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miR-124 function during *Ciona intestinalis* neuronal development includes extensive interaction with the Notch signaling pathway

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SUMMARY

The nervous system-enriched microRNA miR-124 is necessary for proper nervous system development, although the mechanism remains poorly understood. Here, through a comprehensive analysis of miR-124 and its gene targets, we demonstrate that, in the chordate ascidian *Ciona intestinalis*, miR-124 plays an extensive role in promoting nervous system development. We discovered that feedback interaction between miR-124 and Notch signaling regulates the epidermal-peripheral nervous system (PNS) fate choice in tail midline cells. Notch signaling silences miR-124 in epidermal midline cells, whereas in PNS midline cells miR-124 silences *Notch*, *Neuralized* and all three *Ciona* Hairy/Enhancer-of-Split genes. Furthermore, ectopic expression of miR-124 is sufficient to convert epidermal midline cells into PNS neurons, consistent with a role in modulating Notch signaling. More broadly, genome-wide target extraction with validation using an in vivo tissue-specific sensor assay indicates that miR-124 shapes neuronal progenitor fields by downregulating non-neural genes, notably the muscle specifier *Macho-1* and 50 Brachyury-regulated notochord genes, as well as several anti-neural factors including *SCP1* and *PTBP1*. 3′UTR conservation analysis reveals that miR-124 targeting of *SCP1* is likely to have arisen as a shared, derived trait in the vertebrate/tunicate ancestor and targeting of *PTBP1* is conserved among bilaterians except for ecdysozoans, while extensive Notch pathway targeting appears to be *Ciona* specific. Altogether, our results provide a comprehensive insight into the specific mechanisms by which miR-124 promotes neuronal development.

KEY WORDS: miR-124, microRNA targets, Notch signaling, Macho-1, SCP1, Notochord, *Ciona intestinalis*, PTBP1

INTRODUCTION

microRNAs (miRNAs) are a fundamental class of biological molecules with a crucial role in development (Kloosterman and Plasterk, 2006), the dysfunction of which has been linked to cancer (Calin and Croce, 2006), among other biological processes. Genes encoding miRNAs, which are found in most eukaryotes, produce short (~22 nt) RNAs that bind to mRNA transcripts and downregulate their expression either through mRNA destabilization or translational repression (Bartel, 2009). How miRNAs recognize and bind their mRNA targets is still a subject of intense research (Bartel, 2009), although in most cases good complementary binding between the mRNA 3′ untranslated region (UTR) and the first eight nucleotides of the miRNA is necessary, which we will refer to as the 'seed' site.

miR-124 is one of the most abundant miRNAs expressed in the mouse brain (Lagos-Quintana et al., 2002). Subsequent studies showed that miR-124 is also expressed in the nervous systems of *Drosophila* (Aboobaker et al., 2005), *C. elegans* (Clark et al., 2010) and humans (Sempere et al., 2004). Its clinical importance has recently been revealed in a study showing that aberrant downregulation of miR-124 in humans is associated with the formation of brain tumors (Silber et al., 2008). In vertebrates, miR-124 regulates two important anti-neural factors: SCP1 (small C-terminal domain phosphatase), a component of the REST/NRSF neuronal transcriptional repressor complex (Visvanathan et al., 2007), and PTBP1, which represses brain-specific alternative pre-mRNA splicing (Makeyev et al., 2007). A recent in vitro study using FACS-sorted stem cell astrocytes from the neurogenic subventricular zone of the mouse brain showed that miR-124 knockdown increased the number of dividing neural stem cells and reduced the number of postmitotic neurons, whereas conversely, miR-124 overexpression promoted cell cycle exit and increased the expression of postmitotic neuronal markers (Cheng et al., 2009). These results suggest a role of miR-124 in driving neuronal differentiation. However, aside from detailed studies of a few specific targets, the underlying genetic pathways by which miR-124 may drive neuronal differentiation remain largely unexplored.

Here, using computational target extraction with in vivo functional and transgenic assays, we show that, in the chordate ascidian *Ciona intestinalis*, miR-124 plays a multifaceted role in promoting neuronal development. As the sister group of vertebrates (Delsuc et al., 2006), ascidians possess a simplified chordate nervous system. The central nervous system (CNS) has ~100 neurons and consists of a sensory vesicle and a dorsal nerve cord, whereas the peripheral nervous system (PNS) consists primarily of epidermal sensory neurons (ESNs) (Imai and Meinertzhagen, 2007a; Imai and Meinertzhagen, 2007b; Passamaneck and Di Gregorio, 2005). Early in development, specification of the ascidian CNS requires FGF signaling (Akanuma and Nishida, 2004; Bertrand et al., 2003) and is likely to utilize Hedgehog signaling as well (Takatori et al., 2002). In the PNS, both FGF and BMP signaling are required early, and, subsequently, Notch signaling resolves the pattern of sensory neurons within the midline of the larval tail (Pasini et al., 2006). Using our sensor assay, we demonstrate that miR-124 can downregulate genes from all of

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these signaling pathways and show that, in several cases, this regulation is conserved in other species. We also show that miR-124 regulates a number of non-neuronal gene targets, including the muscle-specification factor *Macho-1* (Kobayashi et al., 2003; Nishida and Sawada, 2001) and numerous Brachyury-regulated genes expressed in the notochord (Corbo et al., 1997; Takahashi et al., 1999). These targets reveal a potential role for miR-124 in the cell lineage specification of the nervous system.

MATERIALS AND METHODS

Prediction of miR-124 targets

miR-124 targets were found by searching for transcript 3′UTRs with a canonical seed site at least 14 nt away from the stop codon (so that upon binding, the 3′ end of miR-124 remains within the 3′UTR), a similar approach to Grimson et al. (Grimson, et al., 2007). 3′UTR sequences were derived from the most recently published *Ciona* KH (Kyoto Hoya) gene models (Satou et al., 2008). Note that our definition of the miRNA seed includes all eight nucleotides at the 5′ end of the miRNA, whereas the definition used by Bartel includes only nucleotides 2-7 (Bartel, 2009).

Tissue-specific sensor assays in transgenic embryos

For each miR-124 target, we generated an RFP-expressing transgene containing the 3′UTR of the target gene. A second transgene expressing CFP had a neutral SV40 3′UTR lacking canonical sites. A third transgene expressed both copies of the primary miR-124 transcript. Each of these transgenes was driven in the larval epidermis using an epidermis-specific enhancer of the *Ciona EpiB* gene. Exactly 8 μg of each of the three transgenes was electroporated into experimental embryos (Zeller et al., 2006a). For negative control embryos, a neutral YFP transgene was electroporated in place of the miR-124-expressing transgene as a mass control. For PNS assays, RFP and CFP sensor constructs were driven with a *gelsolin* promoter. After electroporation, embryos developed at 18°C for 11-13 hours. All experiments were performed at least in duplicate, with expression data pooled for analysis. Oligonucleotide sequences for promoters and target genes are listed in supplementary material Table S10.

Image analysis

Images were captured using a Zeiss Axioplan 2E epifluorescence imaging microscope equipped with a 14-bit AxioCam HR camera. Per target, images of all embryos under a given fluorescence channel were captured with the same exposure time and objective. Subsequent image analysis of the 14-bit images was performed using ImageJ. Following equal background subtraction (rolling ball radius=50), target RFP and control CFP fluorescence were measured separately for each embryo. The mean and standard deviation of RFP:CFP expression ratios were calculated for at least 25 miR-124-misexpressing embryos (miR-124$^+$). RFP:CFP expression ratios were then compared with those of negative control (miR124$^-$) embryos. All experiments were performed at least in duplicate for a total of at least 50 assayed embryos per target (average 79 embryos). Statistical significance was calculated using a paired one-tailed Student's *t*-test.

