The miR-124 family of microRNAs is crucial for regeneration of the brain and visual system in the planarian *Schmidtea mediterranea*

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ABSTRACT
Brain regeneration in planarians is mediated by precise spatiotemporal control of gene expression and is crucial for multiple aspects of neurogenesis. However, the mechanisms underpinning the gene regulation essential for brain regeneration are largely unknown. Here, we investigated the role of the miR-124 family of microRNAs in planarian brain regeneration. The miR-124 family (miR-124) is highly conserved in animals and regulates neurogenesis by facilitating neural differentiation, yet its role in neural wiring and brain organization is not known. We developed a novel method for delivering anti-miRs using liposomes for the functional knockdown of microRNAs. *Smed-miR-124* knockdown revealed a key role for these microRNAs in neuronal organization during planarian brain regeneration. Our results also demonstrated an essential role for miR-124 in the generation of eye progenitors. Additionally, miR-124 regulates *Smed-slit-1*, which encodes an axon guidance protein, either by targeting slit-1 mRNA or, potentially, by modulating the canonical Notch pathway. Together, our results reveal a role for miR-124 in regulating the regeneration of a functional brain and visual system.

KEY WORDS: Planarian, *Schmidtea mediterranea*, miR-124, Brain regeneration, Photoreceptors, Notch, miRNA

INTRODUCTION
Brain wiring is a complex process that ensures the formation of an intricate network of intercommunicating neurons. This process involves the precise guidance of axons from the neuron to its targets, which, in most animals, occurs during prenatal and early postnatal development (Tessier-Lavigne and Goodman, 1996; Stiles and Jernigan, 2010). Because of their unique capacity to regenerate a complex nervous system, planarian flatworms provide a suitable context in which to study factors essential for brain organization and neural patterning in adult animals. Planarians have a well-organized nervous system, which consists of a bi-lobed brain (cephalic ganglia) and a pair of ventral nerve cords interconnected by commissural neurons (Umesono et al., 1999; Cebria et al., 2002a, b). The eyes are located dorsal to the cephalic ganglia. The planarian eye consists of pigment cells, which form a cup-shaped organ, and photosensing neurons that project rhabdomeres inside the pigment cup, while their axons form the optic chiasm (Sakai et al., 2000; Yamamoto and Agata, 2011). During planarian regeneration, the brain rudiment and eyes form within 24-48 h post-amputation (hpa). Although the differentiating primordia of the brain and eyes are evident ~3 days post-amputation (dpa), the recovery of the functional brain is completed only after 7 dpa (Inoue et al., 2004; Ong et al., 2016). Axon guidance proteins, such as those encoded by members of the *dscam*, *slit*, *nefrin* and *robo* families, are essential for neural regeneration in planarians, and these factors are required for the patterning of the brain (Agata and Umesono, 2008; Yamamoto and Agata, 2011; Fusaoka et al., 2006; Cebria et al., 2007; Beane et al., 2012). However, the factors that regulate the spatiotemporal expression of these genes, which are crucial for the proper patterning of the planarian visual system and brain, are not known.

MicroRNAs (miRNAs) are small RNA species 21-22 nucleotides in length. They function in multiple biological processes, including neural stem cell proliferation, differentiation and patterning (Almuedo-Castillo et al., 2011; Plasterk, 2006; Hatfield et al., 2005; Thatcher and Patton, 2010). Deep sequencing of *Schmidtea mediterranea* small RNAs from the regenerating tissue (blastema) identified the *Smed-miR-124* family as the most prominent miRNAs enriched in the anterior blastema by 3 dpa (Sasidharan et al., 2013). The miR-124 family is highly conserved across metazoa and is generally enriched in the nervous system (Kosik, 2009). Although the *miR-124* family is highly expressed in the adult brain of vertebrates, its role in adult neurogenesis is not known. Since whole-mount in situ hybridization (WISH) revealed that the miR-124 family is enriched in the nervous system of the adult planarian (Gonzalez-Estevez et al., 2009; Sasidharan et al., 2013), we aimed to explore the function of this family, including its potential roles in neurogenesis.

We developed a novel liposome-based method for efficient delivery of *anti-miR-124* into live planarians to study the function of the *miR-124* family (hereafter collectively referred to as *miR-124*). Extensive characterization of *miR-124* knockdown (KD) phenotypes, which include severe brain and eye defects, revealed an essential role for *miR-124* in the generation and/or maintenance of discrete neural populations and eye progenitors, in addition to the patterning of neurons in the anteriorly regenerating planarian. Furthermore, *miR-124* KD caused an expansion of *Smed-slit-1* expression in the midline.

Target prediction analyses identified a cohort of genes essential for axon guidance, planar cell polarity, and the Notch pathway as likely targets of miR-124. To preliminarily validate our target predictions and gain insights into the mechanism of action of miR-
124, we focused our efforts on the Notch pathway. The KD of Notch pathway genes recapitulated the midline defects observed after miR-124 KD, suggesting that miR-124 might regulate slit-1 expression via the canonical Notch pathway. Altogether, our study uncovered roles for miR-124 in neurogenesis and neuronal organization during adult brain and eye regeneration. Furthermore, we find that miR-124 is likely to be required for midline patterning through a deeply evolutionarily conserved miR-124/Notch regulatory interaction.

