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Drosophila miR-124 regulates neuroblast proliferation through its target *anachronism*

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SUMMARY

MicroRNAs (miRNAs) have been implicated as regulators of central nervous system (CNS) development and function. miR-124 is an evolutionarily ancient, CNS-specific miRNA. On the basis of the evolutionary conservation of its expression in the CNS, miR-124 is expected to have an ancient conserved function. Intriguingly, investigation of miR-124 function using antisense-mediated miRNA depletion has produced divergent and in some cases contradictory findings in a variety of model systems. Here we investigated miR-124 function using a targeted knockout mutant and present evidence for a role during central brain neurogenesis in *Drosophila melanogaster*. *miR-124* activity in the larval neuroblast lineage is required to support normal levels of neuronal progenitor proliferation. We identify *anachronism* (*ana*), which encodes a secreted inhibitor of neuroblast proliferation, as a functionally important target of miR-124 acting in the neuroblast lineage. *ana* has previously been thought to be glial specific in its expression and to act from the cortex glia to control the exit of neuroblasts from quiescence into the proliferative phase that generates the neurons of the adult CNS during larval development. We provide evidence that *ana* is expressed in *miR-124*-expressing neuroblast lineages and that *ana* activity must be limited by the action of miR-124 during neuronal progenitor proliferation. We discuss the possibility that the apparent divergence of function of miR-124 in different model systems might reflect functional divergence through target site evolution.

KEY WORDS: microRNA, CNS, Stem cell, miR-124, *anachronism*, *Drosophila*

INTRODUCTION

MicroRNAs (miRNAs) are short non-coding RNAs that act as post-transcriptional regulators of gene expression. Animal miRNAs interact with target mRNAs via partial complementary base pairing to target sites. In most cases this results in repression of mRNA expression through destabilization of the target mRNA and/or translational repression (reviewed by Bushati and Cohen, 2007; Filipowicz et al., 2008; Bartel, 2009). miRNAs have been implicated in many biological phenomena, particularly those associated with dynamic cellular and/or developmental processes such as embryonic development and stem cell differentiation (Karp and Ambros, 2005; Shcherbata et al., 2006; Stadler and Ruohola-Baker, 2008). Given the complexity of the nervous system and the vast number of genes and signaling molecules required for its proper architecture and wiring, miRNAs are likely to play a significant role in central nervous system (CNS) development and function (Kosik, 2006; Christensen and Schratt, 2009).

Among the animal miRNAs implicated in CNS development, miR-124 has been the focus of considerable interest. *miR-124* is deeply conserved and shows CNS-enriched expression in all animals examined (Stark et al., 2005; Cao et al., 2007; Kapsimali et al., 2007; Makeyev et al., 2007; Visvanathan et al., 2007; Rajasethupathy et al., 2009; Shkumatava et al., 2009). Over the past few years, there have been several reports on the roles of miR-124 in different model organisms. These studies were in large part based on miRNA overexpression or depletion using antisense oligonucleotides and reached different conclusions on whether miR-124 inhibits neuronal precursor proliferation and/or promotes neuronal differentiation (Lim et al., 2005; Cao et al., 2007; Makeyev et al., 2007; Visvanathan et al., 2007; Cheng et al., 2009; Maiorano and Mallamaci, 2009; Qiu et al., 2009; Arvanitis et al., 2010; Clark et al., 2010; Liu et al., 2011). In vivo studies using genetic mutants that completely remove miRNA function might provide the means to resolve the role of miR-124 in CNS development.

Here we report the in vivo analysis of miR-124 function during the development of the CNS in *Drosophila*. Mutants lacking *miR-124* show reduced proliferative activity of neuronal progenitor cells in the developing larval central brain, resulting in a decrease in the production of adult postmitotic neurons. We identify *anachronism* as a functionally important target through which miR-124 controls neuronal stem cell proliferation.

MATERIALS AND METHODS

Fly stocks

Drosophila stocks were obtained from the Bloomington Stock Center. The *UAS-ana-RNAi* line is stock #27515. *EP-ana* is stock #22173. Flies were maintained on standard yeast-cornmeal-agar medium at 25°C unless otherwise stated. Canton S flies were used as the wild-type control.

MARCM analysis

To generate positively labeled MARCM clones, *hsFLP, elav-Gal4, UAS-mCD8::GFP; FRT40A, tubP-Gal80/Cyo* flies were crossed to *FRT40A* or *FRT40A, miR-124*$^{\Delta 177,w+}$*/Cyo* or *FRT40A, miR-124*$^{\Delta 177,w+}$*/Cyo; UAS-miR-124* or *FRT40A, miR-124*$^{\Delta 177,w+}$*, ana*^{1}*/Cyo*. Embryos were collected over a 4-hour time window and raised at 25°C for 21-25 hours before a 60-minute heat-shock treatment at 37°C. The treated L1 larvae were grown at 18°C until dissection. All larvae were dissected at late wandering third larval stage unless otherwise stated.

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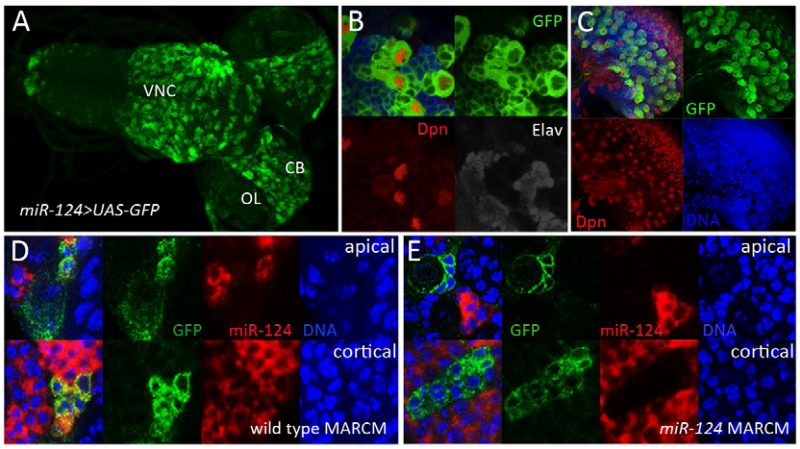

