

1 **A novel miR-99b-5p-Zbp1 pathway in microglia contributes to the pathogenesis of schizophrenia**

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36

## 37 **Abstract**

38 Schizophrenia is a psychiatric disorder that is still not readily treatable. Pharmaceutical advances in  
39 the treatment of schizophrenia have mainly focused on the protein coding part of the human genome.  
40 However, the vast majority of the human transcriptome consists of non-coding RNAs. MicroRNAs are  
41 small non-coding RNAs that control the transcriptome at the systems level. In the present study we  
42 analyzed the microRNAome in blood and postmortem brains of controls and schizophrenia patients  
43 and found that miR-99b-5p was downregulated in both the prefrontal cortex and blood of patients.  
44 At the mechanistic level we show that inhibition of miR-99b-5p leads to schizophrenia-like phenotypes  
45 in mice and induced inflammatory processes in microglia linked to synaptic pruning. The miR-99b-5p-  
46 mediated inflammatory response in microglia depended on *Z-DNA binding protein 1 (Zbp1)* which we  
47 identified as a novel miR-99b-5p target. Antisense oligos (ASOs) against *Zbp1* ameliorated the  
48 pathological phenotypes caused by miR-99b-5p inhibition. In conclusion, we report a novel miR-99b-  
49 5p-*Zbp1* pathway in microglia that contributes to the pathogenesis of schizophrenia. Our data suggest  
50 that strategies to increase the levels of miR-99b-5p or inhibit *Zbp1* could become a novel therapeutic  
51 strategy.

## 53 **Introduction**

54  
55 Schizophrenia (SZ) is a devastating psychiatric disorder, and the difficulties involved in treating and  
56 managing it make it one of the ten most expensive disorders for health care systems worldwide [1]  
57 [2]. SZ is believed to evolve on the background of complex genome-environment interactions that  
58 alter the cellular homeostasis as well as the structural plasticity of brain cells. Thus, genetic  
59 predisposition and environmental risk factors seem to affect processes that eventually contribute to  
60 the manifestation of clinical symptoms [3] [4] [5]. Despite the available pharmacological and non-  
61 pharmacological treatment options, a significant number of patients do not benefit from these  
62 treatments in the long-term, underscoring the need for novel and potentially stratified therapeutic  
63 approaches [6]. So far, drug development has focused on the human transcriptome that encodes  
64 proteins, but the success of this approach is limited [7]. However, most of the transcriptome consists  
65 of non-coding RNAs (ncRNAs) which are recognized as key regulators of cellular functions [8].  
66 Therefore, RNA therapeutics represent an emerging concept that may expand current therapeutic  
67 strategies focused on the protein-coding part of our genome [9] [10]. RNA therapies utilize, for  
68 example, antisense oligonucleotides (ASOs), siRNA, microRNA (miR) mimics or corresponding anti-  
69 miRs to control the expression of genes and proteins implicated in disease onset and progression [11]  
70 [9]. Of particular interest are miRs, which are 19-22 nucleotide-long RNA molecules that regulate

71 protein homeostasis via binding to target mRNAs, leading either to their degradation or reduced  
72 translation [12]. miRs have been intensively studied as biomarkers and therapeutic targets in cancer  
73 [11] and cardiac diseases [13]. There is also emerging evidence from genetic studies in humans as well  
74 as functional data from mouse models that miRs play a role in CNS diseases including SZ [14] [15] [16]  
75 [17]. In addition, several studies reported changes in miR expression in blood samples of SZ patients  
76 using either qPCR analysis of selected targets or genome-wide approaches. The current findings have  
77 been summarized in several review articles [18] [19] [20]. Despite this progress, there are still only few  
78 reports on the function of candidate miRs [21]. Nevertheless, analysis of miRs in liquid biopsies is  
79 highly valuable because one miR can affect many target genes, and thus changes in miR expression  
80 can indicate the presence of multiple pathologies [22] [14] [23]. Moreover, miRs also participate in  
81 inter-organ communication [24] [25], suggesting that alterations of miR expression in liquid biopsies  
82 may inform about relevant pathological processes in other organs, including the brain. This is  
83 important since the analysis of the molecular processes underlying neuropsychiatric diseases in post-  
84 mortem human brain tissue is challenging because it might be affected e.g. by peri-mortem events or  
85 the timing of post-mortem tissue sampling. Furthermore, the onset of the disease often precedes  
86 tissue collection by decades. In contrast, liquid biopsies such as blood samples are easy to collect on  
87 the premise that molecular changes in blood mirror changes in the brain. In this context, the analysis  
88 of the microRNAome in liquid biopsies could be a suitable approach to identify candidate microRNAs  
89 that may play a role in the onset and progression of SZ.

90

91 In the present study we performed small RNA sequencing in blood samples of control participants (n  
92 = 331) and schizophrenia patients (n = 242) of the PsyCourse Study [26] (<http://www.psycourse.de/>).  
93 By cross-correlating our findings with data from post-mortem human brain tissue, we identified miR-  
94 99b-5p as a promising biomarker candidate that is decreased in blood and in the prefrontal cortex of  
95 SZ patients, and correlates with disease phenotypes. Furthermore, we found decreased levels of miR-  
96 99b-5p in the prefrontal cortex of mice to elicit SZ-like phenotypes and activate pathways linked to  
97 innate immunity. In line with these observations, inhibition of miR-99b-5p in microglia increased  
98 phagocytosis and reduced the number of synapses. Finally, we were able to demonstrate that this  
99 effect is controlled by the miR-99b-5p target gene *Zbp1*, an upstream regulator of innate immunity  
100 [27]. Taken together, our data suggest that targeting miR-99b-5p or its target *Zbp1* could provide a  
101 novel approach towards the treatment of SZ patients.

102

## 103 **Results**

104 *miR-99b-5p expression is decreased in SZ patients*

105 To identify microRNAs that play a role in the pathogenesis of SZ, we conducted small RNA sequencing  
106 of blood samples obtained from 573 participants of the PsyCourse Study (<http://www.psycourse.de/>).  
107 We analyzed 331 healthy controls and 242 SZ patients (**Fig. 1a, Fig. EV1, supplemental table 1**) [26].  
108 After data normalization and correction for confounding factors, we performed a weighted gene co-  
109 expression network analysis (WGCNA) and detected 8 co-expression modules that significantly  
110 differed between groups. Three were significantly decreased (**Fig. 1b**) while 5 were increased (**Fig. 1c**)  
111 in SZ patients (**supplemental table 2**). The turquoise, yellow, blue and red modules displayed the most  
112 significant differences among the groups ( $P < 0.0001$ ). We then investigated whether the expression  
113 levels of any of these modules correlated with the clinical phenotypes defined by the positive and  
114 negative syndrome rating scale (PANSS) and Beck depression inventory (BDI-II), both of which are  
115 decreased in SZ patients, and/or the global assessment of functioning (GAF) score, which is increased  
116 in these patients (**Fig. EV1**). Out of the 8 co-expression modules the turquoise, yellow, blue and red  
117 modules were significantly correlated to all disease phenotypes. The turquoise module exhibited a  
118 significant negative correlation to the PANSS and BDI-II score and a positive correlation with the GAF  
119 score (**Fig. 1d**), which is in line with the decreased expression of this module in SZ. The green, yellow,  
120 blue and red modules were positively correlated to the PANSS and BDI-II scores and exhibited a  
121 negative correlation with the GAF score, which is in agreement with their increased expression in SZ  
122 (**Fig. 1d**). The pink module was negatively correlated to the PANSS scores and positively correlated to  
123 the GAF, which is also in agreement with its decreased expression in SZ patients. However, the pink  
124 module was not correlated to the BDI-II score (**Fig. 1d**). The black and the brown modules were not  
125 significantly correlated any of the analyzed phenotypes. Taken together, these data suggest that  
126 especially the microRNAs present in turquoise, pink, green, blue, yellow and red modules warrant  
127 further analysis. When we subjected the confirmed targets of the microRNAs present within each of  
128 these modules to a GO-term analysis, we observed that most modules were linked to inflammatory  
129 processes, which is in agreement with a suggested role of neuro-inflammation in the pathogenesis of  
130 SZ [28] (**Fig. EV2; Supplemental table 3**). While WGCNA is a suitable first approach to identify groups  
131 of candidate microRNAs, we also performed a differential expression analysis to directly compare the  
132 microRNAome in control vs SZ patients. We found 59 microRNA that were significantly increased in SZ  
133 patients while 34 microRNAs were decreased (**Fig 1e, Supplemental table 4**).

134 With the aim to further refine the list of candidate microRNAs that may play a role in the pathogenesis  
135 of SZ, we performed small RNA sequencing from the prefrontal cortex of SZ patients (n=13) and  
136 controls (n=17). We detected 36 microRNAs that were significantly decreased in SZ patients (FDR <  
137 0,01, log<sub>2</sub>FC > 1) and 32 that were significantly upregulated (**Fig. 1f, supplemental table 5**). Next, we  
138 examined whether any of these microRNAs are also found among the differentially expressed

139 microRNAs that were altered in blood samples when compared via differential expression analysis,  
140 and within the co-expression modules decreased in SZ patients. Three miRs of the yellow cluster were  
141 significantly increased in the brain and in the blood when analyzed via differential expression analysis.  
142 In addition, one miR of the blue and one of the green clusters were also increased in brain and blood.  
143 These were miR-101-3p, miR-378a-3p, miR-21-5p, miR-192-5p and miR-103a-3p (**supplemental table**  
144 **6**). MiR- 21-3p has been associated with SZ while the other 4 miRs have been studied in the context  
145 of other neuropsychiatric or neurodegenerative diseases (**supplemental table 6**). When analyzing the  
146 down-regulated miRs we found four microRNAs, namely miR-500a-3p, miR-501-3p, miR-221-5p and  
147 miR-99b-5p, that were detected in the turquoise module and were also decreased in the postmortem  
148 brain of SZ patients (**Fig. 1g**). Of these 4 microRNAs miR-501-3p has been recently linked to  
149 schizophrenia [21] (**supplemental table 6**) while miR-99b-5p was the only candidate that was part of  
150 a significantly downregulated co-expression module and was significantly downregulated in the brain  
151 and blood of SZ patients when analyzed via differential expression analysis.

152 In summary, our data reveal a number of interesting candidate miRs such as miR-501-3p and miR-21-  
153 5p that have been already linked to SZ in previous studies [29] [21]. Most of the other miRs have been  
154 detected in the context of other brain diseases including Alzheimer's disease (AD), Major depression  
155 (MD) or Amyotrophic lateral sclerosis (ALS) (**supplemental table 6**). Except for miR-500a-3p and miR-  
156 221-5p, the expression of all other candidate miRs have is significantly correlated to the PANSSs, GAF  
157 and BDI-II scores (**Fig. 1h, Fig. EV3**). While all of these candidate miRs would warrant further functional  
158 analysis in the context of SZ, we decided to focus on miR-99b-5p since it has not been linked to any  
159 brain disease yet and comparatively little is known about this miR in general.