RESULTS

Expression of the planarian miR-124 family is enriched in the cephalic ganglia and ventral nerve cords

The planarian genome encodes five miR-124 family members (Smed-miR-124a, b, c, d and e), which differ from each other after the tenth nucleotide from the 5’ end (Friedlander et al., 2009; Lu et al., 2009; Palakodeti et al., 2006). High-throughput sequencing of small RNAs from the anterior blastema of regenerating animals detected enrichment for miR-124a, b, c and e (Sasidharan et al., 2013). WISH using locked nucleic acid (LNA) detection probes confirmed the expression of miR-124a, b and c in the cephalic ganglia and ventral nerve cords (Fig. S1A). The miR-124c detection probe was not used for further expression studies because of its weak and inconsistent staining. Colocalization of miR-124a, b and c with the pan-neural marker Smed-pc2 and Hoechst staining revealed their expression in the bi-lobed cephalic ganglia. Unlike miR-124a and b, miR-124c showed a distinct expression pattern in the brain and ventral nerve cord, suggesting that the LNA probes effectively differentiate the expression of miR-124c from that of miR-124a and b (Fig. 1A,B, Fig. S1A-C). Additionally, miR-124c showed broad expression in both the perinuclear soma and the neuronal axon bundles in the bi-lobed cephalic ganglia, as compared with miR-124a and b, which were enriched in the perinuclear soma (Fig. 1B, Fig. S1B,C). Since miR-124a and b showed similar expression patterns, all additional expression studies were performed using the miR-124b probe. Colocalization studies of miR-124b and miR-124c with other neuronal markers, including choline acetyltransferase (Smed-chat), glutamate decarboxylase (Smed-gad), tyrosine hydroxylase (Smed-th) and tryptophan hydroxylase (Smed-tph),
revealed broad expression of miR-124 in all of these neural subtypes (Fig. 1C,D, Fig. S2A,B).

Further examination of miR-124b and miR-124c expression at different regeneration time points (1, 3, 5 and 7 dpa) revealed their enrichment in the brain primordia as early as 3 dpa (Fig. 1E). Enrichment of miR-124b and miR-124c was also observed in the regenerating brain at 5 and 7 dpa, as well as in the anterior commissure that connects the bi-lobed structure (Fig. 1E). The expression of miR-124c in the pre-existing ventral nerve cords close to the regenerating brain was not seen until 3 dpa, whereas the expression of miR-124b was evident by 1 dpa (Fig. 1E). However, weak miR-124c expression was seen throughout the pre-existing ventral nerve cord between 3 and 7 dpa. Prominent expression of miR-124c throughout the ventral nerve cords was observed only after 7 dpa (Fig. S1D). Interestingly, the brain and eye primordia are known to begin forming between 1 and 2 dpa. Since none of the miR-124 genes showed enrichment in the blastema until 3 dpa, this suggests that these miRNAs might contribute to the production of neural lineages born after 3 dpa and/or play roles in later stages of neural regeneration. To test these possibilities, we aimed to perturb the function of miR-124 during planarian regeneration.

Design of non-viral liposomes for enhanced delivery of anti-miRs in planarians

Currently, the study of miRNAs in planarians is limited by the lack of effective methods for delivering anti-miRs into cells. Several methods, such as microinjection and soaking, have been used to deliver double-stranded RNA (dsRNA) into planarians (Orii et al., 2003; Sánchez Alvarado and Newmark, 1999; Martin-Duran et al., 2008; Gonzalez-Estevez et al., 2003). However, these methods have not proved to be successful for delivering anti-miRs (data not shown). To overcome this, we custom-designed cationic, non-viral liposomal vectors to deliver anti-miRs into planarian cells in vivo.

We designed cationic liposomes that are fluidic at lower temperatures (~25°C) to efficiently fuse with planarian cells. Cationic lipids with symmetric (di-octadecyl, Sym-Lip) and asymmetric (octadecyl and oleyl, Asym-Lip) hydrophobic chains were synthesized and characterized by mass spectrometry (Fig. 2A, Fig. S3A-F). The fluidity of liposomes was modulated by their preparation using a single lipid [either Sym-Lip (symmetric liposomes) or Asym-Lip (asymmetric liposomes)] or a 1:1 molar ratio of symmetric-asymmetric lipids (co-liposomes), along with cholesterol. The biophysical characterization of these three liposomes, including their size, surface charge and fluidity, revealed that co-liposomes are the best carriers of nucleic acids compared with symmetric and asymmetric liposomes (Fig. 2B,C). Furthermore, gel retardation studies of the three types of liposomes mixed with miRNAs at different concentrations revealed that all three liposomes efficiently complex with miRNA at a higher lipid-to-miRNA charge ratio of 20:1, inhibiting liposome mobility in the gel (Fig. 2D, Fig. S4A).

To evaluate the fusogenicity of cationic liposomes, we performed fluorescence resonance energy transfer (FRET) by reconstituting the liposome with planarian lipids (see Materials and Methods) using dual fluorophore lipids (NBD-PE and Rho-PE) (Fig. 2E). NBD-PE and Rho-PE in proximity have an emission wavelength of 595 nm due to FRET (Mukthavaram et al., 2009). Fusion of the liposome with the membrane leads to a spatial separation between NBD-PE and Rho-PE lipids, which results in emission at 530 nm instead of 595 nm owing to the absence of FRET. The total percentage of fluorescence recovery at 530 nm, as a direct measure of the fusogenicity of the liposomes (Fig. 2E,F), was 72%, 80% and 93% for the symmetric, asymmetric and co-liposomes, respectively. Additionally, no significant recovery (~5%) was observed for Lipofectamine RNAi Max (Thermo) (Fig. 2F). These data suggest that the cationic liposomes and co-liposomes were particularly efficient at inducing fusion with planarian lipid membranes when compared with Lipofectamine RNAi Max.

Transmission electron microscopy (TEM) was performed on the three liposomal formulations (Fig. 2G) and lipoplexes containing oligonucleotides. This demonstrated the unilamellar stable nature of the liposomes (Fig. S4B). Furthermore, fluorescently labeled oligonucleotide complexes with co-liposomes and Lipofectamine LTX were incubated with regenerating planarians, and confocal imaging revealed that co-liposomes have a better penetration into tissues than Lipofectamine LTX (Thermo) (Fig. 2H, Fig. S5A). Confocal imaging of cell macerates from animals treated with co-liposomes further demonstrated that fluorescently labeled oligonucleotides successfully penetrate into the cytoplasm and nuclei of the cells (Fig. S5B). Cumulatively, these data indicate that these custom-designed liposomes could be highly efficient delivery vehicles for nucleic acids in planarians.