Fig. 1. *miR-124* expression in the *Drosophila* CNS. (**A-C**) GFP expression driven by a 4.7 kb cis-regulatory element from the *miR-124* locus. The fragment is illustrated in Fig. 2A. (A) Ventral view of GFP expression (green) in the brain from a projection of optical sections. CB, central brain; OL, optic lobe; VNC, ventral nerve cord. (B) Single optical section near the surface of the cortex showing several neuroblasts (NBs) labeled with anti-Dpn (red). Elav expression is in blue/gray. (C) Dorsal view showing a projection of optical sections for one brain hemisphere. Most central brain NBs (identified by Dpn expression, red) express *miR-124-GFP* (green). *miR-124-GFP* was detected at lower levels in the NB of the optic proliferation center (upper right). (**D**) Wild-type MARCM clone (green) showing a single NB lineage. (Top) Superficial optical section. (Bottom) Deeper optical section showing differentiating neurons. Mature miR-124 miRNA is in red, DNA in blue (DAPI). (**E**) *miR-124* mutant MARCM clone (green) showing the specificity of the fluorescent in situ hybridization signal in D. (Top) Superficial optical section. (Bottom) Deeper optical section showing differentiating neurons. Mature miR-124 miRNA is in red.

Generation of mutant and other transgenic strains

Gene targeting by ends-out homologous recombination was as previously described (Chen et al., 2011). Two independent knockout alleles of *miR-124*, namely Δ4 and Δ177, were made using modified ends-out gene targeting vectors (Weng et al., 2009). Loss of the *miR-124* hairpin sequence in these two alleles was verified by PCR and TaqMan miRNA qPCR assay (Invitrogen). The *UAS-miR-124* lines were made by cloning a 250 bp genomic fragment containing the miRNA hairpin into the 3′UTR of dsRed in pUAST as described (Brennecke et al., 2005). The *ana* 3′UTR luciferase reporters were made by cloning the 900 bp *ana* 3′UTR downstream of luciferase, under control of the tubulin promoter (Brennecke et al., 2003). *ana* 3′UTR reporters with mutated miR-124 sites were generated by PCR using primers designed to change the seed region from GTGCCTT into GTACATG. PCR products were sequence verified. The piB-miR-124 plasmid was generated by replacing the GFP reporter in piB-GFP (Bateman et al., 2006) with a 430 bp genomic fragment containing the miRNA hairpin in the center using *Sal*I and *Bam*HI sites. The miR-124 RMCE-hairpin-rescued strain was generated using the piB-miR-124 construct as described (Weng et al., 2009). miRNA quantitative real-time PCR (qRT-PCR) was used to verify that the rescued allele produced a normal level of mature miR-124 miRNA.

Cell transfection and luciferase assays

Drosophila S2 cells were transfected in 24-well plates with 250 ng miRNA expression plasmid or empty vector, 25 ng firefly luciferase reporter plasmid, and 25 ng Renilla luciferase DNA as a transfection control. Transfections were performed in triplicate in at least three independent experiments. Sixty hours after transfection, Dual-Luciferase Reporter Assays (Promega) were performed according to the manufacturer's instruction. For luciferase assays on larval CNS, tissues were dissected and immediately lysed in Passive Lysis Buffer (Promega). Luciferase activity was normalized to total protein content measured in the same sample using the Bradford method (Bio-Rad).

RNA analysis and TU tagging

For miRNA qRT-PCR, primer sets designed to amplify mature miR-124 were obtained from Applied Biosystems. Reverse transcription was performed on 20 ng total RNA. miRNA levels were calculated relative to reference genes *snoR442* and *2SrRNA* (Applied Biosystems), after having confirmed that the level of these two small RNAs remains constant in the relevant fly strains. For mRNA qRT-PCR, total RNA was purified using the RNeasy Kit and treated with on-column DNase for 60 minutes (Qiagen) to eliminate DNA contamination. First-strand synthesis used oligo(dT) primers and SuperScript RT-III (Invitrogen). Measurements were normalized to *rp49* (*RpL32* – FlyBase) and *Actin 42A* controls.

TU tagging was performed as described (Miller et al., 2009) with the following modification. Larvae of the indicated genotypes were collected in groups of 20 at 72 hours after larval hatching and transferred to food vials with 4-TU-containing yeast paste at 29°C for 16 hours before CNS dissection.

Immunocytochemistry and imaging

Larval CNS tissues were dissected and fixed in 4% formaldehyde in PBS with 0.1% Triton X-100 for 20 minutes on ice. The following primary antibodies were used: rat anti-Elav at 1:50, mouse anti-Prospero at 1:100 (Developmental Studies Hybridoma Bank); rabbit anti-phospho-histone H3 at 1:200 (Cell Signaling); chicken anti-GFP at 1:2000 (Abcam); and guinea-pig anti-Deadpan (a gift from James Skeath, Washington University, St Louis, MO, USA). Secondary antibodies used were conjugated to Alexa Fluor 555, 633 or 488 (Invitrogen) and used at 1:500, 1:300 or 1:500, respectively. The DNA stain was DAPI (Sigma). Samples were mounted in Vectashield (Vector Laboratories). Quantification of mitotic neuroblasts was performed using projections of confocal sections.

Fluorescent in situ hybridization

The miR-124 locked nucleic acid probe was from Exiqon. Anti-DIG-POD primary antibody (1:200) was detected using the Tyramide Signal Amplification Kit (cyanine 3) from Perkin Elmer following standard protocols.