160

### 161 ***Decreasing miR-99b-5p leads to SZ-like phenotypes in mice and the upregulation of genes linked to*** 162 ***inflammatory processes***

163 The role of miR-99b-5p in the brain has not been intensively studied and thus no data are available in  
164 the context of neuropsychiatric diseases such as SZ, making it a novel candidate in need of further  
165 evaluation. Before performing mechanistic studies, we decided to employ mice in a model system to  
166 test the hypothesis that decreased expression of miR-99b-5p is causatively linked to the development  
167 of SZ-like phenotypes. Therefore, we generated lipid nanoparticles (LNPs) containing locked nucleic  
168 acid (LNA) representing miR-99b-5p inhibitors (anti-miR99b) and injected these into the prefrontal  
169 cortex (PFC) of mice. LNAs representing a scrambled sequence were used as controls (sc-control). MiR-  
170 99b-5p levels were significantly decreased in the PFC when measured 5 or 10 days after the injections  
171 (**Fig 2a**). To test schizophrenia-like behaviors in animals, we injected either anti-miR-99b or sc-control  
172 to the PFC of mice. Explorative behavior measured in the open field test was similar in all groups (**Fig**

173 **2b)**. However, anti-miR-99b-treated mice spent less time in the center of the open field, which is  
174 indicative of increased anxiety (**Fig 2c**), a phenotype commonly observed in schizophrenia patients  
175 [30]. We also analyzed anxiety behavior in the elevated plus maze test. Anti-miR-99b treated mice  
176 spent less time in the open arms, which indicates increased anxiety (**Fig 2d**). Another valid animal  
177 model to test schizophrenia-like behavior in rodents is the pre-pulse inhibition of the startle response  
178 (PPI) which is used to measure sensory-gating function [31]. PPI is impaired in SZ patients, can easily  
179 be assayed in mice and is impaired in mouse models for SZ [32] [33]. We observed that mice injected  
180 with anti-miR-99b displayed significantly impaired PPI responses when compared to the sc-control  
181 group (**Fig 2e**). The basic startle response was unaffected (**Fig. 2f**), suggesting that decreasing the  
182 levels of miR-99b-5p in the PFC of mice can lead to SZ-like phenotypes.

183 To gain first insights into the molecular processes controlled by miR-99b-5p in the brain, we injected  
184 another group of mice with anti-miR-99b and sc-control oligonucleotides and isolated PFC tissue 5  
185 days later for RNA sequencing analysis. Differential expression analysis revealed 147 deregulated  
186 genes (adjusted  $p$ -value  $< 0.1$ , log2FC  $\pm 0.2$ ), of which 113 genes were upregulated and 34 were  
187 downregulated (**Fig. 2g; supplemental table 7**). Gene ontology (GO) analysis revealed that  
188 upregulated genes were linked to processes such as innate immunity and interferon signaling (**Fig.**  
189 **2h**). GO analysis of the downregulated genes did not yield any highly significant pathways but detected  
190 processes linked to voltage-gated potassium channels (**Fig 2h, supplemental table 8**). These data  
191 suggest that miR-99b-5p in the PFC may regulate mRNAs linked to immune-related processes. To  
192 further test this hypothesis, we compared the list of upregulated genes with gene expression data  
193 from neurons, astrocytes and microglia as well as genes present within 3 different immune function-  
194 related gene expression databases. We observed that the upregulated genes were highly enriched in  
195 microglia ( $P = 4 \times 10^{-12}$ ), while no enrichment was observed in astrocytes or neurons. The  
196 downregulated genes were significantly enriched in neurons ( $P = 1.38 \times 10^{-12}$ ). In line with this, the  
197 upregulated genes were significantly overrepresented in 3 databases for genes linked to immune  
198 function, namely the immune, immunome and IRIS databases (**Fig. 2i**). Together, these data suggest  
199 that the levels of miR-99b-5p in the PFC of mice may specifically increase the expression of immune-  
200 related genes in microglia. To further test this we administered anti-miR-99b or sc-control to the PFC  
201 of mice and subsequently isolated CD45<sup>low</sup>/CD11b<sup>+</sup> microglial cells via fluorescence-activated cell  
202 sorting (FACS). While microglia cell numbers did not differ between groups, miR-99b-5p expression  
203 was significantly decreased in microglia isolated from anti-miR-99b-treated mice (**Fig. EV4**). The  
204 expression of selected pro-inflammatory genes *Il1 $\beta$* , *Tgfb1* and *Tnfa* that were upregulated in the RNA-  
205 seq dataset were also increased in microglia obtained from anti-miR-99b-treated mice, although *Tgfb1*  
206 failed to reach significance ( $P = 0.08$ ) (**Fig 2j**).

207 These data suggest that miR-99b-5p controls microglia-mediated immunity in the PFC, which is in  
208 agreement with previous studies linking aberrant microglia function and neuroinflammation to the  
209 pathogenesis of SZ [34].

210

### 211 ***miR-99b-5p controls microglia-mediated immune function and affects dendritic spine number***

212 To further explore the role of miR-99b-5p in microglia, we cultured primary microglia from the cortex  
213 of mice and treated these cells with anti-miR-99b or sc-control LNAs followed by RNA sequencing (**Fig**  
214 **3a**). Differential expression analysis revealed 139 deregulated genes, of which 104 were up- and 35  
215 were downregulated (**Fig 3b, supplemental table 9**). GO-term and KEGG-pathway analysis revealed  
216 that the upregulated genes were linked to immune activation and phagocytosis (**Fig 3c; supplemental**  
217 **table 10**). In agreement with the *in vivo* data, we observed an increased expression of *Il1 $\beta$* , *Tgfb1*, and  
218 *Tnfa*, which could be confirmed via qPCR (**Fig 3d; see also Fig EV4c**). Next, we investigated  
219 phagocytosis, a key function of microglia that is altered during neuroinflammation [35]. In a first  
220 approach we employed immortalized microglia (IMG) cells. Similar to the treatment with the  
221 lipopolysaccharide (LPS) commonly used to induce microglia activation, inhibition of miR-99b-5p  
222 caused an upregulation of proinflammatory cytokines and increased phagocytosis as measured via the  
223 uptake of fluorescent latex beads (**Fig. EV5**). Encouraged by these data we performed similar  
224 experiments in primary microglia isolated from the mouse PFC. In line with the data obtained in IMG  
225 cells, treatment of primary microglia with anti-miR-99b LNAs significantly increased phagocytosis (**Fig**  
226 **3e**).

227 Aberrant microglia activity can have detrimental effects on neuronal plasticity [36]. To test whether  
228 the reduced expression of miR-99b-5p in microglia could affect neuronal plasticity, we performed a  
229 co-culturing experiment. Primary microglia were first treated with sc-control or anti-miR-99b LNAs for  
230 48 h before being harvested and transferred to cortical neuronal cultures (**Fig 3f**). RNA was isolated  
231 from these co-cultures after 48 h and subjected to RNA-seq. Differential expression analysis revealed  
232 366 deregulated genes (155 upregulated and 211 downregulated genes, adjusted p value < 0.05,  
233 log<sub>2</sub>FC +/- 0.1); **Fig. 3g, supplemental table 11**). GO term analysis of the upregulated genes revealed  
234 neuroinflammatory processes, neuron death, neuron apoptotic processes as well as synaptic pruning  
235 (**Fig 3h, supplemental table 12\_UP**), while downregulated genes were associated with processes  
236 indicating loss of synaptic function such as regulation of axon extension or synapse organization (**Fig**  
237 **3h, supplemental table 12\_DOWN**).

238 These data are in agreement with our previous findings suggesting that loss of miR-99b-5p increases  
239 inflammatory processes in microglia. More importantly, the data suggest that microglia lacking miR-  
240 99b-5p may have detrimental effects on synaptic function when co-cultured with cortical neurons. It

241 is particularly interesting that synaptic pruning is detected as a major process increased in the co-  
242 cultures, since synaptic pruning has been linked to SZ [36]. In line with this, key factors of the  
243 complement system known to drive pathological synaptic pruning were increased in primary microglia  
244 treated with anti-miR-99b, as well as in the corresponding co-cultures and also in the postmortem  
245 human prefrontal cortex of schizophrenia patients (**Fig EV6**). When we analyzed the number of  
246 dendritic spines, we observed that spine density was significantly reduced in neurons co-cultured with  
247 microglia that had received anti-miR-99b, when compared to cultures treated with corresponding  
248 control microglia (**Fig 3 i**).

249

### 250 ***miR99b-5p control neuroinflammation via the regulation of Zbp1***

251 The three RNAseq datasets obtained from the PFC of mice, primary microglia and the co-cultures  
252 consistently show that knockdown of miR-99b-5p increases the expression of genes linked to  
253 inflammatory processes. Many of the gene expression changes likely represent secondary effects. To  
254 better understand the mechanisms by which miR-99b-5p controls neuroinflammation, we aimed to  
255 identify direct targets of miR-99b-5p (**supplemental table 13**). When we analyzed the RNA-seq data  
256 obtained from the PFC (see Fig 2), we identified 13 out of 113 genes as potential mRNA targets of  
257 miR99b-5p (**Fig 4A**). GO term analysis was performed for the 13 genes and revealed that they are  
258 linked to inflammatory processes including type I interferon signaling (**Fig. EV7, supplemental table**  
259 **14**), linked to schizophrenia [37] [38]. Seven of these genes were also upregulated in primary microglia  
260 treated with anti-miR-99b, and among them were key regulators of inflammatory processes such as  
261 *Stat1* which was found to be hyperactive in blood samples of SZ patients [39]. A gene that specifically  
262 caught our attention was *Zbp1* because the corresponding protein - also known as the DNA-dependent  
263 activator of interferon regulatory factors (*Dai*) - is a key regulator of pro-inflammatory processes that  
264 result in the activation of inflammatory caspases and the induction of *Il1 $\beta$*  [27]. Thus, *Zbp1*  
265 represented a rather upstream factor in the inflammatory cascade. On this basis, we speculated that  
266 the regulation of *Zbp1* could be a key mechanism by which miR-99b-5p regulates inflammatory  
267 processes and may contribute to the pathogenesis of SZ when it is increased.

268 First, we performed a luciferase assay to directly test the regulation of *Zbp1* by miR-99b-5p. We used  
269 the renilla dual luciferase reporter vector harboring the *Zbp1*-3'UTR. Co-transfection of this vector  
270 with miR-99b-5p LNAs significantly reduced the luciferase activity (**Fig 4b**), but this was not the case  
271 when scramble control LNAs were used (**Fig 4b**). Moreover, we observed that ZBP1 protein levels were  
272 significantly increased in primary microglia treated with anti-miR-99b (**Fig 4c**). These data show that  
273 miR-99b-5p can directly regulate *Zbp1* levels.

274

275 On this basis we decided to investigate whether the inflammatory phenotypes induced in response to  
276 decreased miR-99b-5p levels depend on *Zbp1*. So far, we have found that decreased levels of miR-  
277 99b-5p lead to enhanced phagocytosis and increased expression of pro-inflammatory cytokines such  
278 as *Il1 $\beta$*  which has been associated with *ZBP1* activity [40]. Another important step in ZBP1-mediated  
279 orchestration of inflammation is the activation of pro-inflammatory caspases [41] [42], and therefore  
280 we examined whether reduced miR-99b-5p levels would also affect the activity of pro-inflammatory  
281 caspases. Indeed, when primary microglia were treated with anti-miR-99b, caspase activity was  
282 significantly increased when compared to that in cells treated with sc-control LNAs (**Fig. 4d**). Similar  
283 findings were obtained when protein lysates isolated from the PFC of mice injected with either anti-  
284 miR-99b or sc-control LNAs were analyzed for caspase activity (**Fig. 4e**).