Knockdown of ovo and multiple miRNAs using non-viral liposomes

To validate the effectiveness of our liposome-based nucleic acid delivery method, we performed ovo KD. ovo (RNAi) animals are known to lack eyes. As expected, delivery of ovo dsRNA using liposomes resulted in an eyeless phenotype in 100% of the treated animals (n=15), whereas gfp-treated controls regenerated eyes normally (n=15) (Fig. 3A). This is on a par with the phenotype severity and penetrance obtained with dsRNA feeding or injection delivery methods (Lapan and Reddien, 2012).

Next, we examined the effectiveness of our delivery method for knocking down miRNAs. We administered an anti-miR that was predicted to target all miR-124 family members using co-liposomes in regenerating animals at 1 dpa. Animals were treated with anti-miRs at 10, 50 and 100 µM, and a scrambled anti-miR negative control was used at 10 µM.

The miRNA KD was verified by examining expression of one of the miR-124 family members, miR-124c, by WISH using antisense LNA probes. The animals treated with 50 or 100 µM anti-miRs showed significant decreases in expression of miR-124c (Fig. 3B). Although we observed a reduction in the expression of miR-124c in the 50 µM treatment condition, there was no obvious disorganization of the brain or visual system in these animals (Fig. S5C). This suggests that the reduction in miR-124c levels is not the result of a loss of brain tissue, but is specific to the inhibition of expression by the anti-miRs.

Since the anti-miRs are predicted to target all miR-124 family members, we also examined the expression levels of another member of the family, miR-124b. As expected, we observed a significant decrease in miR-124b expression in the animals treated with 100 µM anti-miRs (Fig. S6A), suggesting that all miR-124 members are likely to be inhibited. To assess whether the decreased levels of miR-124 were due to reduction in brain size, we performed WISH for miR-124a and miR-124c on both control and KD animals after the first round of regeneration, when there were no observable defects in brain morphology (Fig. S6B). We detected a significant reduction in the levels of the two miRNAs, even though the cephalic ganglia were clearly present, suggesting that the reduction in miRNA levels is not the result of a smaller brain.

To confirm this, we performed qPCR for each miR-124 family member after treatment with either the scrambled or anti-miRs.
The anti-miR-treated animals showed a 50-90%, 30-40% and 20-40% decrease in the levels of miR-124a, b and c, respectively (Fig. S6C). This suggested that our liposome-based delivery method is robust.

Liposome-mediated miRNA KD generates highly penetrant and specific regeneration phenotypes

To determine whether the observed KD of miRNA expression translated into robust and specific regeneration defects in planarians, we next examined the live phenotypes of planarians treated with anti-miR-124. Encouragingly, 81% of the animals treated with 100 µM anti-miRs showed various types of eye defects, including small eyes (25%), the complete absence of eyes (10%), cyclopia (13%), and eyes spaced abnormally far apart (14%) (Fig. 3C,D, Fig. S6D). A few animals (19%) also showed lesions in the head region and subsequently lysed (Fig. 3D).

To rule out the possibility that these regeneration defects might be due to unanticipated non-specific effects, we also treated planarians...
with anti-miRs targeting the *miR-10* family. We observed a phenotype entirely different from that of *miR-124* KD animals. No defects in the regeneration of the eyes or brain were observed. Instead, the regeneration of the intestine was disrupted, resulting in fusion of the two posterior gut branches (Fig. S6E,F).

Cumulatively, these validation experiments indicate that our liposome-based technique is highly specific and robust for delivering anti-miRs into planarian cells for functional studies.

**miR-124** is essential for regeneration of the eye in planarians
Since we observed obvious eye defects in live animals, we examined the morphology of the regenerated optic cup in *miR-124* KD animals. Using an anti-ARRESTIN antibody that labels the cell bodies, rhabdomeres and axons of the photoreceptor neurons (PRNs), we observed that *miR-124* KD animals displayed obvious fasciculation and elongation defects of the axons. The optic chiasm appeared highly disorganized (Fig. 4A,B). Furthermore, the aggregation of PRN cell bodies appeared significantly reduced in *miR-124* KD animals compared with scrambled controls (Fig. 4A, arrowheads), suggesting that this differentiated cell population might be missing after loss of *miR-124*.

Since we suspected that the numbers of differentiated cells of the eye were decreased in *miR-124* KD animals, we quantified the size of the regenerated eyes compared with scrambled-treated controls, suggesting a reduction in the number of differentiated cells. To confirm this, we counted individual ARRESTIN\(^+\) PRNs in the regenerated eyes. There were significantly fewer ARRESTIN\(^+\) cells in *miR-124* KD animals than in the scrambled-treated controls, confirming that this cell population was lost (Fig. S7B).

A reduction in the number of differentiated cells could mean that eye progenitors are lost after KD of *miR-124*. Therefore, we quantified the number of eye progenitors at 4 dpa, which are defined as *ovo*\(^+\) ‘trail’ cells posterior to the eye primordia (Lapan and Reddien, 2012). Indeed, the number of *ovo*\(^+\) trail cells was significantly reduced in *miR-124* KD animals (Fig. 4D), suggesting that *miR-124* may be required for the specification, differentiation and/or survival of eye progenitors.

**miR-124** is required for regeneration of the cephalic ganglia
Since *miR-124* family members are highly enriched in the nervous system, we examined whether brain regeneration was affected by loss of these miRNAs. Indeed, some *miR-124* KD animals (16%) displayed a reduction in the overall size of the brain compared with scrambled-treated controls, which was evident by immunostaining for the broad neural marker SYNORF-1 (Fig. 4E,F).