RESULTS

miR-124 is expressed in CNS neuroblasts and postmitotic neurons

In *Drosophila*, *miR-124* is expressed in the CNS from early embryonic stages to adulthood (Stark et al., 2005; Ruby et al., 2006). To further characterize the neuronal expression pattern of *miR-124*, a 4.7 kb genomic region upstream of the *miR-124* locus was used to drive expression of a GFP reporter using the GAL4 system (Brand and Perrimon, 1993). This reporter showed GFP expression in the developing CNS of the embryo. In mature third instar larvae, GFP expression was high in the central brain and

ventral nerve cord, and detectable but somewhat lower in the optic lobes (Fig. 1A). *miR-124-GFP* was co-expressed with neuroblast (NB) markers such as the transcription factor Deadpan (Dpn) and with the postmitotic neuronal marker Embryonic lethal abnormal vision (Elav) (Fig. 1B). Projection from a series of optical sections showed *miR-124-GFP* expression in most Dpn-positive cells (Fig. 1C), suggesting that *miR-124* is expressed in most of the neuronal precursors of the developing larval brain.

We next examined *miR-124* expression using fluorescent in situ hybridization and made use of the MARCM strategy (Lee and Luo, 1999; Wu and Luo, 2006) to compare clones of genetically marked *miR-124* mutant cells with control clones. Mature miR-124 was abundant in postmitotic neurons and present at low levels in NBs (Fig. 1D), but no signal was detected in the *miR-124* mutant tissue, indicating the specificity of the probe (Fig. 1E). These observations indicate that *miR-124* is expressed in proliferating neuronal progenitors as well as in differentiating postmitotic neurons.

Targeted deletion and rescue of a *miR-124* mutant

In order to explore miR-124 function in the neuronal progenitor population, a mutant was generated using an ends-out gene-targeting vector modified to support recombinase-mediated cassette exchange (RMCE) (Weng et al., 2009). The mutant deleted 304 bp encompassing the *miR-124* hairpin sequence and introduced the *mini-white* reporter flanked by inverted *attP* sites to permit subsequent genetic rescue by RMCE (Fig. 2A). Reintroduction of the *miR-124* hairpin sequence into the targeted locus by RMCE restored the expression of mature miR-124 transcript to normal levels (Fig. 2B). Homozygous *miR-124* mutants showed reduced viability when reared under normal conditions, together with their heterozygous siblings. However, when the mutant individuals were isolated as embryos and raised under non-competitive conditions (in groups of 20-30 mutant larvae per vial) they were fully viable and showed no obvious morphological defects. RMCE-rescued mutants were fully viable under normal conditions.

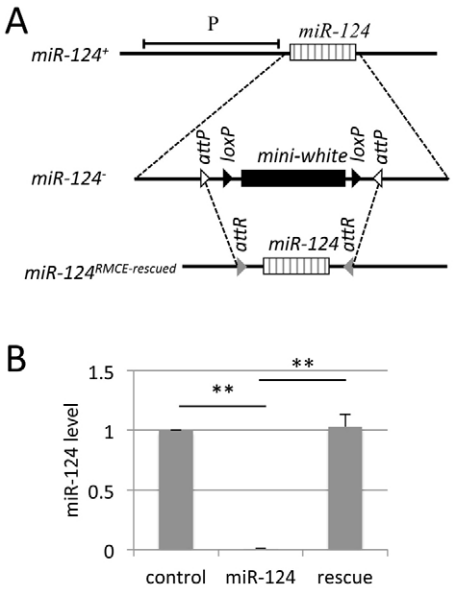

Fig. 2. *miR-124* mutants. (**A**) The *Drosophila miR-124* locus, illustrating the targeting and rescue strategy. Individual elements are not shown to scale. (Top) A 304 bp fragment containing the *miR-124* hairpin sequence is depicted as a hatched box. P indicates the 4.7 kb fragment used to drive GFP expression in Fig. 1. (Middle) Structure of the *miR-124* locus obtained by RMCE-modified gene targeting. The *mini-white* cassette consists of cDNA and genomic sequences from the *white* locus and is flanked by *loxP* and *attP* sites. Left and right extensions indicate the homology arms used in the homologous recombination. (Bottom) Structure of the RMCE-rescued locus, in which the sequences between the *attP* sites have been replaced by a fragment containing the *miR-124* hairpin sequence. (**B**) Quantification of mature miR-124 miRNA by qPCR. RNA samples were extracted from late wandering third instar larval brains of the indicated genotypes. Data show mean ± s.d. based on five independent biological replicates. $^{**}P<0.01$ using the Wilcoxon two-sample test.

Stem cell-like division of *miR-124* mutant neuroblasts

Drosophila central brain NBs undergo repeated rounds of asymmetric division to maintain the stem cell-like NB and to generate intermediate progenitors called ganglion mother cells (GMCs). Multiple rounds of NB division produce clonally related populations of neurons and glia. After an initial phase of proliferation in the embryo, most NBs undergo a period of quiescence but then resume proliferation during larval development. This second phase of NB proliferation generates the neurons that make up the bulk of the adult CNS (Hartenstein et al., 2008). Larval brain NBs are classified as type I or type II depending on their proliferative characteristics. Type I NBs divide repeatedly to produce a series of GMCs that each divide once more to produce two postmitotic progeny. Type II NB lineages differ in that the intermediate GMC progenitors undergo multiple rounds of proliferation. This transit amplification process produces larger clones from type II NBs. Most of the subsequent analysis will focus on type I NBs because these constitute the majority of larval brain NBs.