285 To test whether the miR-99b-5p-mediated increase in caspase activity, *IL-1 $\beta$*  expression and  
286 phagocytosis depends on *Zbp1*, we treated primary microglia with either anti-miR-99b alone or in  
287 combination with an anti-sense oligonucleotide (ASO) targeting *Zbp1* (*Zbp1*-ASO). In agreement with  
288 our previous observation, anti-miR-99b treatment increased caspase activity. This effect was  
289 ameliorated in microglia treated with anti-miR-99b and *Zbp1*-ASO (**Fig 4f**). Similar observations were  
290 made when we analyzed *IL1 $\beta$*  expression (**Fig. 4g**) and phagocytosis (**Fig 4h**). These data suggest that  
291 ZBP-1 plays an important role in mediating the neuroinflammatory processes downstream of miR-  
292 99b-5p.

293 We performed parallel experiments in human iPSC-derived microglia. Similar to the mouse data,  
294 administration of anti-miR-99b increased caspase activity (**Fig 4i**), IL-1 $\beta$  expression (**Fig 4j**) and  
295 phagocytosis (**Fig 4k**) when compared to human iPSC-derived microglia treated with sc-control LNAs.  
296 These effects were attenuated when anti-miR-99 LNAs were co-administered with *Zbp1*-ASOs (**Fig 4i-  
297 k**). These data suggest that in human microglia, miR-99b-5p also controls neuroinflammatory  
298 processes via the regulation of *Zbp-1* expression. In line with this interpretation, IL1 $\beta$  and *Zbp1* mRNA  
299 levels were increased in postmortem human brain samples from SZ patients and controls (**Fig. S8a**).

300 To determine whether knockdown of *Zbp1* would also mitigate the effect of anti-miR-99b treatment  
301 on SZ-like behavior in mice, we injected either anti-miR-99b alone or in combination with *Zbp1*-ASOs  
302 into the PFC of mice before subjecting the animals to behavior testing. Mice injected with sc-control  
303 LNAs served as controls. The corresponding data revealed that *Zbp1* knockdown rescues anti-miR-99b-  
304 mediated impairment of PPI (**Fig. S8**).

305 Our findings suggest that reduced miR-99b-5p levels in microglia contribute to the pathogenesis of  
306 schizophrenia via the regulation of *Zbp1*-controlled neuroinflammation. Therefore, miR-99b-5p may  
307 constitute a novel biomarker for SZ, while targeting miR-99b-5p and/or ZBP1 might represent an  
308 effective SZ treatment

309

## 310 Discussion

311 In this study we combined the analysis of blood samples and postmortem brain tissue to identify  
312 miRs involved in the pathogenesis of SZ. Using WGCNA as well as differential expression analysis in  
313 blood samples, we identified several miRs that differed between patients and controls and were  
314 significantly correlated with SZ phenotypes. GO term analysis of the confirmed target genes of these  
315 miRs hinted at a number of molecular processes of which pathways linked to immune function were  
316 overrepresented. Such a GO term analysis based on miR target genes is, of course, not ultimately  
317 conclusive but our observation is in agreement with previous studies showing that neuroinflammation  
318 plays a role in the pathogenesis of SZ [43] [44]. To further refine the identification of miRs linked to SZ  
319 we also performed a differential expression analysis of the small RNA seq data obtained from blood  
320 as well as from postmortem brain tissue of SZ patients and controls. We eventually identified five  
321 candidate miRs, miR-101-3p, miR-378a-3p, miR-21-5p, miR-192-5p and miR-103a-3p, that were  
322 increased in the blood and brain of SZ patients. All of these miRs have been implicated brain and non-  
323 brain diseases. For example, knock-down of miR-101-3p in the hippocampus impairs learning in mice  
324 [45] while increased circulating levels of miR-101-3p have been observed in patients suffering from  
325 autism [46], attention-deficit hyperactivity disorder [47] but also diabetes [48] [49] or cancer [50].  
326 MiR-378a-3p has been associated with cerebral ischemia [51] is increased in blood samples of Down  
327 syndrome patients [52] and was reported to be part of a blood-miR signature that can distinguish  
328 Alzheimer's disease patients from control [53]. miR-21-5p was found to be increased in blood samples  
329 of SZ patients [29], while antipsychotic treatment is correlated with decreased miR-21-3p expression  
330 [54]. Moreover, miR-21-3p was altered in blood samples of patients suffering from bipolar disease  
331 [55]. However, altered miR-21-3p has also been observed in various other diseases and is for example  
332 increased in patients with lung cancer [56]. Several studies have linked miR-192-5p to cognitive  
333 function and depression. For example, miR-192-5p was decreased in the blood AD patients upon  
334 aerobic exercise [57] and was altered in the brains of patients suffering from major depressive  
335 disorder [58], while increasing the levels of miR-192-5p in a mouse model for depression ameliorated  
336 cognitive impairments [59]. Finally, altered blood levels of miR-103-3p have been linked to childhood  
337 traumatization and depression [60] as well as Autism [61].

338 Four miRs, miR-500a-3p, miR-501-3p, miR-221-5p and miR-99b-5p, that all originated from the  
339 ME\_Turquoise co-expression module which was downregulated in SZ patients and significantly  
340 correlated to SZ phenotypes, were also decreased in the postmortem brains of SZ patients. A recent  
341 study identified miR-501-3p as a schizophrenia-associated miR as it was found to be decreased in  
342 blood samples of monozygotic twins discordant for SZ [21]. The authors went on to show that loss of  
343 miR-501-3p in mice leads to SZ-like phenotypes, a finding that was linked to miR-501-3p-mediated

344 regulation of *metabotropic glutamate receptor 5* expression in the cortex. These data are in  
345 agreement with our observation that miR-501-3p was decreased in SZ patients of the PsyCourse study  
346 as well as in the postmortem brains of SZ patients. As to the other 3 miRs, there are thus far no data  
347 on the role of miR-500a-3p in the CNS, and while miR-221-5p has recently been linked to the regulation  
348 of synaptic processes [62], there are still also no data on the role of miR-221-5p in SZ. As regards to  
349 miR-99b-5p, no functional data are available on its role in the CNS, and no report has as yet implicated  
350 this miR in the pathogenesis of SZ.

351 In summary, we have discovered miRs that have already been linked to the pathogenesis of SZ or  
352 other brain diseases, as well as miRs that have not been extensively studied so far. These data  
353 suggests that our approach may provide a viable means to detect novel SZ-associated miRs.

354 To test this hypothesis, we decided to investigate miR-99b-5p that was part of the ME\_Turquoise  
355 co-expression module but was also decreased in the postmortem brain of SZ patients as well as in the  
356 blood of SZ patients, when the data was analyzed via differential expression. Moreover, essentially  
357 nothing was known about the role of miR-99b-5p in the adult brain. Inhibiting miR-99b-5p in the  
358 prefrontal cortex of mice led to impaired PPI and increased anxiety. PPI is impaired in SZ patients and  
359 in mouse models for SZ [32] [33]. Furthermore, increased anxiety is a phenotype often observed in SZ  
360 patients [30], suggesting that reduced miR-99b-5p levels are indeed linked to the development of SZ-  
361 like phenotypes. Nevertheless, these data cannot establish a clearly causal link between reduced miR-  
362 99b-5p expression and the pathogenesis of SZ in humans, since no animal model can fully recapitulate  
363 the complex processes in human patients due to functional and structural differences in cortical  
364 anatomy [63] [64].

365 However, that miR-99b-5p is involved in the pathogenesis in SZ is further underscored by the  
366 results of our molecular analysis. RNA-seq analysis of the prefrontal cortex of mice revealed that  
367 inhibition of miR-99b-5p mainly led to an increased expression of genes, which is in agreement with  
368 the established action of miRs in controlling mRNA levels. Furthermore, the upregulated genes were  
369 almost exclusively related to immunity pathways in microglia, a process which has been linked to the  
370 pathogenesis of SZ by various means. For example, altered microglia have been observed in  
371 postmortem brain samples of SZ patients [65]. In addition, epidemiological data demonstrated a  
372 correlation between immune diseases and SZ [66], while several neuroimaging studies reported an  
373 increase in activated microglia in the brains of SZ patients [67] [68]. Finally, studies in animal models  
374 have implicated aberrant microglia activation with the onset of SZ-like phenotypes [69] [70]. While  
375 miR-99b-5p has not been studied in microglia so far, these data are in line with previous reports  
376 demonstrating a role of the miR-99b in the modulation of inflammatory responses. For example, miR-  
377 99b levels are decreased in tumor-associated macrophages and re-expression of miR-99b attenuates

378 tumor growth [71]. Furthermore, inhibition of miR-99b in dendritic cells significantly elevated the  
379 levels of proinflammatory cytokines including *Il1 $\beta$*  and *Tnf $\alpha$*  [72]. These findings are in agreement with  
380 our data showing that inhibition of miR-99b-5p in the prefrontal cortex of mice increased the  
381 expression of pro-inflammatory cytokines including *Il1 $\beta$*  and *Tnf $\alpha$*  in microglia that we had isolated  
382 from the brains of these mice via FACS. A strong upregulation of genes linked to inflammatory  
383 processes, including the upregulation of *Il1 $\beta$*  and *Tnf $\alpha$* , was also observed when miR-99b-5p was  
384 inhibited in IMG cells or primary microglia. This is interesting since increased *Il1 $\beta$*  and *Tnf $\alpha$*  levels  
385 have been repeatedly reported in SZ patients [73] and may offer novel therapeutic avenues. For  
386 example, inhibition of TNF $\alpha$  was recently shown to ameliorate disease phenotypes in different mouse  
387 models of SZ [70].

388 Aberrant microglia activation can affect neuronal function via synaptic pruning, a process that is  
389 based on the phagocytic activity of microglia [74]. We observed that inhibition of miR-99b-5p in IMG  
390 cells and in primary microglia increased their phagocytic activity. Moreover, cortical neurons co-  
391 cultured with microglia that were treated with anti-miR-99b oligonucleotides displayed differentially  
392 expressed genes, of which the downregulated genes were linked to GO terms such as synapse  
393 assembly, regulation of synaptic plasticity or dendritic spine organization. As for the upregulated  
394 genes, the most significant GO term was synapse pruning. Since our data also revealed that neurons  
395 co-cultured with anti-miR-99b-treated microglia indeed displayed a reduced number of dendritic  
396 spines, our findings suggest a scenario in which reduced levels of miR-99b-5p lead to an upregulation  
397 of proinflammatory processes in microglia, which eventually impacts on synaptic structure. This  
398 interpretation is in agreement with previous reports suggesting that aberrant microglia activation  
399 leads to pathological synaptic pruning, which in turn leads to plasticity defects which could drive the  
400 pathogenesis of SZ [36] [75]. Notably, the increased expression of several complement factors in  
401 microglia have been implicated in this process [76]. In line with these data, we observed increased  
402 expression of key complement factors in primary microglia and in corresponding microglia/neuron co-  
403 cultures in which miR-99b-5p was inhibited, as well as in postmortem brain samples from SZ patients.  
404 In summary, these findings provide a plausible mechanism on how reduced levels of miR-99b-5p can  
405 contribute to the pathogenesis of SZ, namely the induction of a pro-inflammatory response associated  
406 with synaptic pruning. Nevertheless, we cannot exclude that additional mechanisms within microglia  
407 or other neural cells play a role.