The planarian brain is composed of different neuronal subtypes (Roberts-Galbraith et al., 2016; Cowles et al., 2013; Ong et al., 2016). To explore the neural defects, we examined three of the major neuronal subtypes in planarians after *miR-124* KD: the dopaminergic (*th\(^+\)*), GABAergic (*gad\(^+\)*) and cholinergic (*chat\(^+\)*) neurons. FISH revealed a dramatic decrease in the numbers of *gad\(^+\)* and *th\(^+\)* cells in *miR-124* KD animals compared with scrambled-
treated controls, suggesting a loss of GABAergic and dopaminergic neurons. Interestingly, KD of miR-124 caused a significant increase in the number of chat+ cells, indicating a likely expansion of cholinergic neurons (Fig. 4G, Fig. S7C).

Since a disruption in differentiated neural cell types and a reduction in the size of the brain might indicate defects in the generation and/or maintenance of neural progenitors, we examined the expression of a progenitor marker for the nervous system (Smed-pax6A) (Wenemoser et al., 2012). In miR-124 KD animals, no reduction in the expression of pax6A was observed at 4 dpa (Fig. S7D). However, the KD animals showed mispatterned expression of pax6A+ cells. This suggested that miR-124 might not be required for the initial generation of neural progenitors, but may instead be required later during the progression of the lineage. Cumulatively, these data indicate that miR-124 may be involved in the production of discrete neural subpopulations during planarian brain regeneration.

Fig. 4. See next page for legend.
Fig. 4. miR-124 KD disrupts regeneration of the eyes and brain. (A) Immunostaining of the optic chiasm (anti-ARRESTIN) in scrambled-treated versus anti-miR-124-treated animals (100 μM each) at 7 dpa after the second round of regeneration. 13/16 KD animals showed diverse eye defects. Arrowheads indicate defects in KD animals. (B) FISH showing the expression of ovo in the eye and immunostaining for photosensory neurons (anti-ARRESTIN) in miR-124 KD (n=10) and scrambled-treated (n=7) animals. (C) Quantification of the size of the eyes determined by the ratio of the area of ovo staining to the total body area in scrambled (n=6) versus anti-miR-124-treated (n=5) animals. (D) FISH showing the expression of ovo in the eye and trail cells posterior to the eye in scrambled-treated and miR-124 KD animals at 4 dpa (n=6 animals per treatment condition). ovo+ trail cells were quantified in each condition. (E) Quantification of anti-SYNORF-1 staining of anteriorly regenerating animals normalized to body area in scrambled-treated versus anti-miR-124-treated (100 μM) animals (n=5 each). (F) Organization of the brain, ventral nerve cords, (anti-SYNORF-1, green) and photosensory neurons (anti-ARRESTIN, red) in scrambled (10/10) versus miR-124 KD animals regenerating either a head (10/10; top) or tail (13/16; bottom). Illustrations show the amputation plane (dashed line) and lighter colored areas indicate regenerated tissue. (G) FISH for neuronal subtypes in the brain of scrambled-treated versus miR-124 KD animals, including dopaminergic (th, red; n=6), GABAergic (gad, red; n=6) and cholinergic neurons (chat, green; n=6). Nuclei are stained with Hoechst (blue). Arrowheads indicate the neural subtypes. **P<0.01, ***P<0.001 (Student’s t-test). Error bars indicate s.d. Scale bars: 50 μm in A,B,D; 100 μm in F,G.

We also tested whether miR-124 is required in regeneration contexts that do not involve regenerating organs from scratch. First, we examined the head fragments that only regenerate tissues posterior to the brain de novo. In these animals, the pre-existing brain and eyes undergo remodeling and resizing, which is dependent on tissue turnover (Gurley et al., 2010). miR-124 KD animals regenerating posterior tissue showed no defects in the brain, eyes or optic chiasm, suggesting that miR-124 is not required for brain remodeling in posteriorly regenerating animals (Fig. 4F).

We also examined these organs during homeostatic tissue turnover after miR-124 KD. Intact animals were continuously treated with anti-miR for 1 month. No defects in the organization of the nervous system or eyes were observed after miR-124 KD compared with scrambled-treated controls (Fig. S7E). These results suggest that miR-124 is primarily required during de novo regeneration of the cephalic ganglia, ventral nerve cords and visual system in planarians.

miR-124 eye regeneration defects are independent of brain defects

Brain malformations are known to cause secondary defects in the eyes of planarians (Cowles et al., 2013). Therefore, it is possible that the observed eye defects upon miR-124 KD might be secondary to abnormalities present in the brain. To help address this, we examined the function of miR-124 during eye-only regeneration in the absence of any brain defects. This was achieved by first generating ovo(RNAi) worms, which lacked eyes but did not display brain abnormalities or any other morphological defects (Lapan and Reddien, 2012) (Fig. 5A,B). Next, we stopped the treatment with ovo dsRNA, which allowed the eyes to progressively regenerate over the course of 1 month as the gene expression recovered. During this recovery period, we treated animals with either scrambled or anti-miR-124, and assessed whether the eyes could regenerate in the presence of otherwise normal anatomy (Fig. 5A,B). The scrambled-treated live animals clearly regenerated eyes, whereas the animals treated with anti-miR-124 showed no signs of eye recovery at the same time point (Fig. 5C). To confirm this, we visualized the differentiated PRNs (Smed-opsin), optic chiasm (anti-ARRESTIN) and pigment cells (Smed-tyrosinase) in fixed worms. Consistent with our live animal observations, scrambled-treated worms regenerated both PRNs and pigment cells, in addition to a well-organized optic chiasm. By contrast, miR-124 KD animals possessed significantly fewer mature pigment cup cells and PRNs. Additionally, the axons projected by the few PRNs that were present after miR-124 KD did form an optic chiasm, but mis-targeting of some axons was observed (Fig. 5D). Cumulatively, the results of this eye-specific regeneration paradigm suggest that miR-124 has a direct role in eye regeneration, and this eye phenotype is unlikely to be a secondary effect of a generalized brain defect.