In light of the finding that *miR-124* is expressed in larval NBs, we sought to determine its role in the development of the central brain NB lineages by comparing genetically marked NB clones that were normal or mutant for *miR-124*. MARCM clones were induced randomly in early first instar larvae and analyzed in late third instar larval brains. All labeled control and *miR-124* mutant type I NB lineages contained one large cell, the NB, which expressed Dpn (Fig. 3A,B), as well as many smaller cells. GMCs were identified by expression of the differentiation marker Prospero (Pros) (Fig. 3C,D) and by Partner of numb (Pon) (Fig. 3E,F), which is expressed in the NB and segregated to the GMCs during asymmetric NB division. Pros and Pon were detected in wild-type and mutant GMCs. GMCs were found clustered directly adjacent to the large apical NB in control and mutant clones (Fig. 3C-F). In addition to the NB and GMC precursors, control and *miR-124* clones contained many smaller cells that expressed Elav, representing the neuronal progeny of the GMCs (Fig. 3A-F). The presence of the labeled NB and GMC together with many postmitotic progeny in each clone reflects the successive rounds of asymmetric NB division to produce many GMCs.

To visualize mitotic cells, we made use of an antibody specific for the phosphorylated form of histone H3 (pH3). A subset of NBs and GMCs was labeled with anti-pH3 in control and *miR-124* mutant clones (Fig. 3G,H), indicating that NBs and GMCs were mitotically active. Comparable observations were made for marked *miR-124* mutant type II NB clones. Therefore, loss of *miR-124* does not seem to alter neuronal cell fates in type I or type II larval brain NB lineages.

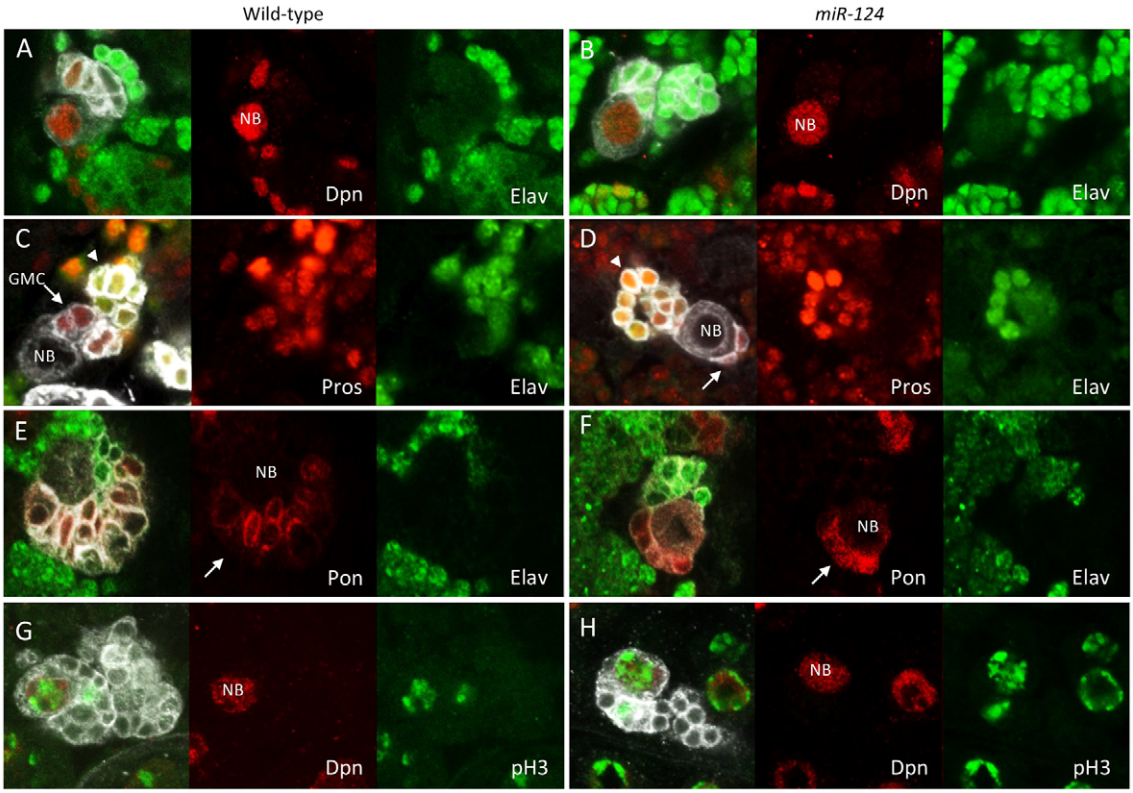

Fig. 3. Cell types in the NB lineages are not altered in *miR-124* mutant clones. (**A-F**) Single optical sections showing GFP-labeled MARCM clones (gray) of the indicated genotypes. (A,B) NB labeled with anti-Dpn (red). Postmitotic neurons were labeled with anti-Elav (green). (C,D) GMCs (arrows) were identified by labeling with anti-Pros (red) and by the absence of the postmitotic marker Elav (green). Arrowhead indicates Elav-positive postmitotic neurons. (E,F) NBs and GMCs labeled with anti-Pon (red) and neurons labeled with anti-Elav (green). (**G**,**H**) NBs labeled with anti-Dpn (red) and with anti-phospho-histone H3 (green).

miR-124 is required for normal levels of neuroblast proliferation

Although loss of *miR-124* did not compromise the ability of type I NBs to undergo asymmetric division, a closer examination revealed a reduction in the number of postmitotic neurons per clone (Fig. 4A). The average number of GFP-labeled Elav-positive cells was determined for control and mutant type I NB clones. Control clones contained an average of 85 cells, versus 66 cells in *miR-124* mutant clones (Fig. 4A; $*P<0.05$). To confirm that this difference was due to the absence of *miR-124*, a clonal rescue experiment was carried out. The MARCM system makes use of *elav-Gal4* to direct *UAS-GFP* expression to positively label clones (note that the *elav-Gal4* driver is expressed in all cells of the NB lineage, in contrast to Elav protein which is limited to postmitotic neurons). To perform genetic rescue, we co-expressed a *UAS-miR-124* transgene along with *UAS-GFP* in control and *miR-124* mutant MARCM clones. This restored mutant type I NB clone size to normal control levels (Fig. 4A; $P=0.44$ for control versus rescued mutant). Comparable results were obtained for type II NB lineages. These findings indicate that *miR-124* is required in the NB lineage to generate the normal number of postmitotic neurons.