408 MiRs mediate their biological action by controlling the expression of specific target mRNAs. Our data  
409 showed that within microglia, miR-99b-5p controls the expression of the *Zbp1* gene that plays an  
410 important role in the innate immune response [27]. ZBP1 acts as sensor for Z-DNA/Z-RNA and controls  
411 inflammatory pathways such as type I interferon-signaling and other pathways, eventually leading to

412 the upregulation of various pro-inflammatory cytokines including e.g. the induction of *IL1 $\beta$*  [77] [78]  
413 [40].

414 These data suggest that reduced levels of miR-99b-5p in microglia contribute to SZ-like  
415 phenotypes because the tight control of *Zbp1* levels is lost. In line with this interpretation, we  
416 demonstrated that the administration of *Zbp1*-ASO rescues the effects of anti-miR-99b treatment on  
417 SZ-like phenotypes in mice as well as in the corresponding cellular alterations observed in primary  
418 microglia from mice as well as in microglia derived from human iPSCs. Interestingly, the cellular  
419 processes we find to be affected by altered miR-99b-5p and *Zbp1* levels have also been implicated in  
420 other brain diseases. Thus, it will be important to investigate the role of miR-99b-5p and *Zbp1* in other  
421 neuropsychiatric diseases. Moreover, aberrant microglia activation and synaptic pruning is observed  
422 in neurodegenerative diseases such as Alzheimer's disease [79], and ZBP1 also controls the NLRP3  
423 inflammasome [78], a key regulator of neuroinflammatory phenotypes in Alzheimer's disease [80]. In  
424 this context it is interesting to note that one study found decreased miR-99b-5p levels in plasma  
425 samples obtained in a mouse model of Alzheimer's disease when measured at 6 and 9 months of age,  
426 while increased levels were reported in older mice [81]. These data might underscore the need for  
427 further study as to the role of *miR-99b-5p* and *Zbp1* in microglia obtained from wild type mice as well  
428 as mouse models for neuropsychiatric or neurodegenerative diseases at different ages. Indeed, it is  
429 well established that microglia undergo age-dependent functional changes and even differ between  
430 brain regions [82] [83].

431 There are other questions we could not address within the scope of this manuscript. It will for  
432 example be interesting to investigate the other candidate miRs we found in addition to miR-99b-5p.  
433 Similarly, it will be important to study the potential miR-99b-5p targets we found in addition to *Zbp-1*  
434 in the context of SZ. Another question relates to the mechanisms that underlie the downregulation of  
435 miR-99b-5p in SZ patients. In future projects it will be interesting to test for example whether miR-  
436 99b-5p is altered in SZ mouse models that are based on either genetic or environmental risk factors  
437 such as early life stress. In addition, it will be important to identify the source of elevated miR-99b-5p  
438 levels in blood samples of SZ patients. It is known that miRs can be transported from the brain to the  
439 periphery within exosomes [84] [25], and recent studies reported the isolation of microglia-derived  
440 exosomes from human blood [85]. While this approach is not undisputed, it will be interesting to apply  
441 such methods to the PsyCourse Study, which is, however, beyond the scope of the current work.  
442 Although our findings that miR-99b-5p is decreased in the brain and the blood of SZ patients support  
443 the idea that the changes in blood may reflect corresponding changes in the brain, we cannot  
444 conclusively answer this question at present. Rather, we suggest that the analysis of miR-99b-5p levels

445 in blood may eventually help stratify patients for treatment, including novel approaches based on RNA  
446 therapeutics towards miR-99b-5p or *Zbp1*.

447 In conclusion, in the present study we identify a miR-99b-5p-*Zbp1* pathway in microglia as a  
448 novel mechanism that likely contributes to the pathogenesis of schizophrenia. Our data also suggest  
449 that strategies to increase the levels of miR-99b-5p or inhibit *Zbp1*, for example via ASOs, could serve  
450 as novel therapeutic strategies for treating SZ patients.

451

## 452 **Material and Methods**

### 453 *Human subjects:*

454 All experiments involving human data were approved by the relevant Ethics committees (see Budde  
455 et al.). Informed written consent was obtained for all subjects. Blood samples (PAXgene Blood RNA  
456 Tubes; PreAnalytix, Qiagen) and behavioral data (supplemental table 1) of control and schizophrenia  
457 patients were obtained from participants of the PsyCourse Study [26]. Psychiatric diagnoses were  
458 confirmed using the Diagnosis and Statistical Manual of Mental Disorders Fourth Edition (DSM-IV)  
459 criteria. Control subjects were screened for psychiatric disorders using parts of the structured clinical  
460 interviews for mental disorders across the lifespan (MINI-DIPS). All subjects were assessed for  
461 psychiatric symptoms through a battery of standard tests including the Positive and Negative  
462 Syndrome Scale (PANSS), the Global Assessment of Functioning Scale (GAF), and the Beck depression  
463 inventory (BDI-II).

464

### 465 *Post-mortem human brain samples:*

466 Postmortem tissue samples (prefrontal cortex A9&24) from controls (n = 17; 5 females & 12 males;  
467 age = 62.3 ± 18.9 years, PMD = 19.7 ± 6.7 h) and schizophrenia patients (n = 13; 5 females & 8 males;  
468 age 57.7 ± 16.8 years, PMD = 21 ± 6.4 h) were obtained with ethical approval and upon informed  
469 consent from the Harvard Brain Tissue Resource Center (Boston, USA). RNA was isolated using Trizol  
470 as described in the manufacturer protocol using the Directzol RNA isolation kit (Zymo Research,  
471 Germany). RNA concentration was determined by UV measurement. RNA integrity for library  
472 preparation was assessed using an RNA 6000 NanoChip in a 2100 Bioanalyzer (Agilent Technologies).

473

### 474 *High throughput small RNAome sequencing:*

475 Small RNAome libraries were prepared with total RNA according to the manufacturer's protocol with  
476 NEBNext® small RNA library preparation kit. All human subject small RNAome libraries were prepared  
477 with 150 ng of total RNA. Briefly, total RNA was used as starting material, and the first strand of cDNA  
478 was generated, followed by PCR amplification. Libraries were pooled and PAGE was run for size

479 selection. For small RNAome, ~150 bp band was cut and used for library purification and  
480 quantification. A final library concentration of 2 nM was applied for sequencing. The Illumina HiSeq  
481 2000 platform was used for sequencing and was performed using a 50-bp single read setup. Illumina's  
482 conversion software bcl2fastq (v2.20.2) was used for adapter trimming and converting the base calls  
483 in the per-cycle BCL files to the per-read FASTQ format from raw images. Demultiplexing was carried  
484 out using Illumina CASAVA 1.8. Sequencing adapters were removed using cutadapt-1.8.1. Sequence  
485 data quality was evaluated using FastQC ([http://www.bioinformatics.babraham.  
486 ac.uk/projects/fastqc/](http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)). Sequencing quality was determined by the total number of reads, the  
487 percentage of GC content, the N content per base, sequence length distribution, duplication levels,  
488 overrepresented sequences and Kmer content.

489

490 *Data processing, QC, and Differential expression (DE) analysis:*

491 Sequencing data was processed using a customized in-house software pipeline. Quality control of raw  
492 sequencing data was performed by using FastQC (v0.11.5). The quality of miRNAs reads was evaluated  
493 by mirtrace (v1.0.1). Reads counts were generated using TEsSmall (v0.4.0) which uses bowtie (v1.1.2)  
494 for mapping. Reads were aligned to the Homo\_sapiens GRCh38.p10 genome assembly (hg38). The  
495 miRNA reads were annotated using miRBase. Read counts were normalized with the DESeq2 (v1.26.0)  
496 package. Unwanted variance such as batch effects, library preparation effects, or technical variance  
497 was removed using RUVSeq for all data (v1.20.0; k = 1 was used for factors of unwanted variation).  
498 DESeq2 was utilized for differential expression analysis and adjustment of confounding factors. In the  
499 DESeq2 model, the PsyCourse data were corrected for sex, age and medication in DESeq2. Volcano  
500 plots were plotted with the R package EnhancedVolcano (v1.4.0).

501

502 *WGCNA analysis:*

503 microRNAome co-expression module analysis was carried out using the weighted gene co-expression  
504 network analysis (WGCNA) package (version 1.61) in R [86]. We first regressed out age, gender, and  
505 other latent factors from the sequencing data, and after that, normalized counts were log (base 2)  
506 transformed. Next, the transformed data were used to calculate pairwise Pearson's correlations  
507 between microRNAs and define a co-expression similarity matrix, which was further transformed into  
508 an adjacency matrix. Next, a soft thresholding power of 8 was chosen based on approximate scale-  
509 free topology and used to calculate pairwise topological overlap between microRNAs in order to  
510 construct a signed microRNA network. Modules of co-expressed microRNAs with a minimum module  
511 size of 10 were later identified using cutreeDynamic function with the following parameters: method  
512 = "hybrid", deepSplit = 4, pamRespectsDendro = F, pamStage = T. Closely related modules were merged

513 using a dissimilarity correlation threshold of 0.25. Different modules were summarized as a network  
514 of modular eigengenes (MEs), which were then correlated with the different psychiatric symptoms  
515 and functionality variables (e.g., PANSS, GAF etc). The module membership (MM) of microRNAs was  
516 defined as the correlation of microRNA expression profile with MEs, and a correlation coefficient  
517 cutoff of 0.5 was set to select the module specific microRNAs. The Pearson correlation of MEs and  
518 psychiatric symptoms and functionality variable was plotted as a heat map.

519

#### 520 *Enriched gene ontology and pathways analysis:*

521 To construct the Gene Regulatory network (GRN) for miRNA-target genes we retrieved validated  
522 microRNA targets from miRTarBase (v 7.0) (<http://mirtarbase.mbc.nctu.edu.tw/>). microRNA target  
523 genes were further filtered based on the expression in the brain. Brain-enriched expression was  
524 examined using the Genotype-Tissue Expression (GTEx) database. (GTEx Consortium). To identify the  
525 biological processes and their pathways in the miRNA-target genes, the ClueGO v2.2.5 plugin of  
526 Cytoscape 3.2.1 was used [87]. In the ClueGo plugin [88] a two-sided hypergeometric test was used to  
527 calculate the importance of each term and the Benjamini-Hochberg procedure was applied for the P  
528 value correction. KEGG (<https://www.genome.jp/kegg/>) and Reactome (<https://reactome.org/>)  
529 databases were used for the pathway analysis. To construct GRN for significantly deregulated mRNAs,  
530 the ClueGO v2.2.5 plugin of Cytoscape 3.2.1 was used. Biological processes (BP) and pathways with  
531 adjusted p value < 0.05 were selected for further analysis. For further analysis, cellular metabolism  
532 and cancer-related biological processes were omitted. Key BPs with low levels of GOLevel (because  
533 terms at lower levels are more specific and terms higher up are more general) were further considered  
534 for data presentation and interpretation.