Fig. 5. miR-124 is required for eye-specific regeneration. (A) Methodology of eye-specific regeneration experiments. ovo dsRNA was administered by injection for 5 days, and on the sixth day the animals were amputated pre-pharyngeally. LNA anti-miRs were administered on alternative days for 8 days after 25 days of head regeneration. (B) Live images and anti-SYNORF-1 staining of ovo KD animals after 25 days of RNAi (n=15 animals). (C) Live images of ovo KD animals showing recovery of eyes after scrambled treatment (n=15/15 recovery) but not after anti-miR-124 treatment (n=12/15 failure). (D) Molecular characterization of eye phenotypes using anti-ARRESTIN (photosensory neurons and axons), opsip (photosensory neuron cell bodies) and tyrosinase (pigment cup cells). miR-124 KD animals (10/12) showed drastic reduction in opsip and tyrosinase staining, along with mispatterned photoreceptor neurons. Scale bars: 100 μm in B; 50 μm in C,D.
The putative targets of mir-124 are crucial for neuronal organization and growth

In an effort to identify the transcripts regulated by mir-124, we performed target prediction using the miRanda program (Enright et al., 2003). Target prediction identified a total of 1699 putative targets that might be regulated by the mir-124 family (Table S1). Although most of the predicted targets were common between all the mir-124 family miRNAs, we identified 128 and 125 predicted targets unique to mir-124b and mir-124c, respectively. Gene set enrichment analysis (GSEA) (Subramanian et al., 2005; Mootha et al., 2003) of mir-124 targets identified genes normally involved in axon guidance (slit-1, dscam, netrin-1), Notch signaling (notch-2, jagged-2) and planar cell polarity (PCP) (dishevelled-2, daam-1) (Table S2, Fig. S8A). Most have been shown to regulate visual neuronal organization and neuronal growth in planarians (Yamamoto and Agata, 2011; Fusaoka et al., 2006; Cebria et al., 2007). We validated some of the predicted targets of mir-124, including dscam, slit-1, ankyrin-3 and notch-2, by cloning their miRNA binding sites into the 3′-UTR region of the Firefly luciferase gene, followed by transfection into HEK293 cells. Upon mir-124 mimic transfection, we observed a 24-50% decrease (notch-2, 24%; dscam, 37%; slit-1, 50%; ankyrin-3, 43%; P<0.05) in Firefly luciferase activity compared with controls (Fig. 6A, Fig. S8B). These results show that the tested 3′-UTRs are likely targets of mir-124 in vivo.

Furthermore, we performed transcriptome sequencing to determine the transcriptional changes following miR-124 KD. The transcriptome sequencing identified 2609 transcripts that were differentially expressed (P<0.05). Of these, 1523 were significantly upregulated (Table S3). We used qPCR to examine the expression levels of 30 of these transcripts, and validated 25 that were significantly upregulated after miR-124 KD (P<0.05; Fig. S8C). The other five were upregulated by more than 2-fold, showing a similar trend to the transcriptome data, although not statistically significant. Among the 25 validated transcripts, nine were predicted as targets by miRanda. Interestingly, our data also show that most of the miRanda predicted targets were either marginally upregulated or show no change in expression upon miR-124 KD. It has previously been reported that miR-124 could potentially function by repressing mRNA translation rather than promoting transcript degradation (Hendrickson et al., 2009). Here we hypothesize that most of the targets of mir-124 that are only marginally upregulated upon miR-124 KD might be regulated by translational repression.

Mediolateral polarity is disrupted by loss of mir-124

The disruption of anteroposterior (A/P) or mediolateral (M/L) polarity might cause the observed eye and brain defects in miR-124 KD animals (Gurley et al., 2008). To determine whether the miR-124 KD phenotype is secondary to a polarity defect, we first investigated the organization of an organ system that is often disrupted by perturbation of these axes: the intestine (Smed-porcn-1) (Rink et al., 2009; Petersen and Reddien, 2009; Gurley et al., 2010; Cebria et al., 2007). We found that the intestines were indistinguishable between miR-124 KD animals and controls (Fig. 6B). No fusion of the posterior gut branches, ectopic or missing pharyngeal cavities, or additional major gut branches were observed, which are typical defects in worms with generalized A/P or M/L disruption.

We next examined whether A/P and M/L markers were likely to be targets of mir-124. Furthermore, we performed WISH to check...
for the expression of Smed-notum, an anterior marker and key regulator of A/P polarity (Petersen and Reddien, 2011), at 2 dpa in scrambled-treated versus anti-miR-124-treated animals (Fig. 6C). No difference in notum expression was observed between the control and KD animals. Cumulatively, this suggests that A/P polarity is not disrupted by miR-124 KD.

By contrast, slit-1, a broad marker for the midline, was a predicted target of miR-124. WISH for slit-1 revealed the presence of ectopic slit-1+ cells in the anterior blastema after KD of miR-124 (Fig. 6D). Interestingly, the loss of slit-1, not its expansion, is typically associated with a reduction in brain size and cyclopia (Cebria et al., 2007) like that we observe after miR-124 KD. These data suggest that the eye and nervous system abnormalities in miR-124 KD animals are unlikely to be secondary to a generalized loss of A/P or M/L polarity.

miR-124 may regulate the slit-1-expressing midline via canonical Notch signaling

Finally, we aimed to provide functional evidence that this list of predicted miR-124 targets can yield insights into the undoubtedly complex functions of this miRNA family during planarian regeneration. As a pilot experiment, we selected notch-2 as a candidate for functional analysis because a previous RNAi screen in the lab indicated that canonical Notch signaling might be involved in regulation of the midline, which we knew to be disrupted after miR-124 KD.