In situ hybridization

Digoxigenin-labeled RNA exon and intron probes (supplementary material Fig. S1) were generated from linearized DNA templates. Custom locked nucleic acid (LNA) probes complementary to the mature *Ciona* miR-124 sequence were obtained from Exiqon (hsa-miR-124, #88066-15). All embryos were reared at 18°C. In situ hybridization was carried out essentially as described previously (Mita and Fujiwara, 2007). LNA in situ hybridizations added an EDC fixation step (Pena et al., 2009).

Gene Ontology (GO) annotation analysis

Blast2GO was used to perform initial GO analysis under standard program settings (Conesa et al., 2005). Enriched terms were found using a two-sided Fisher's exact test with correction for multiple testing using the Benjamini-Hochberg procedure, as performed previously for GO enrichment analysis (Clark et al., 2010). For the independent gene enrichment analysis we used a custom Python script to extract neuronal category genes among all GO annotated miR-124 targets (451 out of 867) by filtering GO terms for neuronal-related keywords. Statistical significance was calculated by comparing the proportion of neural category genes among the annotated miR-124 targets versus all annotated non-miR-124 targets using a two-sided Fisher's exact test. Filtering and analysis for other categories and for conserved targets were performed in a similar manner.

RESULTS

miR-124 is expressed extensively in the ascidian nervous system

Two tandem copies of *miR-124* are located within the second intron of the gene *Ci-Pans*, which is extensively expressed throughout the developing nervous system beginning at the cleavage stage (Fujiwara et al., 2002; Alfano et al., 2007). To determine whether the expression pattern of miR-124 mimics that of its host gene, we compared timecourse in situ hybridizations using an intron probe specific for the primary miR-124 transcript, a locked nucleic-acid (LNA) probe specific to the mature miR-124 product, and a probe for the mature *Ci-Pans* mRNA (supplementary material Fig. S1A). Both the intron and LNA probe in situ hybridizations demonstrate that miR-124 is expressed extensively throughout the developing CNS and PNS (Fig. 1; supplementary material S1B,C). Although the intron probe shows that the primary miR-124 transcript is expressed as early as the 44-cell stage, we were not able to detect early expression using the LNA probe. Indeed, recent deep sequencing efforts have reported that mature miR-124 read counts are relatively low in cleavage and gastrula stage embryos, suggesting that the LNA probe is not sensitive enough to detect the low expression levels at these stages (Shi et al., 2009; Hendrix et al., 2010). Importantly, though, the collective expression pattern using both miR-124 probes is identical to that of *Ci-Pans* throughout development, suggesting that miR-124 and its host gene are expressed concurrently and extensively in the nervous system (Fig. 1; supplementary material Fig. S1B,C).

The ascidian epidermis is neurogenic

The embryonic PNS is induced to form specifically along midline epidermal cells by early BMP and FGF signaling, followed by Notch-Delta signaling that restricts which midline epidermal cells will form ESNs (Pasini et al., 2006). To visualize PNS expression in vivo, we use a PNS-specific *gelsolin* reporter gene (Ohtsuka et al., 2001) and an acetylated-tubulin antibody that labels ciliated ESNs (Crowther and Whittaker, 1994) (Fig. 2A-C). Interestingly, ectopic expression of the PNS-specific *Ciona Pou4* gene (Candiani et al., 2005) in the epidermis induces extensive ectopic gelsolin expression and ectopic ESN cilia formation (Fig. 2D,E), indicating that the entire larval epidermis is neurogenic and can be easily converted to PNS by ectopic Pou4 expression. Interestingly, miR-124 is also ubiquitously expressed in this phenotype (Fig. 2F). Because the epidermis does not normally express miR-124, but is neurogenic, we decided to examine miR-124 function in these cells.

miR-124 overexpression in the epidermis causes ectopic neurogenesis

We first asked whether miR-124, like Pou4, could convert the epidermis into PNS. Previous in vitro studies have shown that ectopic expression of miR-124 can cause increased expression of postmitotic neuronal markers (Cheng et al., 2009; Makeyev et al., 2007), although explicit induction of extra neurons in vivo has not been shown. In *Ciona*, ectopic epidermal expression of miR-124 resulted in the formation of extra ciliated ESNs along the tail

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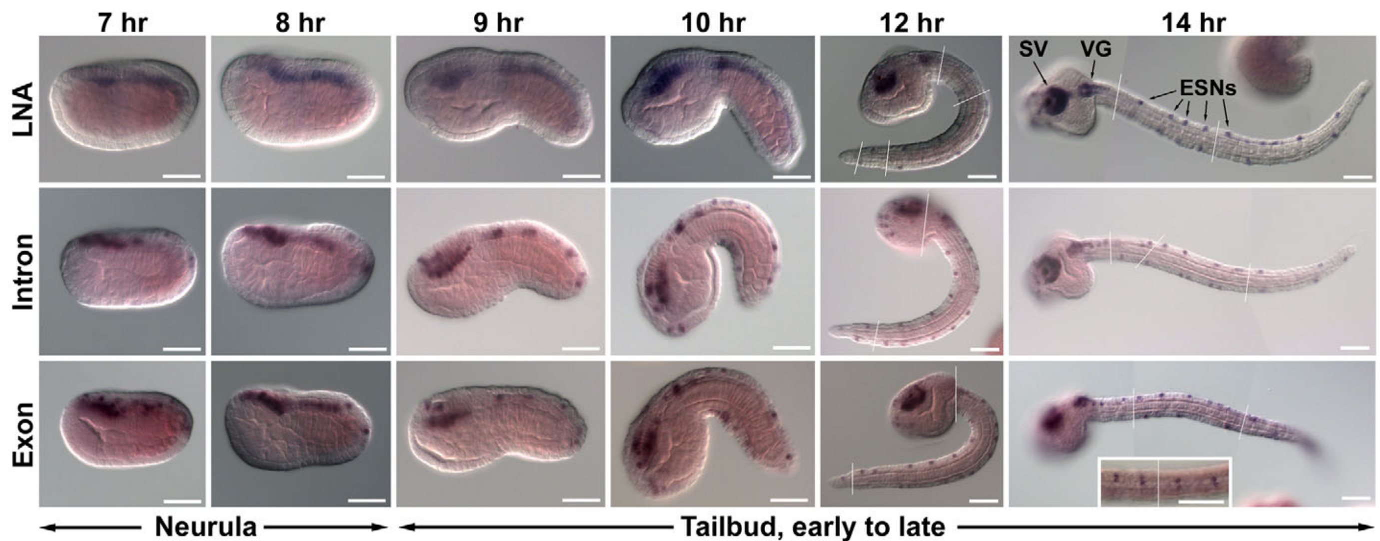

Fig. 1. miR-124 is extensively expressed in the *Ciona* nervous system during development. Whole-mount in situ hybridizations using an exon probe (against *Ci-Pans*, bottom row), an intron probe (overlapping primary miR-124 transcript, middle row) and an LNA probe (against the mature miR-124 product, top row) all show identical expression patterns and demonstrate that miR-124 is expressed extensively in the CNS and PNS. Lateral views of neurula and tailbud stage embryos are shown (see supplementary material Fig. S1 for dorsal views and expression at earlier stages). Sensory vesicle (SV, CNS), visceral ganglion (VG, CNS) and epidermal sensory neurons (ESNs, arrows, PNS) are labeled in the 14-hour LNA-probed embryo. Both neurons in an ESN pair express miR-124 (14 hour exon, inset). White lines within embryos demarcate boundaries between different focal planes. Scale bars: 50 μm.

midlines (Fig. 2G,H). A similar phenotype is also observed when Notch signaling is blocked in the epidermis by expressing a dominant-negative form of Suppressor of Hairless (Paisini et al., 2006) (Fig. 2J,K) and, in these embryos, miR-124 is also ectopically expressed along the tail midlines (Fig. 2L). When Notch signaling is ectopically activated in the epidermis by expressing the Notch intracellular domain (NICD), ESNs (Paisini et al., 2006) (Fig. 2M,N), as well as miR-124 expression (Fig. 2O), are eliminated in the tail midlines. These results suggest that miR-124 expression is regulated by Notch signaling in the midline epidermis. Ectopic miR-124 expression can also convert some non-midline epidermal cells to neurons (Fig. 2I), but this phenotype is not as strong as that observed for Pou4.

miR-124 targets can be found by searching for three types of canonical seed sites

To determine the role played by miR-124 in neuronal development, we first computationally searched for target genes in the *Ciona* genome. Although miRNAs have been shown to bind several categories of target sequences, it is now well established that the majority of target sequences contain one of three types of canonical seed site in their 3′UTRs: an 8mer or [1,0,0] site (perfect complementarity of a transcript with the miRNA seed); a 7mer-m8 or [0,1,0] site (complementarity with miRNA nucleotides 2-8); or a 7mer-A1 or [0,0,1] site (complementarity with miRNA nucleotides 2-7 with an A at mRNA position 1) (Bartel, 2009; Selbach et al., 2008) (Fig. 3A). To identify putative miR-124 targets we extracted 3′UTRs from the most recent *Ciona* KH transcript models (Satou et al., 2008) and found 867 predicted miR-124 targets with at least one canonical seed site (supplementary material Table S1).