535

#### 536 *microRNA and mRNAs lipid nanoparticles preparation:*

537 miR99b-5p inhibitor sequences were used to decrease the expression of miR99b-5p. To decrease the  
538 expression of mRNAs, anti-sense oligos (ASO) were employed. ASOs, inhibitor and negative control  
539 sequences were purchased from Qiagen. MicroRNA inhibitor, or ASOs lipid nanoparticle (LNP)  
540 formulation, was achieved using a proprietary mixture of lipids containing an ionizable cationic lipid,  
541 supplied as Neuro9™ siRNA Spark™ Kit (5 nmol). The miRNA inhibitor or ASOs were encapsulated using  
542 a microfluidic system for controlled mixing conditions on the NanoAssemblr™ Spark™ system  
543 (Precision Nanosystems, Canada). The experiments were performed as described in the  
544 manufacturer's protocol. Briefly, 5 nmol lyophilized microRNA inhibitor or ASOs were dissolved in  
545 formulation buffer 1 (FB1) to a final concentration of 2 nmol. This solution was further diluted to a  
546 final concentration of 930 ug/mL. Formulation buffer 2 (FB 2), microRNA inhibitor/ASOs in FB1, and

547 lipid nanoparticles were added to the cartridge and encapsulated using the NanoAssembler Spark  
548 system.

549

#### 550 *Animals*

551 C57BL/6J mice were purchased from Janvier and housed in an animal facility with a 12-h light–dark  
552 cycle at constant temperature (23 °C) with *ad libitum* access to food and water. Animal experiments  
553 complied with relevant ethical regulations and were performed as approved by the local ethics  
554 committee. All experiments were performed with 3 months old male mice. Pre-frontal cortex (PFC)  
555 region was dissected on day five after stereotaxic surgery for RNA-seq-based experiments.

556

#### 557 *Stereotaxic surgery:*

558 For intracerebral stereotaxic injections of LNPs in the PFC, 3-month-old mice were anesthetized with  
559 Rompun 5mg/kg and Ketavet 100mg/kg. After application of local anesthesia to the skull, two small  
560 holes were drilled into the skull. Mice then received a bilateral injection of LNPs of microRNA  
561 inhibitor/negative control or ASOs (dose: 0.15 ug/mL for microRNA inhibitor/negative control; dose:  
562 0.3 ug/mL for ASO+ microRNA inhibitor mix). LNPs were injected with a rate of 0.3 µl/min per side.  
563 Only 0.9 ul of LNPs were injected per hemisphere (0,5 µl/min). After surgery, all mice were monitored  
564 until full recovery from the anesthesia and housed under standardized conditions.

565

#### 566 *Behavioral phenotyping:*

567 The open field test was performed to evaluate locomotory and exploratory functions. Mice were  
568 placed individually in the center of an open arena (of 1 m length, 1 m width, and side walls 20 cm  
569 high). Locomotory activity was recorded for 5 min using the VideoMot2 tracking system (TSE Systems).  
570 The elevated plus maze test was used to evaluate basal anxiety. Mice were placed individually in the  
571 center of a plastic box consisting of two open and two walled closed arms (10 × 40 cm each, walls 40  
572 cm high). Their behavior was recorded for 5 min using the VideoMot2 system. Time spent in open  
573 versus closed arms was measured to assay basal anxiety phenotype. Prepulse inhibition (PPI) was  
574 performed to test the acoustical startle response (ASR). ASR was completed in an enclosed sound-  
575 attenuated startle box from TSA Systems. In brief, mice were placed individually inside a cage attached  
576 with a piezoelectric transducer platform in a sound-attenuated startle cabinet. These sensory  
577 transducers converted the movement of the platform induced by a startle response into a voltage  
578 signal. Acoustic stimuli were executed through speakers inside the box. The mice were given 3 min to  
579 habituate at 65 dB background noise and their activity was recorded for 2 min as baseline. After the  
580 baseline activity recording, the mice were tested to six pulse-alone trials, at 120-dB startle stimuli

581 intensity for a duration of 40 ms. PPI of startle activity was measured by conducting trials for pre-pulse  
582 at 120 dB for 40 ms or preceding non-startling prepulses of 70, 75, 80, 85, 90 dB.

583

584 *RNA isolation:*

585 Humans: PAXgene Blood RNA Tubes (PreAnalytix/Qiagen) were stored at -80°C. For RNA isolation, the  
586 tubes were thawed and incubated at room temperature overnight. RNA was extracted according to  
587 the manufacturer's protocol using PAXgene Blood RNA Kits (Qiagen). RNA concentrations were  
588 measured by UV measurement. RNA integrity for library preparation was determined by analyzing  
589 them on an RNA 6000 NanoChip using a 2100 Bioanalyzer (Agilent Technologies).

590 Mice: The mice were sacrificed by cervical dislocation on day five after stereotaxic surgery. Unilateral  
591 PFC region was collected and immediately frozen in liquid nitrogen and later stored at -80°C until RNA  
592 isolation. Total RNA was isolated using the trizol method as described by the manufacturer's protocol  
593 using the Directzol RNA isolation kit (Zymo Research, Germany). The RNA concentration was  
594 determined by UV measurement. RNA integrity for library preparation was assessed using a  
595 Bioanalyzer (Agilent Technologies).

596

597 *RNA sequencing:*

598 Total RNA was used for the library preparation using the TrueSeq RNA library prep kit v2 (Illumina,  
599 USA) according to the manufacturer's protocol. 500 ng RNA was used as starting material. The quality  
600 of the libraries was assessed using the Bioanalyzer (Agilent Technologies). Library concentration was  
601 measured by Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific, USA). Multiplexed libraries were  
602 directly loaded onto a HiSeq2000 (Illumina) with 50 bp single read setup.

603 The sequencing data were processed using a customized in-house software pipeline. Illumina's  
604 conversion software bcl2fastq (v2.20.2) was employed for adapter trimming and converting the base  
605 calls in the per-cycle BCL files to the per-read FASTQ format from raw images. Quality control of raw  
606 sequencing data was carried out using FastQC (v0.11.5)([http://www.bioinformatics.babraham.  
607 ac.uk/projects/fastqc/](http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)). Reads were aligned using the STAR aligner (v2.5.2b) and read counts were  
608 generated using featureCounts (v1.5.1). The mouse genome version mm10 was utilized.

609

610 *Publicly available datasets:*

611 Various publicly available datasets were used in this study to explore cell type-specific expression of  
612 differentially expressed genes. Published single cell data [89] were utilized to explore neuron-,  
613 astrocyte-, and microglia-specific expression of genes. Immunome-related genes were retrieved from

614 the Immunome database. The Immune Response In Silico (IRIS) dataset was used to explore immunity-  
615 related genes [90] [91]

616

617 *Primary microglia cultures:*

618 Primary mouse microglia cell cultures were prepared as previously described for wild-type pups [92].  
619 In brief, newborn mice (P1 pups) were used to prepare mixed glia cultures. Cells were grown in DMEM  
620 (Thermo Fisher Scientific) with 10% FBS, 20% L929 conditioned medium and 100 U ml<sup>-1</sup> penicillin–  
621 streptomycin (Thermo Fisher Scientific). Microglia were collected 10-12 days after cultivation by shake  
622 off, counted and plated in DMEM supplemented with 10% FBS, 20% L929 conditioned medium and  
623 100 U ml<sup>-1</sup> penicillin–streptomycin. The microglia were shaken off up to two times.

624

625 *Ex-vivo isolation of microglia:*

626 PFC regions were dissected, mechanically dissociated and digested for 15 minutes with liberase (0.4  
627 U/mL; Roche) and DNase I (120 U/mL; Roche) at 37°C. Subsequently, the cell suspension was passed  
628 through a 70 µm cell strainer. Myelin debris was eliminated by the Percoll density gradient. Single cell  
629 suspension was labeled by using anti-mouse CD45 BV 421 (Clone 30-F11, Biolegend) and CD11b FITC  
630 (Clone M1/70, Biolegend). Antibody-labeled CD45<sup>low</sup> CD11b<sup>+</sup> microglial cells were sorted using a  
631 FACS Aria 4L SORP cell sorter (Becton Dickinson) The purity of the sorted microglial cells was above  
632 90%.

633

634 *Primary neuronal culture:*

635 Primary neuronal cultures were prepared from E17 pregnant mice of CD1 background (Janvier Labs,  
636 France). Briefly, mice were sacrificed and the brains of embryos were taken out, meninges removed,  
637 and the cortex dissected out. The cortexes were washed in 1× PBS (Pan Biotech, Germany). Single-cell  
638 suspensions were generated by incubating them with trypsin and DNase before careful disintegration.  
639 One hundred and thirty thousand cells per well were plated on poly-D-lysine-coated 24-well plates in  
640 Neurobasal medium (Thermo Fisher Scientific, Germany) supplemented with B-27 (Thermo Fisher  
641 Scientific, Germany). Primary cortical neurons were used for experiments at DIV10-12.

642

643 *Cell lines*

644 All human iPSCs used in this study are commercially available and reported to be derived from material  
645 obtained under informed consent and appropriate ethical approvals.

646

647 *Differentiation of microglia from induced pluripotent stem cells:*

648 Human induced pluripotent stem cells lines (hiPSCs) (Cell line IDs: KOLF2.1J [93] were obtained from  
649 The Jackson Laboratory; BIONi010-C and BIONi037-A were both from the European bank for Induced  
650 Pluripotent Stem Cells) were differentiated to microglia as previously described [94]. In brief,  $3 \times 10^6$   
651 iPSCs were seeded into an Aggrewell 800 well (STEMCELL Technologies) to form embryoid bodies  
652 (EBs), in mTeSR1 and fed daily with medium plus 50 ng/ml BMP4 (Miltenyi Biotec), 50 ng/ml VEGF  
653 (Miltenyi Biotec), and 20 ng/ml SCF (R&D Systems). Four-day EBs were then differentiated in 6-well  
654 plates (15 EBs/well) in X-VIVO15 (Lonza) supplemented with 100 ng/ml M-CSF (Miltenyi Biotec), 25  
655 ng/ml IL-3 (Miltenyi Biotec), 2 mM Glutamax (Invitrogen Life Technologies), and 0.055 mM beta-  
656 mercaptoethanol (Thermo Fisher Scientific), with fresh medium added weekly. Microglial precursors  
657 emerging in the supernatant after approximately 1 month were collected and isolated through a 40  
658  $\mu$ m cell strainer and plated in N2B27 media supplemented with 100 ng/ml M-CSF, 25 ng/ml interleukin  
659 34 (IL-34) for differentiation.

660

661 *Quantitative PCR experiment:*

662 cDNA synthesis was performed using the miScript II RT Kit (Qiagen, Germany) according to the  
663 manufacturer's protocol. In brief, 200 ng total RNA was used for cDNA preparation. HiFlex Buffer was  
664 used so that the cDNA could be used for both mRNA and microRNA quantitative PCR (qPCR). A  
665 microRNA-specific forward primer and a universal reverse primer were used for quantification. The  
666 U6 small nuclear RNA gene was employed as an internal control. For mRNA quantification, gene-  
667 specific forward and reverse primers were used. The relative amounts of mRNA were normalized  
668 against GAPDH. The fold change for each microRNA and mRNA was calculated using the  $2^{-\Delta\Delta Ct}$   
669 method<sup>18</sup>. The Light Cycler® 480 Real-Time PCR System (Roche, Germany) was used to perform qPCR.