To determine whether this difference in clone cell number might be due to reduced proliferative activity in the *miR-124* mutant clones, the number of MARCM clones containing type I NBs labeled with the mitotic marker pH3 was scored: 35% of *miR-124* mutant clones had pH3-positive NBs compared with 46% of control clones (Fig. 4B; $**P<0.01$). The mitotic index was restored to normal levels in the *UAS-miR-124* clonal rescue experiment (Fig. 4B; $P=0.28$ for control versus rescued mutant; $*P<0.05$ for mutant versus rescued mutant). A similar experiment using co-expression of UAS-p35 to block apoptosis in the *miR-124* mutant MARCM clones did not restore the mitotic index. Together, these experiments suggest that reduced proliferative activity could account for the reduction in mutant clone size and suggest that *miR-124* is necessary for maintaining proliferation of neuronal progenitors in the developing CNS.

miR-124 negatively regulates the expression of *anachronism*

Computational predictions have suggested that miR-124 targets mainly epithelial-specific or non-neuronal genes, including the glial-specific genes *repo* and *Gliotactin* (Stark et al., 2005). Intriguingly, the list of predicted targets of miR-124 includes *anachronism* (*ana*), which has been reported to encode a negative regulator of NB proliferation that is expressed specifically in a subset of glial cells (Ebens et al., 1993). As a first step to determine whether *ana* might be a functionally important target of miR-124, we examined *ana* transcript levels by qRT-PCR in RNA samples from dissected third instar brains of control and *miR-124* mutants. *ana* transcript levels were elevated in the mutant brains and were restored to normal in the RMCE-rescued mutant (Fig. 5A; $**P<0.01$). This indicates that the endogenous *ana* transcript is affected in a manner consistent with regulation by miR-124.

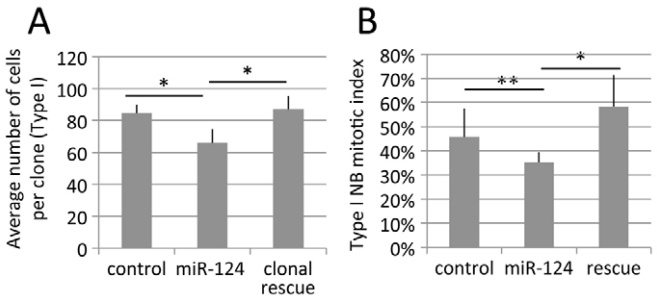

Fig. 4. Proliferative status of *miR-124* mutant type I NB clones. (**A**) Average cell number in wild-type control type I NB MARCM clones, as compared with *miR-124* mutant and rescued *miR-124* clones. s.d.=5 (control), 9 (miR-124) and 8 (clonal rescue). (**B**) Mitotic index of type I NBs in wild-type, *miR-124* mutant and rescued *miR-124* MARCM clones, shown as the percentage of NBs that were immunopositive for the mitotic marker phospho-histone H3 in late wandering third instar larval brains. s.d.=12% (control), 4% (miR-124) and 13% (clonal rescue). Data show mean ± s.d. **P*<0.05, ***P*<0.01, using two-tailed unpaired Student's *t*-test (*n*>100 for each genotype).

The *ana* 3′ untranslated region (UTR) is predicted to contain two potential miR-124 binding sites (Fig. 5B). To address whether *ana* is a direct target of miR-124, we generated luciferase reporter constructs carrying the full-length *ana* 3′UTR or a mutant version in which three nucleotides of one of the predicted miR-124 sites were mutated to compromise pairing with the miRNA seed region (Fig. 5B, gray). Co-expression with *miR-124* in S2 cells significantly reduced luciferase activity from the reporter carrying the intact target site but had little effect on the mutant form of the *ana* reporter (Fig. 5C; ***P*<0.01). This suggests that miR-124 can act directly via this target site to repress *ana* expression.

To further assess the functionality of the miR-124 target site in vivo, transgenic flies expressing the *ana* 3′UTR luciferase reporter were generated. Luciferase activity levels were compared in brains dissected from late third instar control larvae and *miR-124* mutants. We observed elevated luciferase activity in *miR-124* mutant brains carrying the intact *ana* reporter transgene, as compared with the level of expression of this transgene in the control brains (Fig. 5D; **P*<0.05). The UTR reporter carrying the mutated version of miR-124 site 1 did not show statistically significant upregulation in *miR-124* mutant brains. Taken together, these experiments provide evidence that miR-124 can act directly via the site identified in the 3′UTR to regulate *ana* mRNA levels in vivo.

Elevated *ana* levels contribute to the *miR-124* mutant NB proliferation phenotype

As a first step to assess whether elevated *ana* expression contributes to the *miR-124* mutant phenotype, we made use of the MARCM system to generate clones of cells overexpressing *ana* in the neuronal lineage. This resulted in a reduced proliferative index (Fig. 6A; **P*<0.05). This experiment shows that upregulation of *ana* is sufficient to mimic the effects of the *miR-124* mutant on NB proliferation.