We next designed an in vivo assay using transgenic embryos to quantify the downregulation of individual miR-124 target genes in whole, live ascidian larvae (Fig. 3B). A CFP-expressing transgene containing a neutral SV40 3′UTR (Zeller et al., 2006b) served as a reference for fluorescent protein expression. A transgene expressing miR-124 containing both copies of miR-124 in their natural intronic location was also generated. RFP-expressing transgenes for each miR-124 target gene contained the 3′UTR of each gene. These transgenes were all driven with the same epidermal tissue-specific promoter (*EpiB*). To determine whether miR-124 mediated downregulation of a target gene, we compared RFP/CFP expression level ratios in embryos with (miR-124^{+}) or without (ctrl, miR-124^{-}) miR-124 overexpression. Experiments were performed at least in duplicate, comparing on average 80 embryos from each sample per target gene.

We quantified downregulation of 25 predicted miR-124 targets among a wide range of canonical seed types. We tested five 8mer targets (one with two sites), seven 7mer-m8 targets (two with two sites), and seven 7mer-A1 targets, with the remaining targets having combinations of these sites. From each seed type, we selected at least one ‘strong’ and one ‘weak’ target, based on a scoring method established by Grimson et al. (Grimson et al., 2007) (supplementary material Table S2). Remarkably, all 25 of our tested targets showed significant downregulation (P<0.005, one-tailed paired Student’s t-test) relative to the SV40 3′UTR control (Fig. 3C; supplementary material Table S3). Moreover, we were able to detect as little as 9.0% downregulation, an improvement in sensitivity over large-scale approaches (Baek et al., 2008; Karginov et al., 2007; Lim et al., 2005). Importantly, all tested targets from every class of canonical seed site showed significant downregulation. Because we tested both ‘strong’ and ‘weak’ targets from each class of seed site, thus covering the range of all possible targets, our results indicate that transcripts with at least one miR-124 canonical site are bona fide miR-124 targets in vivo (Fig. 3D-F). Interestingly, the degree of downregulation for our targets varied considerably, ranging from 9.0% (gene ID KH.C13.2, P=0.0027) to 88.7% (*Macho-1*, P=4.8×10^{-92}).

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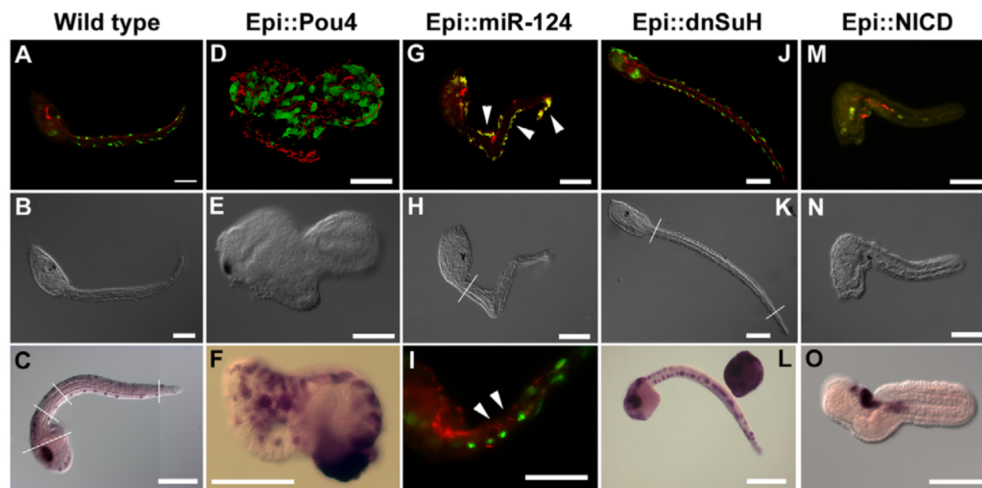

Fig. 2. miR-124 and Notch signaling feedback interaction specifies the *Ciona* PNS. Immunohistochemical (IHC) staining and associated in situ hybridizations show that miR-124 and Notch signaling interact to specify the tail midline PNS. All IHC images (A,D,G,I,J,M) are of representative larval stage embryos stained for acetylated tubulin, which labels ESN cilia (red), and for gelsolin, which labels PNS cells (green in A,D,I,J,M; yellow in G). DIC images (B,E,H,K,N) are shown below their corresponding IHC images. All in situ hybridizations (C,F,L,O) are stained against miR-124. (**A-C**) Representative wild-type larva showing punctate PNS ESN expression along the dorsal and ventral tail midlines (A,B), with corresponding miR-124 in situ staining showing that these ESNs express miR-124 (C). (**D-F**) Ectopic expression of the early PNS gene *Pou4* shows that the entire epidermis (Epi::Pou4) can be converted into PNS. (**G-I**) miR-124 misexpression in the epidermis (Epi::miR-124) expands the PNS along the tail midlines with extensive ectopic ESN formation (G, arrowheads), with modest ESN formation outside the midlines as well (I, arrowheads). (**J-L**) Disruption of Notch signaling with a dominant-negative *Suppressor-of-Hairless* (dnSuH) transgene in the epidermis (Epi::dnSuH) also expands the PNS along the tail midlines, with the corresponding in situ (L) showing that the domain of miR-124 expression is likewise expanded. (**M-O**) Conversely, epidermal ectopic activation of Notch signaling with a transgene expressing the intracellular domain of Notch (Epi::NICD) eliminates tail midline ESN formation (M), with a corresponding repression of miR-124 expression (O). White lines demarcate boundaries between different focal planes. Scale bars: 50 µm.

Incidentally, the degree of downregulation did not always correlate with the predicted strength of targets (Grimson et al., 2007) (supplementary material Table S2), indicating that further experimentation is required to understand the parameters involved in miRNA-mediated target downregulation.

miR-124 targets pathways throughout *Ciona* nervous system development

We next performed a gene ontology (GO) enrichment analysis of our miR-124 canonical targets. From this, we discovered an overrepresentation of genes among three broad categories of biological processes: neural, development and cell cycle/apoptosis (supplementary material Fig. S2, Table S4). Among these processes are many important pathways throughout *Ciona* nervous system development, ranging from initial neuronal specification to cell cycle exit (supplementary material Table S5), many of which are conserved targets in human (supplementary material Table S6).

First, we discovered that miR-124 targets are enriched for FGF, MAPK and Ets genes (eight genes total; supplementary material Table S5), which are involved in induction of the ascidian brain (sensory vesicle) (Akanuma and Nishida, 2004) and PNS (Bertrand et al., 2003). Although these pathways have other functions in the developing ascidian, we note that miR-124 expression appears specifically in sensory vesicle precursors in the early gastrula immediately after FGF-MAPK-Ets brain induction is complete (Akanuma and Nishida, 2004; Alfano et al., 2007). We thus hypothesize that miR-124 expression in the CNS modulates the inductive signals that specify the brain.