670

671 *Caspase 1 activation assay:*

672 Caspase-Glo® 1 Inflammasome Assay (Promega, Germany) was used to detect caspase 1 activation as  
673 described in the manufacturer's protocol. In brief, microglia, treated with ASO/inhibitor or primed  
674 with LPS and stimulated with ATP, were seeded on opaque, flat-bottom 96-well plates (Cellstar,  
675 Germany) at 50,000 per well in 100  $\mu$ l DMEM supplemented with 10% FBS, 20% L929 conditioned  
676 medium and 100 U ml<sup>-1</sup> penicillin–streptomycin. 100  $\mu$ l of Caspase-Glo buffer was mixed with cell  
677 medium. Plates were incubated at room temperature for 1 h. Luminogenic caspase activity was  
678 measured using a FLUOstar Omega plate reader (BMG Labtech).

679

680 *Microglia phagocytosis assay:*

681 The microglia phagocytosis assay was performed as described<sup>19</sup>. Primary microglia cultures were  
682 plated at a density of  $18 \times 10^4$  in poly-D-lysine-coated 24-well plates in DMEM supplemented with 10%  
683 FBS, 20% L929 conditioned medium and 100 U ml<sup>-1</sup> penicillin–streptomycin. Immortalized microglia  
684 (IMG) cultures were plated at a density  $5 \times 10^3$  in poly-D-lysine-coated 24-well plates in DMEM  
685 supplemented with 10% FBS, 1X Glutamine (Millipore), and 100 U ml<sup>-1</sup> penicillin–streptomycin. To  
686 evaluate phagocytosis, treated microglia were incubated with fluorescent latex beads of 1  $\mu$ m  
687 diameter (green, fluorescent 496/519; Sigma-Aldrich) for 1 h at 37°C, rinsed, and fixed with 4%  
688 formaldehyde. Cells were stained using the Iba1 (CD68) antibody (1:500; Wako) and DAPI. A confocal  
689 microscope was used for imaging at a low magnification (10x). ImageJ was used to quantify fluorescent  
690 latex beads. Region of interests (ROIs) were selected as microglial cells outlined with the Iba1  
691 immunostaining to quantify beads. An intracellular section of the cell was selected to assure  
692 engulfment of latex beads by microglia. Similar acquisition parameters were used for each individual  
693 experiment. The results were expressed as the percentage of phagocytic index (# of total engulfed  
694 beads in an image / # of total cells identified in an image; n = 13 independent experiments).

695

#### 696 *Synaptic pruning in primary microglia neural co-culture:*

697 Primary cortical neurons were seeded at a density of 130,000 on poly-D-lysine-coated 13 mm  
698 coverslips in 24-well plates in Neurobasal medium supplemented with B-27. Primary cortical neurons  
699 were used for experiments at DIV10-12. Treated primary microglia cultures were harvested from T-75  
700 flasks and 4000 cells were seeded to each neural culture well. Plates were kept at 37°C for three days.  
701 On the third day, the cells were washed and fixed with 4% PFA (Sigma Aldrich, Germany) and 100  
702 mM NH<sub>4</sub>Cl (Merck, Germany) respectively, at room temperature for 30 minutes. Next, the cells were  
703 washed in permeabilization and blocking buffer (0.1% Triton-X [Merck, Germany] + 3% bovine serum  
704 albumin (BSA) [AppliChem GmbH, Germany]) on a shaker. The cells were then incubated with primary  
705 antibodies for 1 hour at room temperature. The antibodies used included synaptophysin 1 (guinea pig,  
706 SySy), PSD-95 (rabbit, Cell Signaling,), and Iba1 (goat, Abcam). After incubation, the cells were washed  
707 in PBS and then incubated with a secondary antibody for 1 hour at room temperature. As secondary  
708 antibodies, Cy3 (donkey, anti- guinea pig, Jackson Imm.), Abberior STAR 635p (goat, anti-rabbit) were  
709 used. Mowiol (Merck, Germany) and DAPI were used as a mounting medium. Images were taken with  
710 a multicolor confocal STED microscope (Abberior Instruments GmbH, Göttingen, Germany). Analysis  
711 of colocalization of pre- and post-synaptic markers were performed using SynQuant plugins in Fiji (v  
712 2.0.0).

713

#### 714 *Dendritic spine analysis:*

715 As described above, primary cortical neurons and primary microglia were co-cultured and fixed with  
716 4%PFA. Dendritic spines were labeled as described [95]. In brief, the cells were aspirated and 2-3  
717 crystals of Dil stain (Life Technologies-Molecular Probes) were added to each culture well and  
718 incubated on a shaker for 10 minutes at room temperature. Cells were washed with PBS until no  
719 crystals were visible and incubated overnight at room temperature. On the following day, the cells  
720 were washed and mounted with Mowiol. For high-magnification images, a multicolor confocal STED  
721 microscope with a 60× oil objective was used. Spine density and total spine length were measured by  
722 using ImageJ.

723

#### 724 *Protein extraction of primary microglia:*

725 Primary microglia cell lysates were used to detect ZBP1 in RIPA fractions. Primary microglia were  
726 seeded in a 6-well plate at a density of  $1 \times 10^6$  in each well. Cells were collected in a RIPA buffer  
727 supplemented with 1 x protease inhibitor. Samples were kept on ice for 15 minutes and vortexed  
728 every 5 minutes and then centrifuged at 5000 rpm for 15 minutes at 4°C before supernatants were  
729 transferred to a new tube and stored at -20°C. The protein concentration was measured using a BCA  
730 assay.

731

#### 732 *Immunoblot analysis:*

733 For standard immunoblot analysis, 20 ug of samples were mixed with 1× Laemmli buffer (Sigma,  
734 Germany), heated for 5 min at 95°C and loaded onto 4–15% Mini-PROTEAN® TGX™ Precast Protein  
735 Gels (Bio-Rad, Germany). Proteins were transferred on nitrocellulose membranes and membranes  
736 were blocked with 5% BSA in PBS-Tween. Membranes were incubated with primary antibodies in 5%  
737 BSA in PBS-Tween. Fluorescent-tagged secondary antibodies (LI-COR) were used for visualization of  
738 proteins. Imaging was performed using a LI-COR ODYSSEY. HSP-70, GAPDH were used as a loading and  
739 run on the same gel.

740

#### 741 *Treatment of microglia:*

742 Microglia activation by LPS was used as a positive control. For this, microglia cells were first primed  
743 with 100 ng/ml ultrapure LPS (E. coli 0111:B4, Invivogen) and then incubated at 37°C. After this, 5 mM  
744 ATP were added to the culture and incubated for 30 minutes. Caspase 1 assay and phagocytosis assay  
745 were performed from these cultures. For immunoblot, cell lysate was prepared. For miR99b-5p-  
746 related analysis, microglia were either treated for two days with miR99b-5p inhibitor/negative control  
747 or ASOs in T-75 after first harvesting or after harvesting cells were seeded in a 24-well culture plate.

748

749 *Luciferase assay:*

750 Seed sequences of miR-99b-5p and pairing 3'UTR sequences of Zbp1 were generated with TargetScan.  
751 Cloned 3'UTR sequence of Zbp1 and scrambles UTR were purchased from Gene Copoeia  
752 (<https://www.genecopoeia.com/product/mirna-target-clones/mirna-targets/>). UTR was cloned  
753 downstream to firefly luciferase of pEZX-MT06 Dual-Luciferase miTarget™ vector. The pEZX-MT06-  
754 scrambled UTR or pEZX-MT06-Zbp1 3'UTR construct and miR99b-5p mimic or negative control were  
755 co-transfected into HEK293-T cells cultured in 24-well plates using EndoFectin™ Max Transfection  
756 Reagents (Gene Copoeia) according to the manufacturer's protocol. 48 hours after transfection, Firefly  
757 and Renilla luciferase activities were measured using a Luc-Pair™ Duo-Luciferase HS Assay Kit (for high  
758 sensitivity) (GeneCopoeia). Firefly luciferase activity and Renilla luciferase activity were normalized.  
759 The mean of luciferase activity and of Firefly/Renilla was considered for the analysis.

760

761 *Statistical analysis:*

762 Unless otherwise noted, statistical analysis was carried out with GraphPad Prism software version 8.0.  
763 Statistical measurement is shown as mean  $\pm$  SD. Each n represents a biological sample. Either a two-  
764 tailed unpaired t-test or a two-way ANOVA with Tukey's post hoc test were applied to analyze the  
765 data. Enriched gene ontology and pathway analysis was performed using Fisher's exact test followed  
766 by a Benjamini-Hochberg correction.

767

768 **Conflict of interest**

769 The authors declare no conflict of interest.

770

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788 and L.K., F.S., and A.F. wrote the paper with input from other co-authors.

789

## 790 **Figure legends**

791 **Figure 1: Identification of microRNAs that play a role in the pathogenesis of SZ.** **a.** Experimental  
792 scheme. **b.** Violin plots showing the results of WGCNA analysis. Depicted is the comparison of the  
793 eigenexpression in of the 3 co-expression modules in SZ patients and in control, showing a decrease  
794 in SZ patients. **c.** Violin plots showing the results of WGCNA analysis. The eigenexpression of the 5 co-  
795 expression modules was higher in SZ patients than in controls (for b & c: unpaired t test; \*\*P < 0.01,  
796 \*\*\*P < 0.001; \*\*\*\*P < 0.0001, a P value < 0.01 was considered as significant). **d.** Heat map showing  
797 the correlation of the eigenexpression of the co-expression modules shown in (a) and (b) with the  
798 corresponding clinical phenotypes. The numbers in each rectangle represent the correlation (upper  
799 number) and the corresponding p-value (lower number). A P value < 0.01 was considered as  
800 significant. **e.** Volcano plot depicting the results of the differential expression analysis when comparing  
801 SZ patients and controls shown in (a). **f.** Volcano plot demonstrating the results of the differential  
802 expression when comparing postmortem brain samples from SZ patients (n=13) and controls (n=17).  
803 **g.** Venn diagram comparing the microRNAs detected in blood samples when performing differential  
804 expression analysis (Blood DESEQ2\_DE), the microRNAs of the ME\_Turquoise and ME\_Pink co-  
805 expression modules and the microRNAs differentially expressed when comparing postmortem brain  
806 tissue (brain\_DE). miR-99b-5p is the only microRNA decreased in all comparisons. **h.** Heat map  
807 showing the correlation of miR-99b-5p expression levels to the clinical phenotypes for the individuals  
808 as analyzed in (a). The numbers in each rectangle represent the correlation (upper number) and the  
809 corresponding p-value (lower number).

