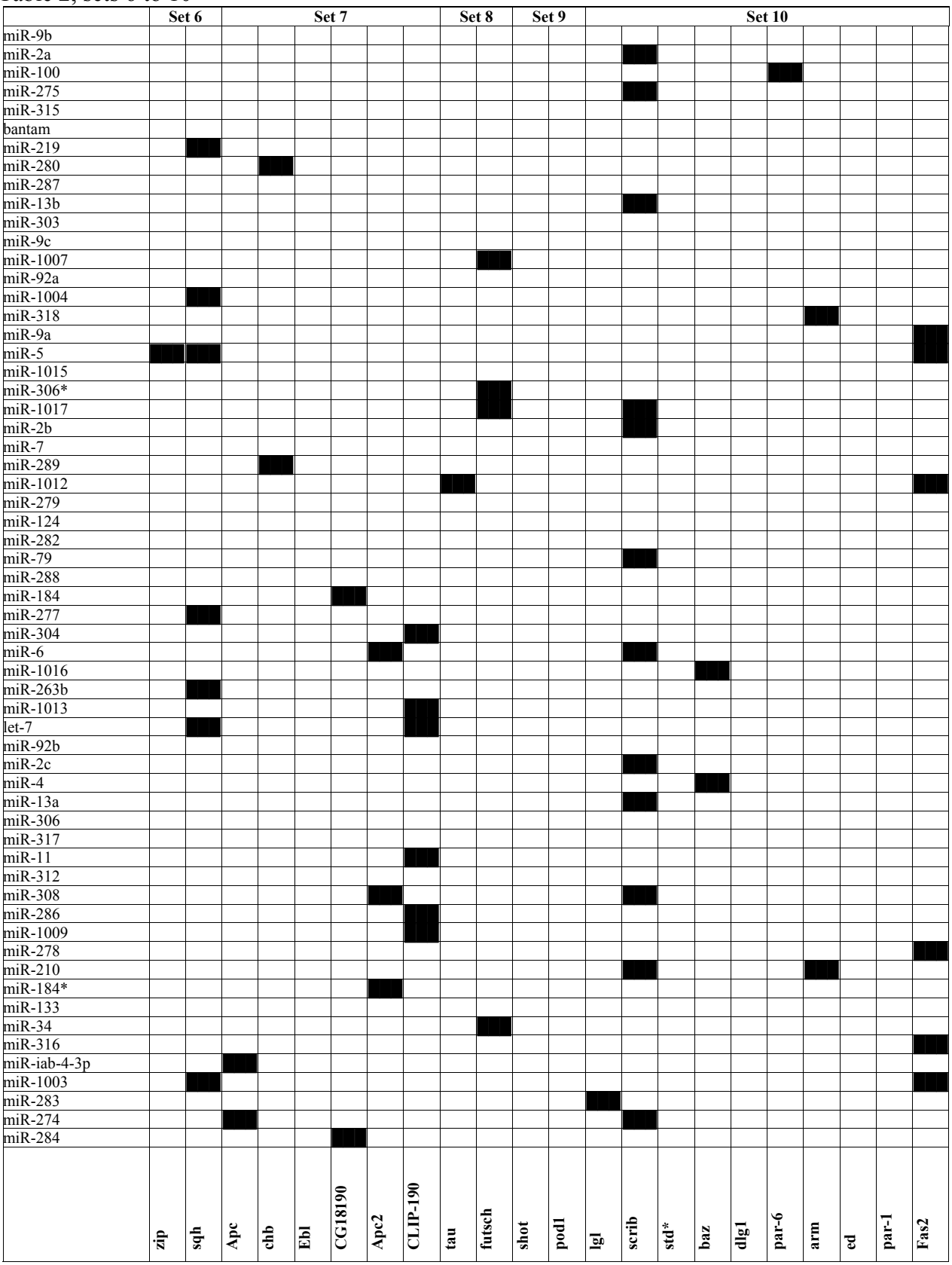


Table 2, sets 6 to 10



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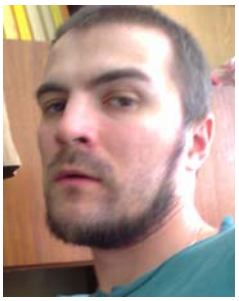

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