Second, we discovered that miR-124 targets many genes known to be involved in cell cycle progression (*CDK6*, *CDK7*, *Cullin-3*, *Cyclin-E*) and control (*p53/73*, *TFAP4*) (supplementary material Table S5). We also observed several genes involved in the induction of apoptosis (*Caspase3/7*, *AMID*, *Traf3*). Incidentally, miR-124 targeting of these processes is conserved in vertebrates (Makeyev et al., 2007; Cheng et al., 2009). These results suggest that, when a dividing neuronal precursor transitions into a postmitotic neuron, miR-124 plays a conserved role in promoting cell cycle exit and preventing apoptosis.

Third, miR-124 targets genes that pattern the CNS in the neck region and dorsal nerve cord (Fuccillo et al., 2006; Passamaneck and Di Gregorio, 2005; Takatori et al., 2002). *Sonic hedgehog* (*SHH*), *Pax2/5/8* and *GLI1* [a confirmed downstream target of *Pax2/5/8* (Satou et al., 2005) and a reported downstream target of SHH in vertebrates (Fuccillo et al., 2006)] were all identified as miR-124 seed targets, with *SHH* regulation verified in vivo (Fig. 3C; supplementary material Fig. S3).

Fourth, we discovered that *Ciona* miR-124 targets several conserved neuronal gene repressors, including *SCP1* and *PTBP1* (supplementary material Table S5). Using our sensor assay, we verified miR-124 targeting of *SCP1* and *PTBP1* in *Ciona* (Fig. 3C; supplementary material Fig. S3). Incidentally, *SCP2*, which might have a similar function to *SCP1* (Yeo et al., 2005), is also a verified *Ciona* miR-124 target (Fig. 3C; supplementary material Fig. S3).

miR-124-mediated PNS specification through regulation of Notch signaling

The observations that epidermal expression of miR-124 produced ectopic ESNs along the tail midlines (Fig. 2G-I) and that miR-124 expression is regulated by Notch signaling (Fig. 2L,O) suggested that miR-124 might interact with Notch signaling to regulate PNS specification. We first searched through our predicted targets for known conserved Notch pathway genes (Lai, 2004) and found

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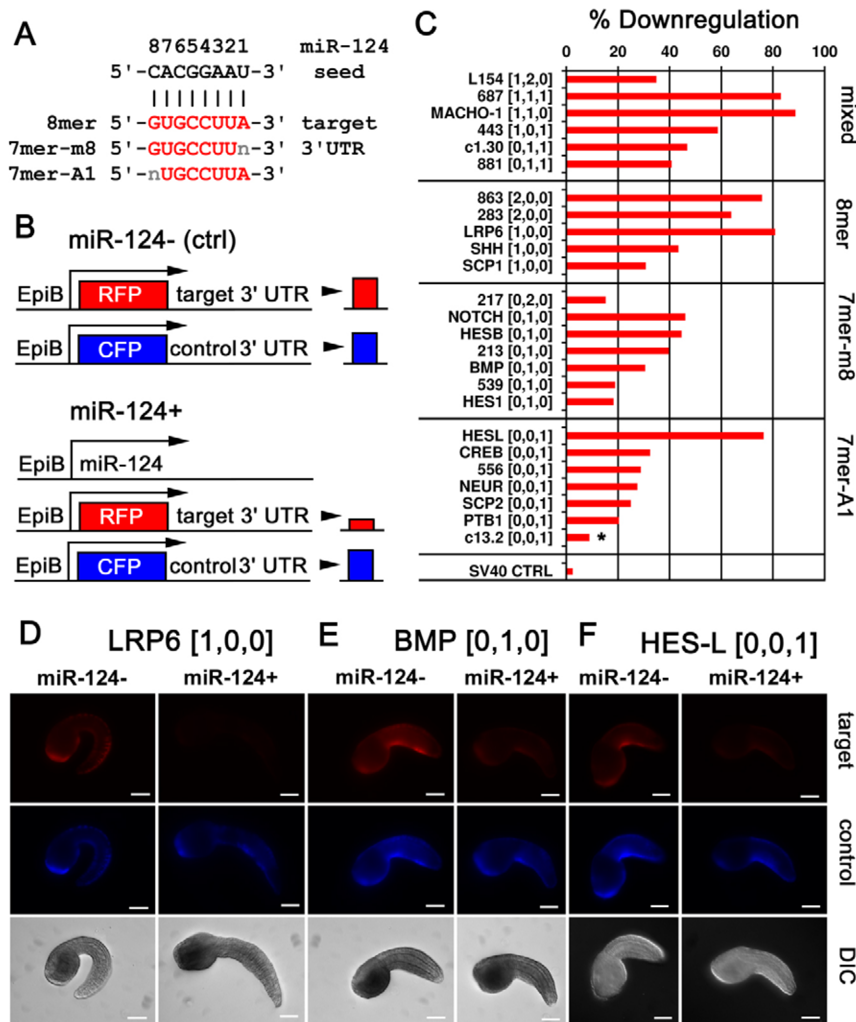

Fig. 3. The presence of a canonical seed site is sufficient for miR-124 target downregulation. (**A**) The miR-124 seed sequence (nt 1-8) putatively binds to three types of canonical site in target 3′UTRs: perfect 8mer, 7mer-m8 and 7mer-A1. (**B**) Schematic of in vivo sensor assay. All embryos express a target 3′UTR RFP sensor ('target') and a control SV40 3′UTR CFP sensor ('control'). Experimental (miR-124^{+}) embryos co-express a miR-124 construct, whereas control (miR-124^{-}) embryos do not. All constructs are driven by an epidermal tissue-specific *EpiB* promoter. RFP/CFP expression ratios are expected to decrease compared with control embryos when miR-124 is co-expressed with the reporters. In our PNS sensor assays, the *EpiB* promoter is replaced with a PNS-specific *gelsolin* promoter (not shown). (**C**) Verification of miR-124 downregulation of 25 targets with 8mer [#,0,0], 7mer-m8 [0,#,0], 7mer-A1 [0,0,#], or mixed combinations of canonical sites. [#,#,#] is a notation for indicating how many of each type of canonical site are found in a given target. Percentage downregulation is calculated based on the ratio of RFP/CFP expression in miR-124^{+} versus control embryos. All experiments were performed at least in duplicate with a mean total of 79 embryos used for quantifying percentage downregulation for each target. All targets were statistically significant at the $P<5\times10^{-6}$ level (one-paired Student's *t*-test), except for c13.2, which was significant at the $P<0.005$ level (asterisk). Full gene IDs and data are provided in supplementary material Tables S2 and S3. (**D-F**) Verification of miR-124-mediated downregulation of targets containing each type of canonical site. In each six-panel montage, a representative control embryo (miR-124^{-}) is shown in the left three panels, and a miR-124^{+} embryo is shown in the right three panels. Note that in each case target RFP expression is lower in the miR-124^{+} embryo than in the control, while control CFP expression remains relatively constant (middle row). Scale bars: 50 μm.

miR-124 canonical seed sites in the 3′UTRs of all three *Ciona* Hairy/Enhancer-of-Split genes (*Hes1*, *HesB*, *HesL*), in the only gene encoding the Notch receptor, and in *Neuralized*, a ubiquitin ligase that degrades the Notch ligand Delta. Using our epidermal sensor assay we verified significant downregulation of these targets in vivo (average 42.6% downregulation) (Fig. 4A), with *HesL* showing the greatest downregulation (76.4%) (Fig. 4B). Downregulation was abrogated upon mutation of the miR-124 seed sites of these 3′UTRs, showing that miR-124 regulates these Notch pathway genes specifically through binding their respective target sites (Fig. 4A,B). Using a PNS-specific *gelsolin* promoter we expressed each of these Notch pathway UTR sensors specifically in the ESNs where miR-124 is endogenously expressed and measured miR-124 downregulation of these sensor constructs in the PNS (Fig. 4C,D). A computational analysis of the miR-124 cis-regulatory region detected three Hes-binding N-box (CACNAG) sites and at least ten general basic helix-loop-helix transcription factor (bHLH-TF) binding E-box sites, suggesting that repression of miR-124 transcription might occur immediately downstream of Hes genes and activated Notch (supplementary material Fig. S4).