Knockdown of notch-2, which is broadly expressed throughout the planarian body (Fig. 7A), resulted in conspicuous regeneration defects that are typically associated with disruption of the midline. Specifically, notch-2(RNAi) animals displayed cyclopia and fusion of the two posterior gut branches (Fig. 7B,C). Since these defects are similar to those reported after RNAi of slit-1 (Cebria et al., 2007), we examined the expression of slit-1 after RNAi of notch-2. As expected, notch-2(RNAi) animals displayed a significant reduction in the number of slit-1+ cells (Fig. 7D). Interestingly, RNAi of other components of the canonical Notch pathway, including the core transcription factor Su(H) and the ligand delta-3, resulted in very similar midline defects. These included cyclopia, fusion of the posterior gut branches, and loss of slit-1 expression (Fig. S9A-J). Furthermore, RNAi of a negative regulator of Notch signaling, numb-1, yielded the opposite phenotype: a conspicuous increase in slit-1+ cells (Fig. 7E,F), which appeared strikingly similar to the phenotype observed for slit-1 expression after miR-124 KD.

Next, we investigated whether the effects on slit-1 expression are likely to be direct effects of notch-2/miR-124 regulation. We first examined colocalization of the notch-2 receptor and the delta-3 ligand in the slit-1+ population. This revealed that notch-2 and delta-3 are both expressed in discrete subpopulations of slit-1+ cells and in cells directly adjacent to slit-1+ cells (Fig. S10). Since Notch/Delta signaling is cell-autonomous, this suggests the possibility of a direct role for Notch signaling in regulating the slit-1-expressing midline.

Fig. 7. Regulation of notch-2 and slit-1 by miR-124. (A) Colorimetric WISH shows broad expression of notch-2 in uninjured planarian. (B-C′) notch-2 KD leads to cyclopia (B′ versus C′; 14 dpa) and fusion of posterior gut branches (B′ versus C′; mat, green; DAPI, blue; 17 dpa). (D) Colorimetric WISH for slit-1 after long-term notch-2 RNAI: slit-1 is significantly reduced in notch-2(RNAI) planarians compared with controls. (E) Colorimetric WISH shows broad expression of numb-1 in uninjured planarian. (F) Colorimetric WISH for slit-1 in control versus numb-1(RNAI) planarians at 23 dpa. Higher magnification images of the head show ectopic slit-1+ cells in numb-1(RNAI) planarians. (G,G′) Double FISH showing colocalization (arrows) of miR-124c (red) and notch-2 (green). (H,H′) Double FISH for slit-1 and miR-124c during anterior regeneration. miR-124c colocalizes with slit-1 (arrows) at 3 dpa, but not at 7 dpa. Scale bars: 100 μm.
We also examined the expression of miR-124 in both notch-2+ and slit-1+ cells. We found co-expression of notch-2 and miR-124c in the periphery of the brain in uninjured animals (Fig. 7G,G′). During head regeneration, a subset of miR-124+ cells colocalized with slit-1 at 3 dpa, but not at 7 dpa (Fig. 7H,H′, Fig. S9K). These expression dynamics suggest that miR-124 has the potential to function cell-autonomously in notch-2+ and slit-1+ cells.

Cumulatively, these phenotype and expression data suggest that the expansion of the slit-1+ expressing cell population observed after miR-124 KD may be mediated via direct regulation of notch-2 and subsequent canonical Notch signaling in planarians. Moreover, this indicates that our list of predicted targets of miR-124 promises to be extremely useful for further dissecting the complex roles of this miRNA family in the regeneration of the brain, visual system and midline of planarians.

DISCUSSION

Although several genes crucial for anterior fate determination and nervous system regeneration have been identified (Vasquez-Doorman and Petersen, 2014; Fraguas et al., 2014; Blassberg et al., 2013; Roberts-Galbraith and Newmark, 2013; Agata and Umesono, 2008; Beane et al., 2011; Higuchi et al., 2008; Gurley et al., 2008; Rink et al., 2009; Bonar and Petersen, 2017; Seebeck et al., 2017; Currie et al., 2016; Wang et al., 2016; Roberts-Galbraith et al., 2016; Adell et al., 2010), how these genes are coordinated in time and space to establish the developmental transitions necessary for form and function of the nervous system remains to be fully elucidated. Our study has revealed enrichment for the miR-124 family of miRNAs in the nervous system of planarians during homeostasis and discrete phases of head regeneration. The expression of miR-124c and miR-124b observed in anteriorly regenerating tissue at 3 dpa (Fig. 1E) suggests that these miRNAs might play a role in the organization of the brain and the patterning of visual neurons, rather than in specification of the brain primordium. We tested this hypothesis by perturbing the function of miR-124 during planarian regeneration.

To perform loss-of-function studies for miR-124 in planarians, we developed a novel vehicle to deliver anti-miRs using co-liposomes (Fig. 2). This method proved superior to soaking and injection, and we observed a comparably higher success rate for miRNA-mediated and also dsRNA-mediated KD using co-liposomes (Fig. 3, Fig. S5). miR-124 KD animals regenerating anterior tissue had either reduced or absent photoreceptors. Interestingly, the posterior regenerating tissue and the intact animals did not show any regeneration defects. However, the lack of homeostatic defects in anti-miR-124 KD animals might simply reflect slow turnover rates for neural cell types and/or the targeted miRNAs and proteins.