810

811 **Figure 2: Decreasing miR-99b-5p levels in the PFC of mice leads to SZ-like phenotypes and increases**  
812 **the expression of genes linked to microglia activation.** **a.** Left panel: Experimental design. Right panel:  
813 Bar graph showing qPCR results for miR-99b-5p in tissue obtained from the PCF of mice 5 or 10 days  
814 after injection of anti-miR-99b or sc-control oligonucleotides. (n=4/group; \*\*\*\*P < 0.0001, unpaired t  
815 test). **b.** Bar graph showing the distance traveled in the open field test of mice injected to the PFC with

816 either anti-miR-99b or sc-control oligonucleotides (n=10/group; unpaired t test. **c.** Bar graph showing  
817 the time spent in the center of the open field in mice injected to the PFC with either anti-miR-99b or  
818 sc-control oligonucleotides (n=10/group; \*P < 0.05; unpaired t test). **d.** Bar graph showing the time  
819 spent in the open arms when an elevated plus maze test was performed in mice injected to the PFC  
820 with either anti-miR-99b or sc-control oligonucleotides (n=10/group; \*P < 0.05; unpaired t test). **e.** Bar  
821 graph showing the results of a PPI experiment of mice injected with either anti-miR-99b or sc-control  
822 oligonucleotides. PPI is impaired in anti-miR-99b injected mice (n=10/group) unpaired t test; \*P < 0.05;  
823 \*\*P < 0.01, \*\*\*P < 0.001; \*\*\*\*P < 0.0001). **f.** Bar graph showing the basic startle response among  
824 groups. **g.** Volcano plot showing the differentially expressed genes (upregulated in red, downregulated  
825 in blue) when RNA-seq was performed from the PFC of mice injected with either anti-miR-99b or sc-  
826 control oligonucleotides. Genes with log2-fold change  $\pm$  0.5 and adjusted p value < 0.05 are  
827 highlighted. **h.** GO-term analysis of the upregulated genes found in (e). **i.** Heat maps showing the  
828 enrichment of the upregulated genes as determined in (e) in various datasets. Left panel shows that  
829 the upregulated genes are enriched for microglia-specific genes, while the downregulated genes are  
830 enriched for neuron-specific genes. The right panel shows that the upregulated genes are over-  
831 represented in 3 different databases for immune function-related genes. **j.** Bar graph showing the  
832 qPCR results of the *Il1 $\beta$* , *Tgfb1* and *Tnfa* genes in FACS-sorted microglia collected from the PFC of mice  
833 injected with anti-miR-99b or sc-control oligonucleotides. (n=4 or 5/group; unpaired t test; \*P < 0.05).  
834 Error bars indicate SD.

835

836 **Figure 3: Decreasing miR-99b-5p levels in microglia increases phagocytosis and reduces synapse**  
837 **number in cortical neurons** **a.** Left panel: Experimental design. **b.** Volcano plot showing differential  
838 expressed genes when comparing microglia treated with anti-miR-99b or sc-control LNAs. Genes with  
839 statistical significance are highlighted. **c.** Bar chart showing the top GO terms represented by the up  
840 regulated genes shown in (b). **d.** Bar charts showing qPCR results for *Tgfb1*, *Il1 $\beta$*  and *Tnfa* comparing  
841 microglia treated with anti-miR-99b or sc-control LNAs (n=6/group; unpaired t test; \*P < 0.05;  
842 \*\*P < 0.01, \*\*\*\*P < 0.0001). **e.** Bar chart showing the results of a phagocytosis assay performed in  
843 microglia treated with anti-miR-99b in comparison to cells treated with sc-control LNAs. The  
844 percentage of phagocytic index represents (# of total engulfed beads in an image / # of total cells  
845 identified in an image; n = 13 independent experiments; unpaired t test; \*\*P < 0.01). **f.** Experimental  
846 scheme illustrating the co-culture experiment. **g.** Heat map showing the differentially expressed genes  
847 from the experiment described in (f). **h.** Plot showing the results of a GO term analysis for the up- and  
848 downregulated genes displayed in (g). **i.** Left panel: Representative image showing DIL dye staining to  
849 visualize dendritic spines in co-cultures as illustrated in (f). Scale bar 5  $\mu$ m. Right panel: Bar chart

850 showing the statistical quantification of the data depicted in (i). Each dot represents a spine density  
851 for a dendritic segment. \*P < 0.05; \*\*P < 0.01, \*\*\*P < 0.001; \*\*\*\*P < 0.0001). Error bars indicate SD.

852

853 **Figure 4: miR-99b-5p regulates neuroinflammatory phenotypes via *Zbp1*** a. Venn diagram comparing

854 the genes upregulated in the PFC of mice and in primary microglia when injected or treated with anti-

855 miR99b vs. sc-control LNAs, respectively. The data is further compared to the identified 13 miR-99b-

856 5p target mRNAs detected in the PFC dataset. The left panel shows the gene names of the 13 miR-

857 99b-5p target mRNAs. Red indicates miR-99b-5p targets upregulated in the PCF and in primary

858 microglia upon anti-miR-99b treatment. **b.** Bar graph showing the results of the luciferase assay. In

859 comparison to sc-control LNAs, administration of miR-99b-5p mimic decreases luciferase activity when

860 cells express the *Zbp1*-3'UTR. This effect is not observed when a control 3'UTR that does not bind miR-

861 99b-5p is used. (n=6/group). The upper right panel shows the predicted binding of miR-99b-5p to the

862 3'UTR of *Zbp1*. **c.** Left panel: Representative immunoblot image showing ZBP1 levels in microglia

863 treated with sc-control LNAs or anti-miR-99b. HSP70 was used as a loading control. Right panel: Bar

864 graph showing the quantification of the data depicted in the left panel. n=4/group. **d.** Bar graph

865 showing quantification of caspase activity in primary microglia treated with sc-control LNAs or anti-

866 miR-99b (n=6/group). **e.** Bar graph showing quantification of caspase activity in protein lysates

867 isolated from the PFC of mice injected with anti-miR-99b or sc-control (n=4/group). **f.** Bar graph

868 showing quantification of caspase activity in primary microglia treated with either sc-control LNAs,

869 anti-miR-99b or anti-miR-99b together with *Zbp1*-ASOs. (n=6/group). **g.** Bar graph showing qPCR

870 results for *Il1 $\beta$*  in primary microglia treated with either sc-control LNAs, anti-miR-99b or anti-miR-99b

871 together with *Zbp1*-ASOs (n=6/group). **h.** Bar graph showing the results of a phagocytosis assay

872 performed in primary microglia treated with either sc-control LNAs, anti-miR-99b or anti-miR-99b

873 together with *Zbp1*-ASOs (n=16 independent experiments). **i.** Bar graph showing quantification of

874 caspase activity in human iPSC-derived microglia treated with either sc-control LNAs, anti-miR-99b or

875 anti-miR-99b together with *Zbp1*-ASOs (n=13-16 samples/group). **j.** Bar graph showing qPCR results

876 for IL1 $\beta$  in human iPSC-derived microglia treated with either sc-control LNAs, anti-miR-99b or anti-

877 miR-99b together with *Zbp1*-ASOs (n=6/group). **k.** Bar graph showing the results of a phagocytosis

878 assay performed in human iPSC-derived microglia treated with either sc-control LNAs, anti-miR-99b

879 or anti-miR-99b together with *Zbp1*-ASOs. The percentage of phagocytic index represent (# of total

880 engulfed beads in an image / # of total cells identified in an image; n = 9 independent experiments.

881 Error bars indicate SD; unpaired t test; \*P < 0.05; \*\*P < 0.01, \*\*\*P < 0.001; \*\*\*\*P < 0.0001). RI: relative

882 immunofluorescent,

883

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885

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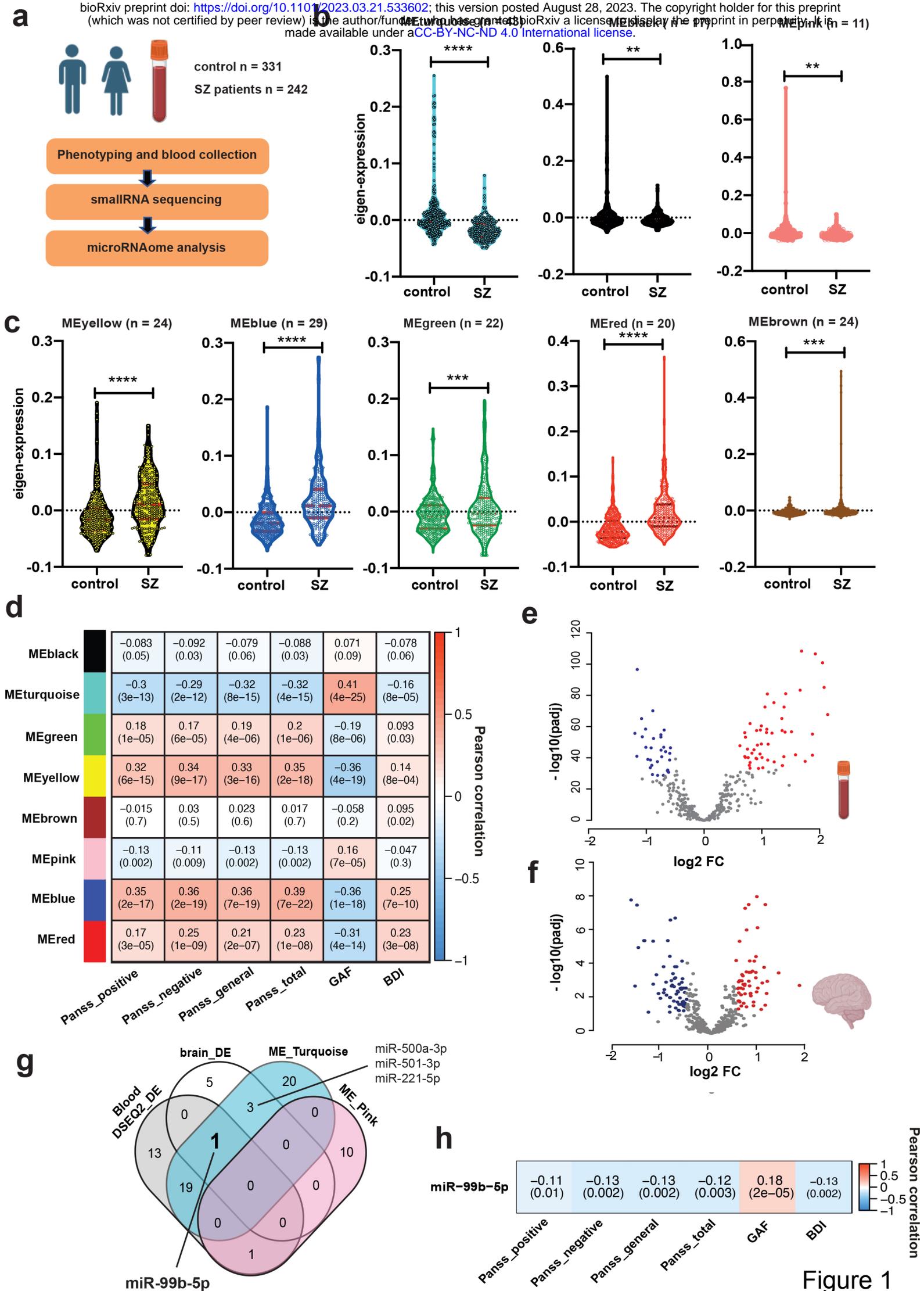
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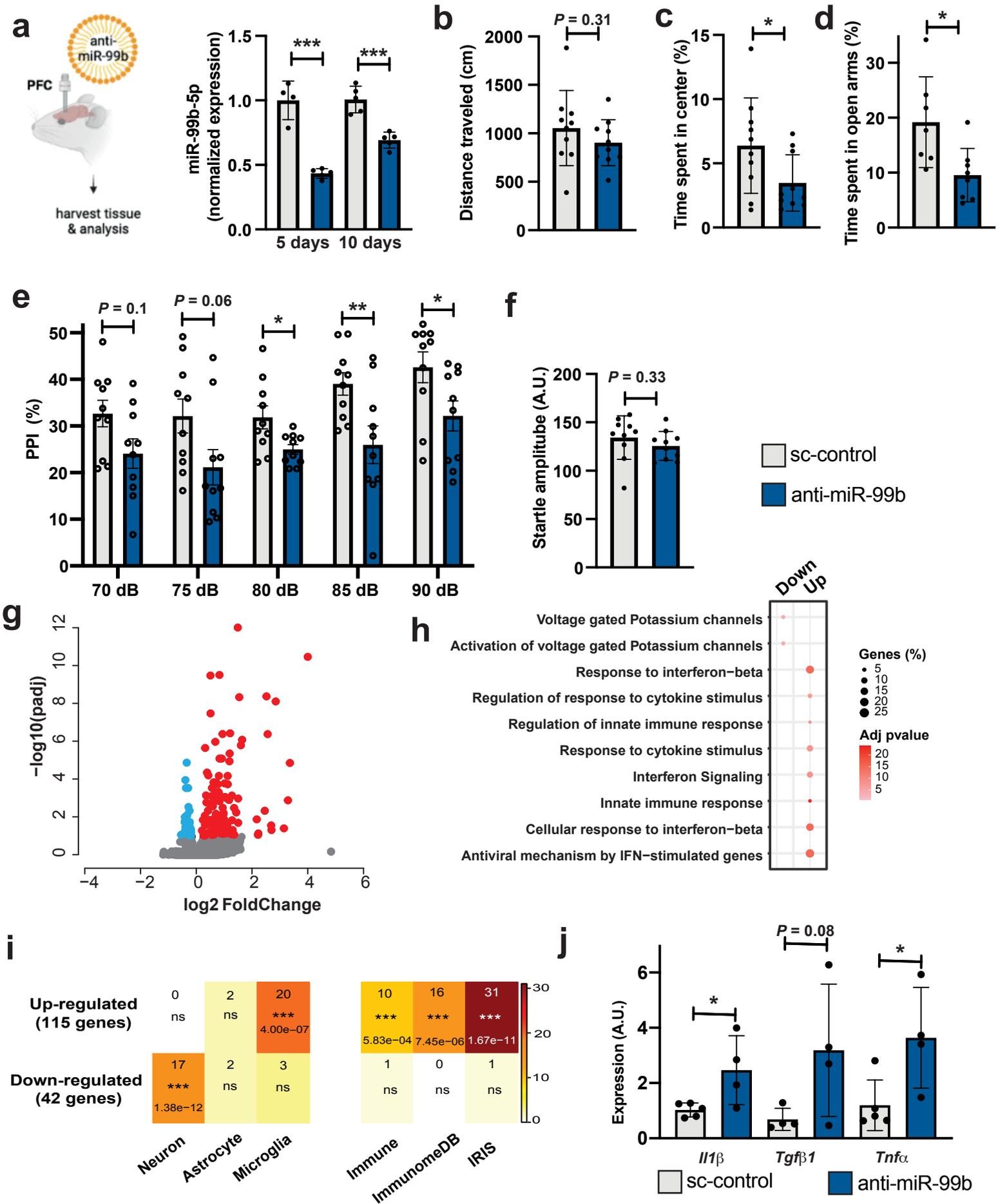