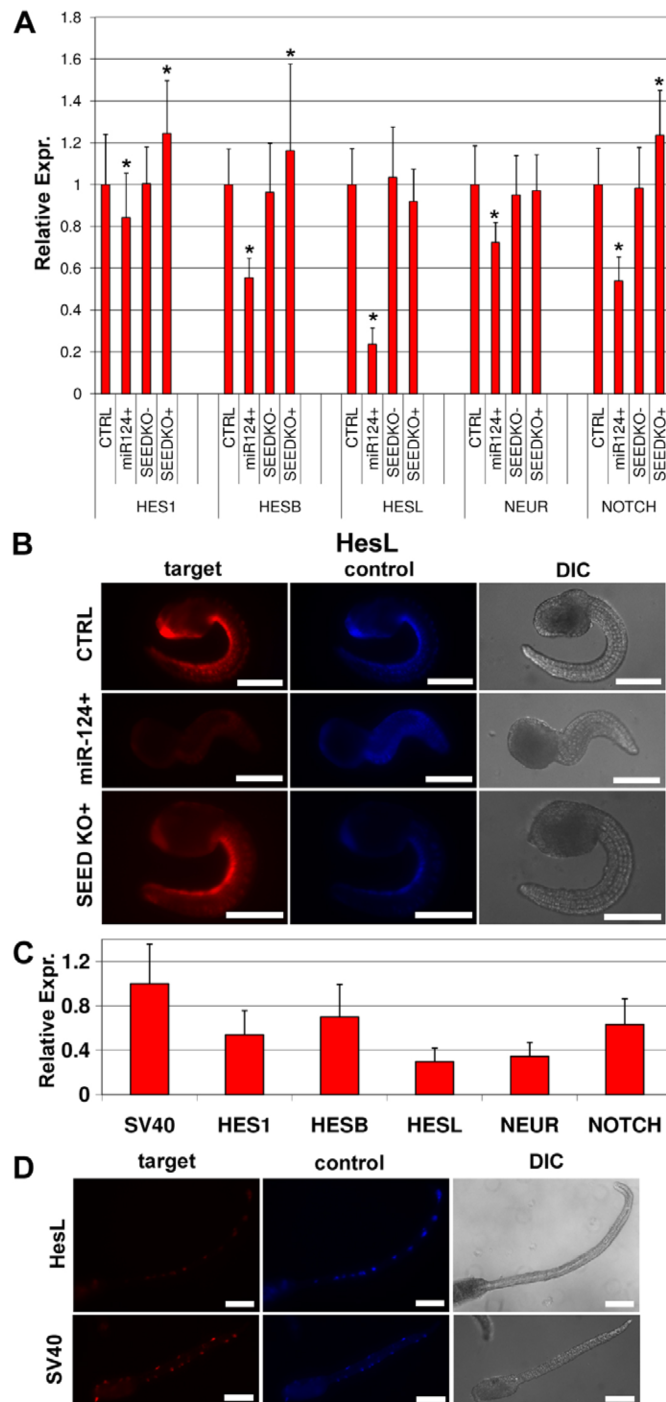

Fig. 4. Extensive miR-124-mediated regulation of Notch pathway genes in the *Ciona* PNS. Tissue-specific in vivo sensor assays in the epidermis (A,B) and PNS (C,D) demonstrate that miR-124 downregulates major Notch pathway genes. (**A**) Mean RFP/CFP expression of endogenous Notch pathway target sensors without (CTRL) and with (miR-124$^+$) epidermal miR-124 overexpression, showing significant miR-124-mediated downregulation of all five predicted targets. Expression of corresponding miR-124 seed knockout sensors without (SEEDKO$^-$) and with (SEEDKO$^+$) miR-124 overexpression show that downregulation of these targets is eliminated by mutation of the miR-124 seed site, indicating that miR-124 regulates these Notch pathway genes specifically through the seed site. *, $P<0.05$; $n \geq 60$ embryos per sensor. (**B**) Representative embryos showing downregulation of HesL sensor expression in the presence of miR-124. Target downregulation is abrogated upon mutation of the miR-124 seed site (SEED KO$^+$). (**C**) Mean RFP/CFP expression of endogenous Notch pathway target sensors specifically expressed in the PNS, normalized to a non-target SV40 sensor. Note that all Notch pathway targets are significantly downregulated in the PNS relative to the SV40 control ($P<0.05$; $n=100$ ESNs per target). (**D**) Representative embryos showing that PNS-specific HesL sensor expression is downregulated (top row) as compared with an SV40 control sensor (bottom row). Statistical significance was calculated using a one-tailed paired Student's *t*-test. Error bars indicate s.d. Scale bars: 50 μm.

Together, these results suggest that miR-124 plays a direct role in the modulation of Notch signaling activity in the PNS. Furthermore, since the targeted Notch pathway genes all function to inhibit proneural activity (Lai, 2004), miR-124 activity promotes the expression of proneural genes at least in the ESNs, consistent with its presumed role in vertebrates (Lai, 2004).

miR-124 regulates *Macho-1* and many Brachyury-regulated notochord genes

Within our predicted list of miR-124 targets, we noticed two classes of targets that are particularly interesting given what is known about early nervous system development. In ascidians, Macho-1 is a potent specifier of muscle tissue and *Macho-1* mRNA is localized to the most posterior B-line vegetal cells at the 64-cell stage (Kobayashi et al., 2003). Experiments have shown that when Macho-1 is ectopically present in the anterior embryo, the cells that would normally give rise to the CNS are instead converted into muscle (Nishida and Sawada, 2001). Here, we discovered that the *Ci-Macho-1* 3′UTR contains multiple miR-124 seed sites (Fig. 5A). Among targets tested using our epidermal sensor assay, *Macho-1* was downregulated the most (88.7%; Fig. 5B,D). We also misexpressed our *Macho-1* UTR sensor specifically in PNS tissue using a *gelsolin* promoter, to assay regulation by endogenous miR-124. Again, *Macho-1* expression was almost completely abolished (93.9% downregulation, $P=2.9\times10^{-37}$) (Fig. 5C). To determine if this effect was mediated specifically by miR-124, we repeated the assay using *Macho-1* mutant 3′UTRs with scrambled miR-124 seed sites (Fig. 5A). Mutating the seed sites restored target expression levels to ~75% of that of control embryos expressing a neutral 3′UTR reporter (Fig. 5C,D), showing that *Macho-1* downregulation is predominately regulated by miR-124. Submaximum restoration suggests that *Macho-1* could be regulated by other neuronal miRNAs, and, indeed, a putative seed sequence for miR-182 is present in the 3′UTR (supplementary material Table S7). We hypothesize that *Macho-1* is a strong miR-124 target because this would allow the nervous system to still form in the event of aberrant anterior Macho-1 mRNA localization.

Among our 867 predicted miR-124 targets we identified 50 genes that are also regulated by the notochord specifier Brachyury (Corbo et al., 1997; Takahashi et al., 1999) (supplementary material Table S8). By comparison, only 29 genes of a control set of 867 randomly selected *Ciona* genes were among the Brachyury-regulated gene set (1.72:1, signal-to-noise ratio). The fact that almost 6% of our miR-124 targets are regulated by Brachyury suggests that one of the major classes of genes silenced by *Ciona* miR-124 are notochord genes. Prior to the 64-cell stage, the notochord and A-line CNS cells share a common cell lineage.