As a more rigorous functional test of whether *ana* overexpression contributes to the reduced NB proliferation observed in *miR-124* mutants, we asked if limiting *ana* levels could suppress the mutant phenotype. As a first approach, we examined

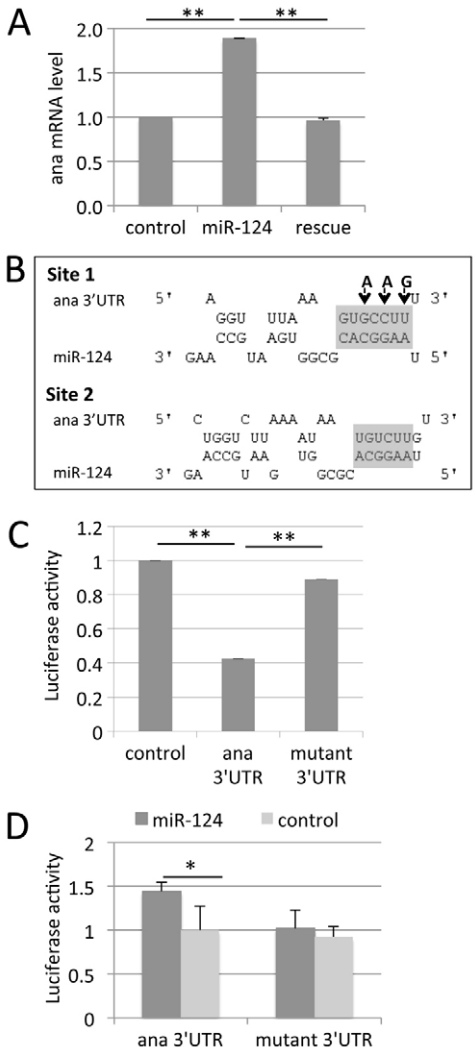

Fig. 5. miR-124 regulates *ana* expression. (**A**) Normalized *ana* mRNA levels measured by qRT-PCR in RNA from late wandering third instar larval brains of the indicated genotypes. Data are presented as mean ± s.d. based on six independent biological replicates. ***P*<0.01 using the Wilcoxon two-sample test. (**B**) Predicted miR-124 target sites in the *ana* 3′UTR. Arrows indicated residues changed in the site 1 mutant. (**C**,**D**) Luciferase assays showing regulation of a firefly luciferase reporter containing the *ana* 3′UTR or a version of the *ana* UTR with site 1 mutated as indicated in B. Data show the ratio of firefly to Renilla luciferase activity and represent mean ± s.d. based on three independent biological replicates. **P*<0.05, ***P*<0.01, using two-tailed unpaired Student's *t*-test. (C) S2 cells were transfected to express the *ana* 3′UTR reporter or the site 1 mutant version of the reporter. Cells were cotransfected to express *miR-124* or a vector-only control, and a Renilla luciferase reporter as a control for transfection efficiency. (D) Normalized luciferase activity from the *ana* 3′UTR reporter transgene in control or *miR-124* mutant larval brain lysates.

proliferation of *miR-124* mutant clones in flies heterozygous for a null allele of *ana* (Park et al., 1997). Limiting the capacity to overexpress *ana* in this way restored the proliferative activity of *miR-124* mutant NBs to near normal levels (Fig. 6B; **P*<0.05). Together, these experiments suggest that the elevated level of *ana* transcript that we observed in the *miR-124* mutant brain contributes to the mutant phenotype.

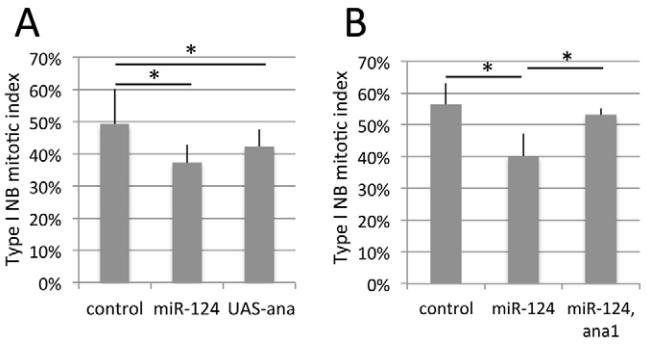

Fig. 6. Genetic evidence that *miR-124* acts via regulation of *ana*. The percentage of type I NB MARCM clones that that were immunopositive for phospho-histone H3 in late wandering third instar larval brains. **P*<0.05 using two-tailed unpaired Student's *t*-test (*n*>100 for each genotype). Data show mean ± s.d. (**A**) Genotypes: control (Canton S); *miR-124* mutant; and Canton S flies that co-expressed the *UAS-ana* transgene to overexpress ana mRNA selectively in the marked NB clones. s.d.=11% (control), 6% (miR-124) and 5% (UAS-ana). (**B**) Genotypes: control (Canton S); *miR-124* mutant; and *miR-124* mutant clones that carrying one copy of the ana^1 mutant allele. s.d.=7% (control), 7% (miR-124) and 2% (miR-124, UAS-ana^1).

miR-124 controls *ana* levels in NB lineages

The data presented thus far provide evidence that *ana* is a functionally significant target of miR-124 in vivo. However, *miR-124* is expressed in the neuronal lineage, whereas *ana* has been reported to be glial-specific in expression (Ebens et al., 1993). This apparent conundrum might be resolved if *ana* transcript were in fact present in the neuronal lineage, perhaps at low levels.

To explore this possibility we made use of the TU-tagging method (Miller et al., 2009) to selectively label and purify RNA from neuronal cells of the NB lineage. The method is based on cell type-specific expression of the uracil phosphoribosyltransferase (UPRT) enzyme, which permits incorporation of a 4-thiouracil base into newly synthesized mRNA. *elav-Gal4* was used to drive expression of *UAS-UPRT* in all neuronal cells of the larval brain. As a positive control for neuronal mRNA, we performed RT-PCR for *elav* mRNA. The glial-specific transcript *repo* was used as a control for glial cell mRNA. *elav* mRNA was recovered in the *elav-Gal4* samples, whereas *repo* transcript was not detectably recovered (Fig. 7A). *ana* mRNA was detected using primers specific for the mature spliced form of the transcript, indicating that *ana* was synthesized in the *elav-Gal4*-expressing cells of wild-type neuronal lineages (Fig. 7A).