Extensive characterization of miR-124 KD planarians using various brain- and eye-specific markers revealed diverse defects in the organization of the brain and visual neurons. The diverse phenotypes observed might be attributed to variability in the extent of KD or to changes in the large cohort of genes with varied functions in miR-124 KD animals. Our study also revealed the key role of miR-124 in regulating neural subtype specificity and organization during planarian regeneration. For instance, miR-124 KD led to a reduction in the number of GABAergic and dopaminergic neurons, in addition to an increase in cholinergic neurons. However, the pathway through which miR-124 regulates neural subtype specificity is not known. Nonetheless, our data provide robust evidence for an important role for miR-124 in brain organization, the generation and/or maintenance of eye progenitors, and visual system patterning in regenerating planarians (Fig. 8A).

Furthermore, our work also highlights the need to invest time and effort in developing novel liposome vehicles, which might help advance the development of transgenic methods in planarians.

Target prediction revealed a cohort of genes involved in PCP (dishevelled-2, daam-1), axon guidance (netrin-1, slit-1, dscam) and the Notch pathway (notch-2, delta-3, serrate-1) as predicted targets of miR-124. Interestingly, miR-124 target conservation across the metazoans revealed that some of the planarian miR-124 targets, particularly notch-2, netrin-1 and slit-1, are also targets of miR-124 in vertebrates, but not in flies and nematodes (Fig. S11, Table S4). This is a notable difference given the extensive functional conservation in neurogenesis ascribed to miR-124 (Cao et al., 2006, 2007). There might be several biological and
methodological reasons for this difference. First, it might be that only vertebrates have evolved the ability for miR-124 to target notch, netrin and slit orthologs, and that the target predictions obtained for planarians might be an artifact. The evolutionary argument seems unlikely given that, in the invertebrate Ciona intestinalis, a feedback interaction between miR-124 and Notch signaling regulates the epidermal-peripheral nervous system (PNS) fate choice in tail midline cells (Chen et al., 2011). These findings suggest that our target predictions are not only biologically relevant, but also that they point to an ancestral origin of the regulation of the Notch pathway by the miR-124 family of miRNAs. Such a regulatory nexus might have been lost in C. elegans and D. melanogaster.

Among the putative miR-124 targets, three genes encoding axon guidance proteins were uncovered. netrin-1 and dscam are expressed in the planarian brain, while slit-1 is expressed in the midline along the length of the A/P axis of planarians (Yamamoto and Agata, 2011; Fusaoka et al., 2006; Cebria et al., 2007). The spatial and temporal expression of these genes is essential for proper patterning of the brain and the visual neurons. For instance, perturbation of slit-1 expression in the midline resulted in the collapse of the photoreceptors, demonstrating the key role for slit-1 in proper patterning of the visual neurons to form the optic chiasm. Furthermore, our expression study based on double FISH for slit-1 and miR-124 in regenerating animals revealed spatial and temporal changes in the expression of these genes in the midline region of anteriorly regenerating tissue. Thus, we hypothesize from these data that miR-124 expression in the brain might be essential for regulation of slit-1 expression during anterior regeneration. This hypothesis was supported by the miR-124 KD studies, which showed increased levels of slit-1 expression in the anterior region of the regenerating planarian. However, the expression of miR-124 is neuronal, except at 3 dpa when miR-124 was seen in slit-1+ cells, whereas the expression of slit-1 is non-neuronal. This raises an important question regarding the molecular mechanism by which miR-124 regulates the expression of slit-1. Our study has demonstrated that KD of notch-2 and delta-3 led to downregulation of slit-1, and KD of its negative regulator numb-1 caused ectopic expression of slit-1. The notch/numb KD studies were supported by colocalization studies, which showed co-expression of delta-3 and notch-2 in slit-1+ cells, suggesting that a NOTCH-2–DELT A-3 interaction might regulate slit-1 expression. Similarly, miR-124 KD led to increased levels of notch-2 expression and ectopic expression of slit-1 in the anterior region of the planarians (Fig. S8C, Fig. S6D). These results were further supported by colocalization studies using FISH, which revealed co-expression of notch-2 and miR-124 in the periphery of the brain. Together, these results suggest a role for miR-124 in modulating notch-2 expression, which is required for regulating slit-1 in the midline of the anterior regenerating tissue. However, it is still not clear how miR-124 expression in the brain regulates the notch-2 domain crucial for slit-1 expression. Recent studies have shown that miRNAs can be secreted from cells after packaging into exosomes, allowing delivery to adjacent cells and target suppression (Lee et al., 2017). The possibility of an exosome-mediated mechanism to deliver miR-124 to the slit-1+ cells needs to be investigated further. Nonetheless, based on our results, we propose a mechanism by which miR-124 regulates slit-1 expression via modulation of canonical Notch signaling (Fig. 8B).

The target prediction data from miR-124 KD animals also revealed the PCP genes dishevelled-2 and daam-1 as potential targets of miR-124. In planarians, vang-1, daam-1 and ROCK KD results in supernumerary eye formation and hyperplasia of neurons throughout the body (Beane et al., 2012). Our results suggest that miR-124 KD animals behave like gain-of-function mutants of the PCP pathway, with premature termination of neural growth that leads to a significant decrease in the size of the cephalic ganglia and eyes. However, a rigorous target validation and dissection of the PCP pathway in planarians is necessary to establish whether the miR-124 family plays a role in regulating this important developmental pathway.

In summary, we report on the development of a novel vehicle to deliver exogenous nucleic acids into planarian cells consisting of co-liposomes designed to have high fusogenic properties with planarian cell membranes. By combining these liposomes with anti-miRs, we were able to robustly target and knock down the function of miR-124, resulting in cephalic regeneration phenotypes. Our study reveals the key role of miR-124 in neuronal organization and axon guidance during regeneration of the brain and visual system in planarians. Such a role for miR-124 might be found in other neuronal regeneration paradigms.

MATERIALS AND METHODS

Animal maintenance

Schmidtea mediterranea planarians were maintained in 1× Montjuic solution at 20°C. Animals were starved for a week prior to the experiments, as described previously (Sasidharan et al., 2013).