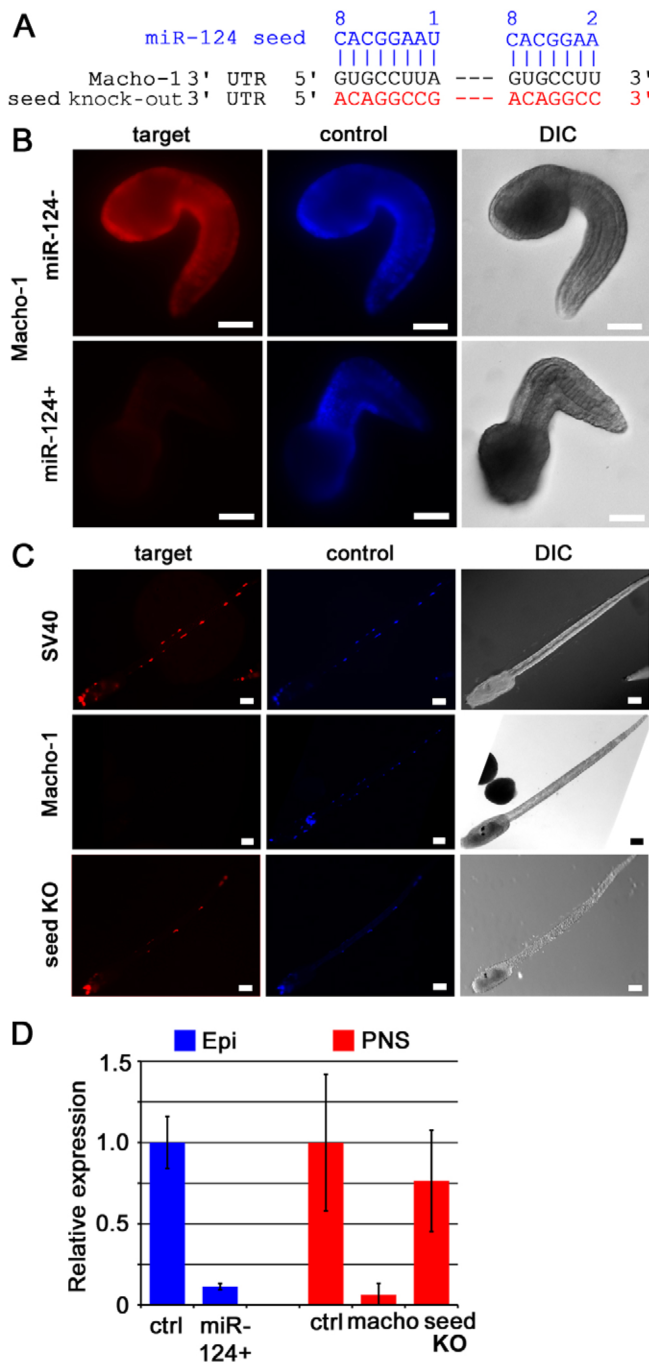

Fig. 5. miR-124 downregulates the muscle specifier *Macho-1*. (**A**) The *Macho-1* 3′UTR contains a miR-124 8mer (left) and a 7mer-m8 (right) seed site. A *Macho-1* seed knockout 3′UTR contains mutated miR-124 seed regions. (**B**) Epidermal sensor assay verifies miR-124-mediated *Macho-1* downregulation. (**C**) Activity of endogenous miR-124 using PNS-specific Macho-1 reporter. For the 'target' column, a PNS-specific RFP sensor expresses either a control SV40 3′UTR (top), *Macho-1* 3′UTR (middle) or seed mutant *Macho-1* 3′UTR (bottom). Macho-1 sensor expression is almost completely absent compared with the control (SV40), but is restored when the miR-124 seed sites are mutated (seed KO). (**D**) Epidermal (Epi) Macho-1 sensor expression with (miR-124$^+$) and without (ctrl) epidermal miR-124 expression (n=118 embryos each; P=4.8×10^{-92}). PNS target expression is shown for the endogenous miR-124 assays. Expression levels of Macho-1 (n=100 ESNs; P=2.9×10^{-37}) and seed KO (n=141; P=1.2×10^{-5}) embryos are normalized to those of SV40 control embryos (n=176). Statistical significance was calculated using a one-tailed paired Student's t-test. Error bars indicate s.d. Scale bars: 50 μm.

These two cell lineages are separated at the 64-cell stage, and this is the first time that miR-124 (*Ci-Pans*) expression is observed within neuronal lineages (supplementary material Fig. S1). Together, our *Macho-1* and notochord results suggest that, in *Ciona*, miR-124 plays a role in ensuring that the neuronal cell lineages are properly segregated and specified.

Conservation of miR-124 targeting and function in humans

Next, we determined whether miR-124 targets in *Ciona* and their annotated functions are conserved in humans. Two datasets from microarray studies (Lim et al., 2005; Karginov et al., 2007) have verified a total of 337 miR-124-downregulated genes in humans. Of our 867 *Ciona* miR-124 targets, 607 (75.3%) showed sequence homology with human peptides (NCBI build 36.3, BLASTP e-value<1×10^{-10}), consistent with the fact that ~80% of all *Ciona* genes have human homologs (Dehal et al., 2002). Of these 607 targets with human homologs, 144 (23.7%) exhibited homology with human miR-124-downregulated genes (BLASTP e-value<1×10^{-10}). Interestingly, these 144 conserved targets are highly enriched for neural-specific, cell cycle and development GO processes (Fig. 6A). In addition, we found a statistically significant number of genes involved in neural-related, cell cycle and development-related processes compared with a control set of 144 random miR-124 target genes (P<0.05, Wilcoxon sum rank test with continuity correction; Fig. 6B).

A drawback of the human miR-124 target studies is the use of microarrays in assaying targets. Although useful for target finding on a global scale, microarrays lack sensitivity and miss targets that are only minimally downregulated at the mRNA level. Thus, in order to gain a more comprehensive picture of target conservation between ascidian and human, we also analyzed conservation with all predicted canonical miR-124 human targets. Among the 2418 identified canonical human targets (supplementary material Table S6), we found homologs for 288 of our 867 ascidian targets (33.2%), 125 of which had already been found through comparison with the microarray-derived human targets (supplementary material Table S6). The other 163 targets represent canonical targets conserved between ascidian and human that were not detected in the microarray assays. When we annotated these 163 'missing' targets, however, we found that very few were involved in neural development or the cell cycle (data not shown). This suggests that the 'stronger' conserved canonical targets found through comparison with the microarray datasets are more enriched for neural developmental processes than the 'weaker' targets, which were not detected with microarrays.

Conservation and divergence of select miR-124-mediated pathways across bilaterian phylogeny

Finally, we examined the extent to which putative miR-124 regulation of select biological pathways in *Ciona* is conserved across bilaterian phylogeny. First, we discovered that few Notch

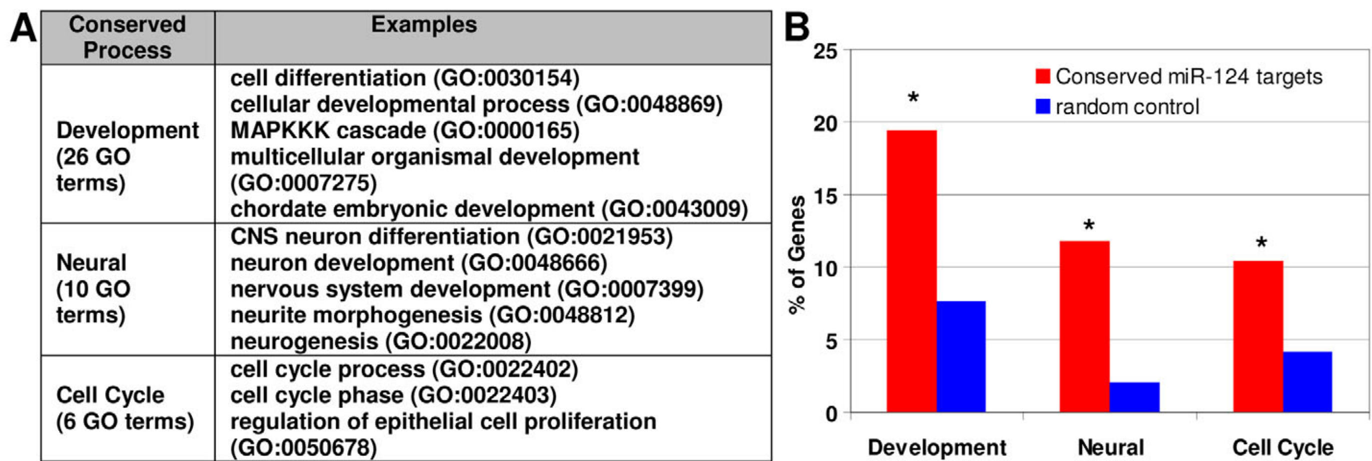

Fig. 6. *Ciona* miR-124 targets conserved in human are enriched for neuronal developmental processes. (**A**) 144 *Ciona* miR-124 targets conserved as verified human miR-124 targets (Lim et al., 2005; Karginov et al., 2007) were enriched with development, neural and cell cycle GO terms. (**B**) Conserved *Ciona* miR-124 targets have a significantly greater number of genes involved in development, neural and cell cycle (including apoptosis) processes compared with a control group of 144 random miR-124 targets. Shown is the percentage of genes out of all conserved targets involved in the respective processes. *, *P*<0.05, based on a Wilcoxon rank sum test with continuity correction.