Next, we used qRT-PCR to measure *ana* transcript levels by TU tagging RNA from *miR-124*-expressing cells, using *miR-124-Gal4* to direct *UAS-UPRT* expression. Samples were prepared from control brains and *miR-124* mutant brains and PCR was normalized to the level of *actin* mRNA. The level of *elav* transcript was unchanged, but *ana* transcript was increased ~2-fold in the *miR-124* mutant cells (Fig. 7B). Again, *repo* transcript was not detected, confirming the absence of glial cells in this population. These findings indicate that (1) *ana* transcript is expressed in neuronal cells and (2) the level of *ana* transcript increased in *miR-124*-expressing cells in the *miR-124* mutant.

Having established that *ana* transcript levels were elevated in the *miR-124*-expressing cells in the *miR-124* mutant, we used the MARCM system to selectively reduce *ana* levels in these cells. We first monitored the efficacy of depletion of *ana* transcript in *miR-124*-expressing cells. The level of *ana* transcript was compared by qRT-PCR of RNA from *miR-124* mutant brains expressing *miR-124-Gal4* alone or together with a *UAS-ana-RNAi* transgene. *ana* transcript levels in the mutant were reduced to near normal control levels by the RNAi treatment (Fig. 7C; ***P*<0.01). MARCM clones mutant for *miR-124* were produced with or without expression of the *ana* RNAi transgene and assayed for proliferative capacity. We found that selectively lowering *ana* levels in the *miR-124* mutant NB clones was sufficient to restore proliferation to near normal levels (Fig. 7D; **P*<0.05).

Given that *ana* and *miR-124* are co-expressed in the NB lineage, we next asked whether the role of miR-124 was to tune *ana* expression to optimal levels in the NB. Tuning relationships have been shown previously: in miR-430 regulation of Nodal activity

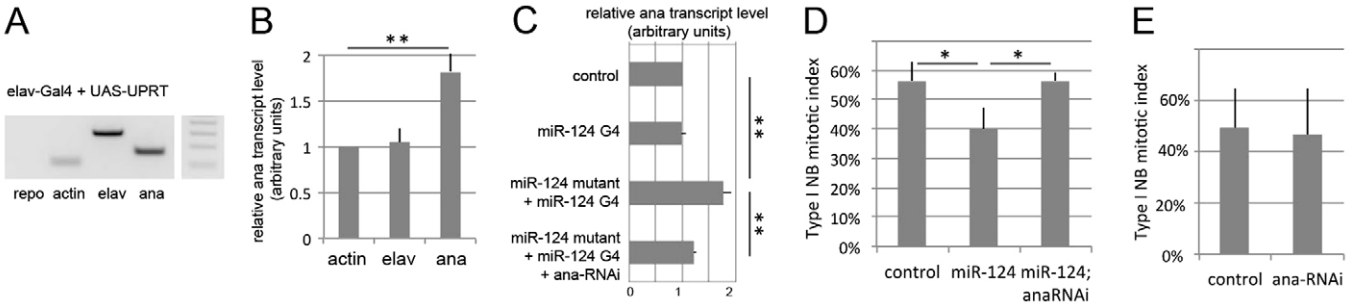

Fig. 7. *ana* expression and function in *miR-124*-expressing neuronal lineages. (**A**) The indicated transcripts were amplified by RT-PCR and resolved on an agarose gel. Note that the intensity of the bands cannot be directly compared owing to differences in the efficiency of the primers for different transcripts. *repo* was amplified from the input RNA, indicating that the primers were functional and that the absence of a product from the RNA from *elav-Gal4*-expressing neurons is meaningful. (**B**) Changes in mRNA level of the indicated genes measured by qRT-PCR in RNA isolated from *miR-124-Gal4*-expressing neurons in *miR-124* mutant versus wild-type larvae. Data were normalized to the level of *actin* mRNA and are presented as mean ± s.d. based on three independent biological replicates. ***P*<0.01 using Student's *t*-test. (**C**) Quantitative RT-PCR to assess the efficacy of *ana* transcript depletion in *miR-124*-expressing cells. Genotypes as indicated. Data are presented as mean ± s.d. based on three independent biological replicates. ***P*<0.01 using Student's *t*-test. (**D**) Functional test of the effects of *ana* depletion in *miR-124* mutant clones. Genotypes as indicated. Data are presented as mean ± s.d. based on three independent biological replicates. s.d.=7% (control), 7% (miR-124) and 3% (miR-124, UAS-ana-RNAi). **P*<0.05 using Student's *t*-test. (**E**) Genotypes: Canton S control; and Canton S flies that co-expressed the *UAS-ana-RNAi* transgene to deplete *ana* mRNA selectively in the marked NB clones. Data are presented as mean ± s.d. based on three independent biological replicates. s.d.=11% (control) and 5% (UAS-ana-RNAi). The difference was not statistically significant.

(Choi et al., 2007); in miR-8 regulation of Atrophin in the CNS (Karres et al., 2007); and in miR-14 regulation of Sugarbabe in insulin-producing neurosecretory cells (Varghese et al., 2010). In the latter two cases, it was shown that the level of target expression that remained after miRNA-mediated downregulation was functionally significant in the miRNA-expressing cells. To address this issue we performed an experiment similar to that in Fig. 7D, except that the MARCM system was used to express the *UAS-ana-RNAi* transgene along with the *UAS-GFP* marker in wild-type control NB clones that expressed *miR-124* at normal levels. RNAi-mediated depletion of *ana* in these clones did not alter NB clone size (Fig. 7E). Thus, further reduction of *ana* levels below that achieved by miR-124 appears to be without consequence for NB proliferation. On this basis, we suggest that miR-124 reduces *ana* to functionally insignificant levels in the NB lineage.

Taken together, these experiments provide evidence that miR-124 acts to regulate *ana* expression in larval central brain NB lineages and that this regulation is important for the control of NB proliferation.