In situ hybridization and immunostaining

Whole-mount in situ hybridization (WISH) was performed as previously described (Pearson et al., 2009; Sasidharan et al., 2013; King and Newmark, 2013). miRNA-based WISH was carried out using digoxigенин-labeled miRCURY LNA probes obtained from Exiqon. For immunostaining, animals were fixed using Carnoy’s solution as described (Sánchez Alvarado and Newmark, 1999). Rabbit anti-ARRESTIN (1:5000, clone VC-1; gift of Dr Kiyokazu Agata, Kyoto University, Japan) and mouse anti-SYNORF-1 (1:100, DHSB) primary antibodies were used. Species-specific secondary antibodies were obtained from Molecular Probes (1:400). Hoechst 33342 (25 μg/ml; Sigma) was used as a nuclear counterstain. Animals were mounted with Mowiol mounting medium (Sigma) and stored at 4°C until imaging.

Preparation and biophysical characterization of liposomes

Liposomes were prepared with 1:1 molar ratios of lipid and cholesterol at a total concentration of 2 mM. Dry thin films of lipids were prepared in glass vials, followed by overnight hydration with deionized water to produce hydrated films. Vortexing vials for 2-3 min produced multilamellar vesicles. Subsequently, bath sonication (Qsonica sonicator, Q700) produced small unilamellar vesicles. The size and zeta potentials of liposomes (2 mM) were measured using a Zetasizer Nano ZS (Malvern). Using the same method, liposomes with planarian lipids and dual fluorescent lipids (1% of FRET pair, NBD-PE and N-Rho-PE) were made. Fluorescence intensities were recorded by excitation at 485 nm and emission at 530 nm. For the gel retardation assay, Cy3-labeled miRNA (2 μM) was complexed with liposomes in a total volume of 30 μl in HEPES buffer (pH 7.4). The samples were electrophoresed at 80 V for 20 min and the miRNA bands were visualized using an agarose gel documentation unit. Each of the methods used for the biophysical characterization of liposomes is described in more detail in the supplementary Materials and Methods.

Knockdown of miR-124, ovo and Notch pathway genes

For miR-124 and ovo KD, sexual planarians were pre-pharyngeally amputated, and the head and tail fragments were treated separately with liposome–complexed anti-miRs and scrambled miRs. Notch pathway gene KD was by injecting and feeding dsRNA to intact planarians followed by amputation. Detailed protocols are provided in the supplementary Materials and Methods.

Transcriptome analysis

Total RNA was isolated from scrambled-treated and anti-miR-treated animals (n=15) using Trizol (Invitrogen) after two rounds of regeneration. The transcriptome library was prepared from the isolated total RNA using

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the TruSeq RNA Library Prep Kit v2 (Illumina) following the manufacturer’s instructions. The transcriptome library was subject to paired-end sequencing (150 bp) on a NextSeq 500 next-generation sequencing machine (Illumina). We obtained 54.47 and 91 million reads from mir-124-let KD and scrambled-treated samples, respectively. We used a reference-based transcriptome assembly algorithm TopHat v2.0.8, Cufflinks v1.0.3 pipeline to assemble transcripts, with the Os_Smed_v1 transcriptome as the reference annotation to guide RABT (reference annotation based transcript) assembly. Please refer to the supplementary Materials and Methods for a detailed protocol.

**Real-time PCR**

Sequencing data were validated by real-time PCR on an ABI PRISM 7900HT. Total RNA used for next-generation sequencing was further used for qPCR validation. Primers are detailed in Table S5. A detailed protocol is provided in the supplementary Materials and Methods.

**miRNA target prediction and validation**

We used miRNA v3.3a (Enright et al., 2003) to predict targets of miR-124a, b and c. The targets were filtered based on the complementarity score (score ≥140) and free energy (ΔG≤-14 kcal/mol). Target validation was performed in HEK293T cells transfected with pMIR-REPORT plasmid (Addgene) containing the 3′-UTR of the target and prLV-SV40 containing the Renilla luciferase gene (Promega) as a transfection control. miRNA mimics were obtained from Eurogentec and diluted as per manufacturer’s instructions. A detailed protocol is provided in the supplementary Materials and Methods.

**LNA detection probes, miR-124 inhibitors and qPCR primers**

Anti-miR-124 was designed against 16 nucleotides at the 5′ end of miR-124. Short probes were used to facilitate better penetrance and substantially reduce cell toxicity, as recommended by the manufacturer. This anti-miR was predicted to KD all miR-124 family members because these miRNAs share the same seed region and differ by only two or three nucleotides outside the seed region. Please refer to the supplementary Materials and Methods for a detailed list of detection and qPCR probes.

**Acknowledgements**

We thank the D.P. lab members, Dr Yashoda Ghanekar, Dr Vineetha Raghavan and Dr Praveen Anand for proofreading the manuscript; Aradhya Jain and L. S Divya for help with confocal imaging; Drs Jochen C. Rink and Kyle A. Gurley for invaluable scientific discussions, technical advice, and for preparing preliminary Notch pathway RNAi screens; and Dr Kyokazu Agata for sharing the VC-1 antibody.

**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**


**Funding**

D.P. was supported by Wellcome Trust/Department of Biotechnology, Ministry of Science and Technology (DBT) India Alliance Interim Fellowship (5001062/09/2) that also supported V.S. and V.L.. P.K.V. was supported by a Ramalingaswami Fellowship, DSR and India. A.S.A. is an investigator of the Howard Hughes Medical Institute and an Investigator of the Stowers Institute for Medical Research. Deposited in PMC for immediate release.

**Data availability**

RNA-seq data generated in this study have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under accession number SRP078730. Original data underlying some of the figures in this manuscript can be accessed from the Stowers Original Data Repository at http://www.stowers.org/research/publications.

**Supplementary information**

Supplementary information available online at http://dev.biologists.org/lookup/doi/10.1242/dev.144758.supplemental

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