pathway genes in other species contain miR-124 seed sites compared with *Ciona*. In vertebrates, only mouse *Hes1* has been verified as a miR-124 target (Wang et al., 2010). When we searched for canonical seed sites among five Notch genes, four Neuralized genes, and the other anti-neural Hes homologs (*Hes3*, *Hes5*) in mouse (Kageyama et al., 2007), we found a seed site in only one gene (*Hes3*) (Table 1). The only human Notch pathway gene containing a miR-124 seed sequence is *JAG1*, a Notch ligand (supplementary material Table S9). Among many other invertebrate genomes representing the span of invertebrate phylogeny, we again found only limited evidence of miR-124 targeting (Table 1; supplementary material Table S9). Thus, the extensive interplay between miR-124 and Notch signaling might be a unique evolutionary adaptation specific to *Ciona*.

Second, for *SCP1* and *PTBP1*, we found conserved miR-124 seed sequences among all representative vertebrate genomes (Table 1), in agreement with previous experimental studies (Yeo et al., 2005; Makeyev et al., 2007; Visvanathan et al., 2007). Interestingly, besides *Ciona*, no other invertebrate EST-verified *SCP1* homologs contain a miR-124 canonical seed site in their 3′UTR (Table 1), suggesting that miR-124 targeting of *SCP1* arose as a shared, derived trait in the vertebrate/tunicate ancestor. We hypothesize that miR-124-mediated regulation of *SCP1* might be an important adaptation in the evolution of the chordate nervous system. For *PTBP1*, we observed that miR-124 seed sequences are conserved among all representative bilaterian genomes except for the ecdysozoans (*C. elegans* and *D. melanogaster*), suggesting a very fundamental role of miR-124-mediated regulation of *PTBP1* in bilaterian nervous systems.

Third, for *SHH* and *GLI1*, we found conserved miR-124 targeting only in humans (Karginov et al., 2007) (Table 1). None of the other representative genomes we examined has miR-124 target sequences in the 3′UTRs of the *SHH* or *GLI1* genes, suggesting that *Ciona* might be an ideal animal model for functional studies investigating miR-124 targeting of the SHH pathway.

DISCUSSION

In *Ciona*, two copies of *miR-124* are present within the second intron of the *Ci-Pans* gene. As in other organisms (Aboobaker et al., 2005; Clark et al., 2010; Visvanathan et al., 2007), we found that miR-124 is expressed extensively in the developing CNS of ascidians. Interestingly, we also observed extensive miR-124

Table 1. Conservation and divergence of select miR-124 targets

	Notch		*Neuralized*		Hes		*SHH*		*SCP1*		*PTBP1*	
Organism	Tar	Ver	Tar	Ver	Tar	Ver	Tar	Ver	Tar	Ver	Tar	Ver
H. sapiens	×		×		×		[1,0,0]		[0,4,1]	✓	[1,0,2]	✓
M. musculus	×		×		*Hes1*, *Hes3*	*Hes1*	×		[0,5,0]	✓	[1,0,0]	✓
G. gallus	×		×		×		×		×‡	✓	[1,0,1]	
C. intestinalis	[0,1,0]	✓	[0,0,1]	✓	*Hes1*, *HesB*, *HesL*	✓	[1,0,0]	✓	[1,0,0]	✓	[0,0,1]	✓
B. floridae	×		×		×		×		×*		[0,1,0]	
S. purpuratus	×		×		×		×		×		[1,0,0]	
D. melanogaster	×		×		×		×		×		×	
C. elegans	×		×		×		n/a		×		×	
L. gigantea	×		×		*Hes13*, *Hes15*		×		×		[0,1,0]	

Conservation of Notch pathway genes (*Notch*, *Neuralized* and Hes genes), *SHH*, *SCP1* and *PTBP1* across nine organisms, listed from top to bottom in order of decreasing phylogenetic complexity. The 'Tar' column designates whether the gene was found as a canonical miR-124 target. The 'Ver' column indicates whether the gene has been experimentally verified as a miR-124 target in a particular organism. *SHH* was not found in *C. elegans*, as indicated by 'n/a'. All 3′UTRs were EST verified.
**B. floridae SCP* 3′UTRs were EST verified using the amphioxus cDNA database (Yu et al., 2008).
‡For *G. gallus SCP1* 3′UTR, a seed site was not found, although it is a verified miR-124 target (Visvanathan et al., 2007).

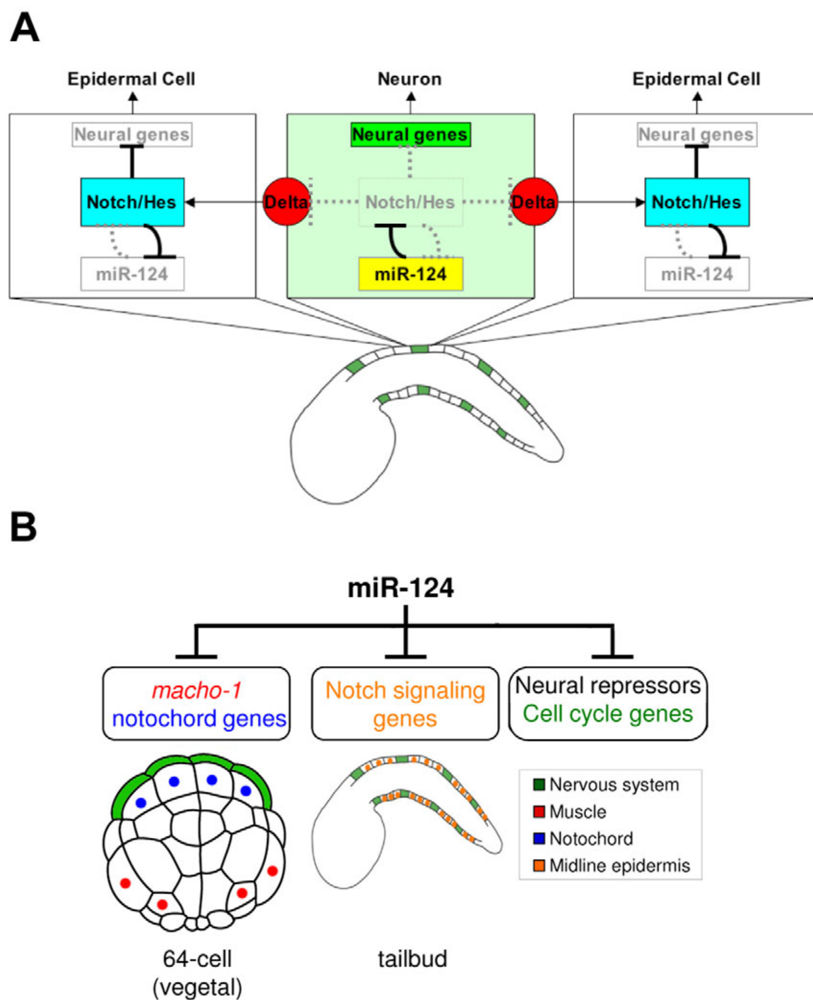

Fig. 7. Model of miR-124 role in *Ciona* neuronal development. (**A**) Model for the interaction between miR-124 and Notch signaling along the tail midlines. In ESNs, miR-124 expression is predicted to silence Notch signaling, whereas in non-ESN cells Notch activation results in the repression of miR-124 expression. If Notch signaling is blocked, ectopic ESNs expressing ectopic miR-124 are formed along the midlines. (**B**) Model for miR-124 function based on the expression pattern of miR-124 (cells shaded green) and the characterization of identified miR-124 target genes in *Ciona*. Relevant tissues and associated pathways are color coded. Note that nervous system expression is more extensive than shown (only neuronal cells relevant to targeted pathways are shaded green). Notochord genes refer to those genes that are modulated by the notochord-specifier Brachyury. The right-hand list includes targeted genes and pathways that cannot confidently be assigned to a particular embryo stage.