DISCUSSION

A previous report has provided evidence that *ana* acts on quiescent larval NBs to control their entry into the proliferative phase (Ebens et al., 1993). Loss of *ana* function resulted in premature onset of NB proliferation during larval development. That study reported that *ana* expression was specific to a subset of glial cells, which are developmentally unrelated to the glial progeny of the NB lineages. *ana* encodes a secreted glycoprotein, so its apparent glial-specific expression led to the proposal that Ana protein acts non-autonomously from glia to regulate NB proliferation. However, our experiments have demonstrated that *ana* is expressed in neuronal cells and that miR-124 acts to control *ana* expression in the NB lineage.

Our findings indicate that miR-124 reduces *ana* to functionally inconsequential levels in the central brain NB lineage, and that failure to do so impairs NB proliferation. Normal levels of *ana* expression in early larval brains are required to control the timing of onset of NB proliferation. It might be that the functional source of Ana protein at this stage is the previously described cortex glia. However, once the larval NBs have entered proliferation, *ana* is expressed in the NB lineage and its expression there must be limited by miR-124 to prevent impairment of proliferation. The levels of *ana* expression that are needed to impair proliferation of these cells might be much lower than those found in the cortex glia, perhaps because *ana* is being expressed by the cells upon which it acts within the NB lineage. Whether this reflects the non-autonomous activity of a secreted protein or an autocrine effect on the NB and GMC to affect their proliferation remains to be explored.

Evolutionary conservation versus divergence of function

The finding that *miR-124* activity is required in the NB lineage to support proliferation contrasts with findings from vertebrate systems that have suggested a role for miR-124 in limiting neuronal progenitor proliferation and in promoting neuronal differentiation. Several independent studies have reported that *miR-124* promotes neuronal differentiation in mouse neural progenitor cells in culture by downregulating inhibitors of differentiation including the RNA splicing regulator PTB1, the SCP1 phosphatase, the transcription factor SOX9 and the ephrin B1 receptor (Makeyev et al., 2007; Visvanathan et al., 2007; Cheng et al., 2009; Arvanitis et al., 2010). Depletion of miR-124 using antisense oligonucleotides has been reported to promote proliferation and reduce differentiation of neuronal progenitors isolated from the subventricular zone (SVZ) of the mouse embryonic CNS (Cheng et al., 2009). Comparable results were obtained by injection of a pump to deliver antisense oligonucleotides to deplete miR-124 in vivo in the SVZ of the mouse brain (Cheng et al., 2009). In the chick spinal cord Visvanathan et al. found that depletion of miR-124 by antisense injection led to reduced neuronal differentiation and ectopic proliferation (Visvanathan et al., 2007). However, a contemporaneous study that undertook similar experiments did not observe these results (Cao et al., 2007). Taken together, the analysis of vertebrate miR-124 function mainly lends support to the idea that its expression in differentiating neurons acts to turn off negative regulators of differentiation and that loss of the miRNA supports proliferation of neuronal precursors.

A possible basis for the difference between our findings and those in vertebrate systems is that the expression of *miR-124*, although broadly CNS specific, differs in detail between insects and vertebrates. We observed expression in neural progenitors, which has not been generally reported in the vertebrate systems (Wienholds et al., 2005; Cao et al., 2007; Makeyev et al., 2007; Visvanathan et al., 2007; Cheng et al., 2009), although the presence of the miR-124 primary transcript in the NB and GMC is consistent with the expression of the *miR-124-GFP* reporter. In situ hybridization does not reveal significant levels of mature miR-124 in vertebrate neural progenitors, but one report using an in vivo sensor for miRNA activity has indicated that miR-124 activity can be detected in mouse neural progenitor cells (De Pietri Tonelli et al., 2006). Taken together, these findings prompt the question of whether there might be a corresponding function in the neural progenitors of the vertebrate CNS that might have been overlooked owing to low-level expression of the mature miRNA.

We have also considered the possibility that the difference between our findings and those reported using vertebrate models reflect methodological differences, i.e. the use of a genetic null mutant versus miRNA depletion using injected or transfected antisense oligonucleotides to reduce miRNA activity. Previous analysis of genetic mutants (Bushati and Cohen, 2007) has not supported the conclusions of antisense injections to deplete miRNA function in *Drosophila* embryos (Leaman et al., 2005). Antisense methods allow partial reduction of function and might introduce a degree of experimental variability. It will be of interest to learn whether mouse knockouts of *miR-124* support the findings reported using antisense methods.

A third and perhaps more interesting possibility is that the different effects of miR-124 on neuronal progenitor proliferation reflect evolutionary divergence of miR-124 function. miR-124 has hundreds of potential targets as identified by computational prediction, by expression profiling of miRNA overexpression and depletion and by immunopurification of miRNA-containing ribonucleoproteins (Lim et al., 2005; Ruby et al., 2006; Ruby et al., 2007; Clark et al., 2010; Chi et al., 2009; Friedman et al., 2009). There is little evidence of conservation of the identified or predicted targets between insects, nematodes and vertebrates. Furthermore, each of the reports on miR-124 function in vertebrates has attributed its role to the regulation of different targets. This might reflect subtly different roles for the miRNA in different neuronal progenitor cell models in vitro, in different regions of the developing CNS or at different stages of development. Evidence has been presented for different miR-124 functions at different stages in *Xenopus* eye development (Qiu et

al., 2009; Liu et al., 2011). It is also possible that miR-124 acts via several functionally significant targets that each serve as repressors of neuronal differentiation in vivo.

In *Drosophila*, we have identified *ana* as a target of miR-124 in vivo and provided direct genetic evidence that downregulation of *ana* expression in neuronal progenitors is required to support a normal level of proliferation within the larval central brain. The *ana* gene is not conserved beyond the *Drosophila* family. This leads to the intriguing proposal that miR-124 might have acquired a novel target in *Drosophila*, which has led to an entirely distinct function in the control of CNS proliferation from that found in vertebrates. It might also suggest that an evolutionarily ancient and presumably conserved role of miR-124 awaits discovery.

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Competing interests statement
The authors declare no competing financial interests.

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