Figure 2

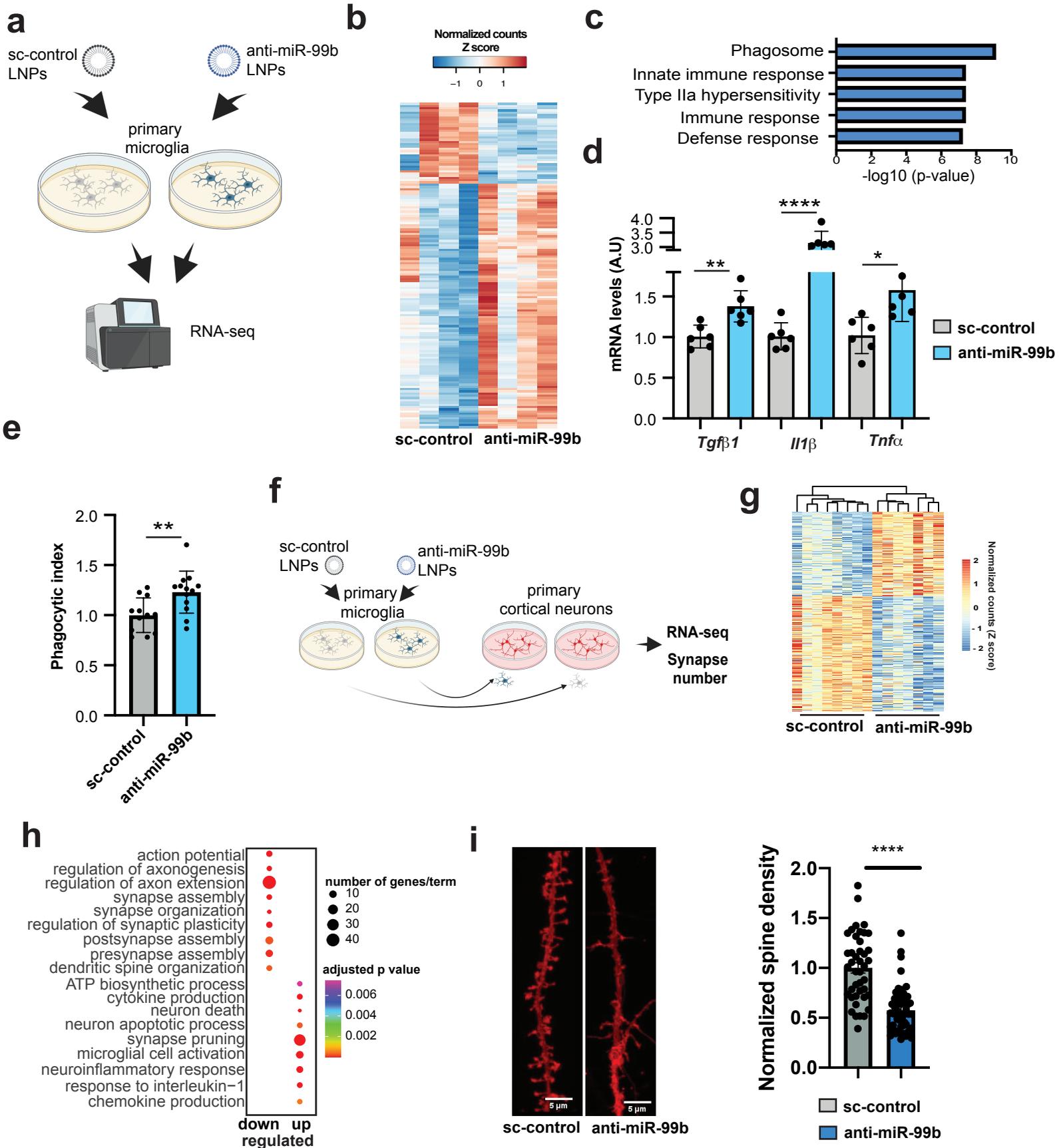


Figure 3

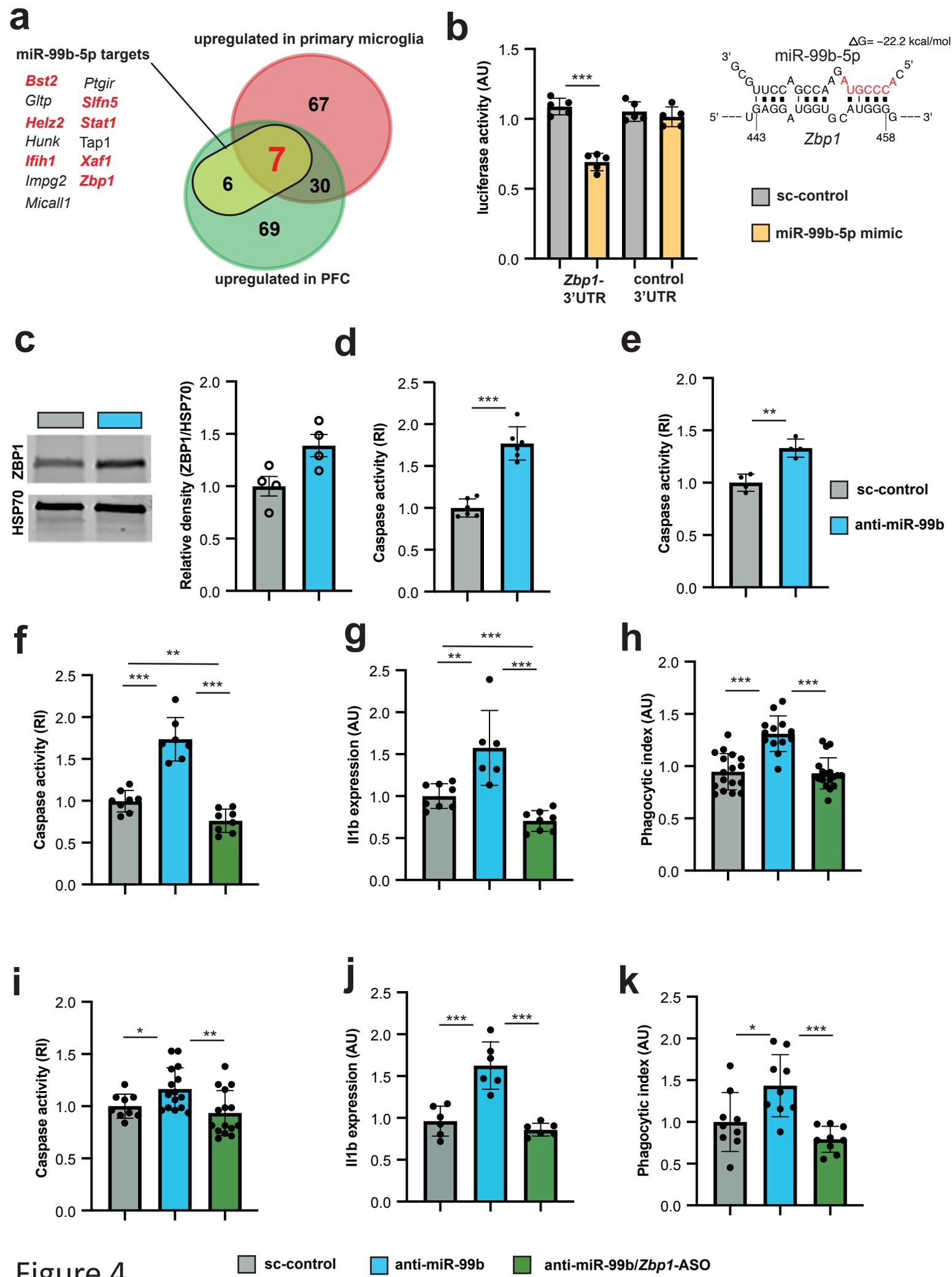


Figure 4