expression in the PNS, which has only been reported in two other organisms studied to date: *C. elegans* and *Aplysia* (Clark et al., 2010; Rajasethupathy, 2009). We used in situ hybridization probes that were specific to (1) the intron containing the two *miR-124* copies, (2) the mature *Ci-Pans* mRNA and (3) the mature miR-124 product. Our results in later embryos (Fig. 1) demonstrate that all three probes report identical expression patterns. In early embryos we were unable to detect specific signals with the LNA probe targeting the mature miR-124 sequence, although expression of the *Ci-Pans* mRNA and the miR-124 primary transcript are clearly observed (supplementary material Fig. S1). Since mature miR-124 read counts are low at the cleavage and gastrula stages (Shi et al., 2009; Hendrix et al., 2010), we suspect that the LNA probe is not sensitive enough to detect the mature miR-124 product in these embryos using the detection method that we employed.

We computationally identified over 800 predicted canonical targets of *Ciona* miR-124 and, using an in vivo tissue-specific sensor assay to verify several weak and strong targets from each type of canonical seed site, deduced that these are in fact bona fide targets. Our analysis differs from previous miR-124 target studies (Cheng et al., 2009; Clark et al., 2010; Karginov et al., 2007; Lim et al., 2005; Makeyev et al., 2007; Visvanathan et al., 2007) in that we established an expression pattern and phenotype for miR-124 in the whole animal, verified direct targeting of representative genes from important targeted processes in vivo, investigated the functions of our targets in the context of neuronal development and provided evolutionary insights into the conservation and divergence of several miR-124 targets. Of particular interest is an apparent interaction between miR-124 and Notch signaling along the tail midlines. Ectopic expression of miR-124 in the epidermis (Fig. 2G-I) produces ectopic midline ESNs reminiscent of the phenotype produced when Notch signaling is blocked by the epidermal expression of a dominant-negative form of Su(H) (Fig. 2J,K). In embryos in which Notch signaling is blocked, ectopic miR-124 expression is observed (Fig. 2L), whereas miR-124 expression is lost in embryos with ectopically activated Notch (Fig. 2O). These results suggest that miR-124 expression in the tail ESNs is regulated by Notch signaling. Five Notch pathway genes (*Notch*, *Neuralized* and the three *Ciona* Hes genes) are targets of *Ciona* miR-124. When tested in our in vivo assays, all five Notch pathway gene sensors were significantly and specifically downregulated by miR-124 (Fig. 4). We hypothesize that miR-124 functions within the ESNs to silence Notch signaling, thus ensuring that these cells will produce the sensory neurons of the larval PNS. We present a model of this interaction in Fig. 7A. Furthermore, our conservation analysis suggests that the extensive interaction between miR-124 and Notch signaling might be unique to ascidians. Interestingly, miR-9 in *Drosophila* appears to regulate Notch signaling in a somewhat analogous fashion (Herranz and Cohen, 2010), suggesting that different organisms have evolved to use different miRNAs to perform similar regulatory activities of Notch signaling.

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Of all the miR-124 targets tested in vivo, the most downregulated gene was *Macho-1*, a maternally expressed muscle determinant, the mRNA of which is normally sequestered to the posterior cells of the embryo (Nishida and Sawada, 2001). When Macho-1 is overexpressed in the anterior of the ascidian embryo, the cells that normally give rise to the CNS are instead converted into muscle (Nishida and Sawada, 2001). In normal embryos, miR-124 expression in the neuronal lineages may function to robustly inhibit Macho-1 translation, should any of these transcripts mislocalize to the anterior regions of the embryo. Interestingly, *Ci-Pans* also appears to be briefly expressed in mesenchyme and mesoderm lineage precursors in the early gastrula (Fujiwara et al., 2002) (supplementary material Fig. S1), which are also converted into muscle upon ectopic Macho-1 expression (Nishida and Sawada, 2001; Fujiwara et al., 2002; Alfano et al., 2007). miR-124-mediated *Macho-1* repression in the mesenchyme lineage (B7.7) might likewise prevent aberrant muscle specification in these cells. Lastly, *Ci-Pans* is also briefly expressed in the three muscle lineage precursors (B8.7, B8.8 and B8.15) (Fujiwara et al., 2002), where miR-124 might also play a role in modulating Macho-1 expression levels. Mesodermal expression of miR-124 has not been reported in any other organisms to date.

In ascidians, the notochord and A-lineage CNS cells are sister cell lineages. The Brachyury transcription factor is first expressed in notochord cells at the 64-cell stage, after these cells have separated from the A-lineage neuronal precursors (Corbo et al., 1997), and subsequently specifies notochord cell fate (Takahashi et al., 1999). It is at this stage that miR-124 expression is first observed within the CNS lineages (supplementary material Fig. S1). Almost 6% of our miR-124 targets are Brachyury-regulated notochord genes. This is about twice the number of notochord genes if selected at random. The high incidence of notochord genes as putative miR-124 targets suggests that one role of miR-124 might be to ensure that notochord genes are silenced within the developing CNS.

From our overall analysis of putative and tested miR-124 targets we can derive a model for the activity of this miRNA in the developing ascidian embryo (Fig. 7B). In ascidians, miR-124 probably regulates a number of biological pathways that are also targets of miR-124 in other organisms. One factor that interferes with neuronal differentiation is the NRSF-REST neuronal repressor complex (Visvanathan et al., 2007; Yeo et al., 2005), which silences neuronal genes in non-neuronal cells. In vertebrates, miR-124 negatively regulates this complex via the SCP1 protein, and *SCP1* is also a target of miR-124 in *Ciona*. A second conserved target is PTBP1, which represses brain-specific alternative pre-mRNA splicing in vertebrates (Makeyev et al., 2007). Genes that regulate the cell cycle in postmitotic neurons are also common targets of miR-124. In *Ciona*, miR-124 uniquely targets the maternal muscle specifier *Macho-1* and significant numbers of notochord-expressed genes. miR-124 also targets genes that encode signaling pathway components important for neuronal specification in *Ciona*, including the FGF pathway (Akanuma and Nishida, 2004; Bertrand et al., 2003) and the SHH pathway (Fuccillo et al., 2006; Takatori et al., 2002). Lastly, *Ciona* miR-124 targets five genes of the Notch pathway, suggesting that regulation of Notch signaling by miR-124 is an important function of this miRNA.

We note that there might be other classes of miRNA 3′UTR targets, such as miRstar (Okamura et al., 2008), 3′ compensatory (Bartel, 2009) and center site (Shin et al., 2010) targets. Recent studies have also demonstrated the presence of functional miRNA target sites in coding regions (Baek et al., 2008; Zisoulis et al., 2010). Although, in general, these appear to play a lesser role in miRNA-mediated regulation (Baek et al., 2008; Okamura et al., 2008; Bartel, 2009; Shin et al., 2010), we cannot exclude the possibility of functionally relevant non-canonical miR-124 targets. Nonetheless, our comprehensive characterization of canonical miR-124 targets in *Ciona* and their conservation in other organisms should provide a foundation for future studies on miR-124.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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