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RESEARCH ARTICLE

Prevention of medulla neuron dedifferentiation by Nerfin-1 requires inhibition of Notch activity

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ABSTRACT

The *Drosophila* larval central nervous system comprises the central brain, ventral nerve cord and optic lobe. In these regions, neuroblasts (NBs) divide asymmetrically to self-renew and generate differentiated neurons or glia. To date, mechanisms of preventing neuron dedifferentiation are still unclear, especially in the optic lobe. Here, we show that the zinc-finger transcription factor Nerfin-1 is expressed in early-stage medulla neurons and is essential for maintaining their differentiation. Loss of Nerfin-1 activates Notch signaling, which promotes neuron-to-NB reversion. Repressing Notch signaling largely rescues dedifferentiation in *nerfin-1* mutant clones. Thus, we conclude that Nerfin-1 represses Notch activity in medulla neurons and prevents them from dedifferentiation.

KEY WORDS: Nerfin-1, Medulla neurons, Dedifferentiation, Notch signaling

INTRODUCTION

Drosophila larval central nervous system (CNS) comprises central brain (CB), ventral nerve cord (VNC) and optic lobe (OL) (Fig. 1A). In contrast to the rest of the nervous system, which develops from neuroectoderm (Hartenstein et al., 2008), *Drosophila* optic lobe originates from a cluster of epithelial cells that invaginate from the posterior procephalic region of the embryonic head (Green et al., 1993). These neuroepithelial (NE) cells, also named the optic lobe placode, develop on both sides of the brain and then grow into the lateral half of the two brain hemispheres at the end of larval development (Green et al., 1993) (Fig. 1A). Later, they give rise to the lamina, medulla, lobula and lobula plate, which play important roles in the visual system at adult stage (Fischbach and Dittrich, 1989; Hofbauer and Camposortega, 1990). During development, the optic placode gradually separates into two parts: the inner optic anlagen (IOA) and the outer optic anlagen (OOA) (Green et al., 1993; Hofbauer and Camposortega, 1990; Nassif et al., 2003). At third instar larval stage, NE cells of lateral OOA differentiate into lamina neurons after receiving Hedgehog signals from retinal axons (Huang and Kunes, 1996; Kunes, 2000), while NE cells of medial OOA differentiate into neuroblasts under the control of multiple signaling pathways, such as Notch, Hippo, JAK/STAT and EGFR (Egger et al., 2010; Kawamori et al., 2011; Orihara-Ono et al., 2011; Reddy et al., 2010; Yasugi et al., 2010; Yasugi et al., 2008). Such NBs then divide asymmetrically and perpendicularly to the surface, producing a self-renewed NB and a ganglion mother cell (GMC), which further divides into two medulla neurons, pushing the older ones inward (Egger et al., 2007; Nassif et al., 2003; Toriya et al., 2006; Yasugi et al., 2008) (Fig. 1B). Based on these sequential events, it is then possible to judge the age of these neurons by their spatial location.

In *Drosophila* CNS, the mechanism of medulla neuron dedifferentiation remains unexplored. To date, only Lola, a BTB zinc-finger transcription factor, has been reported to maintain the differentiation of medulla neurons in the optic lobe (Southall et al., 2014). Lola functions as a co-factor of Prospero (Pros) and prevents neuron-to-NB reversion by repressing the activity of NB and cell-cycle genes in post-mitotic neurons. Interestingly, loss of Lola induces neuron-to-NB reversion only in medulla neurons, implicating differential mechanisms that inhibit dedifferentiation in the optic lobe and the rest of the CNS. Based on these observations, it is therefore crucial to study the mechanism of medulla neuron dedifferentiation.

Here, we show that the zinc-finger transcription factor Nervous fingers 1 (Nerfin-1) is expressed in early-stage medulla neurons and is essential for maintaining their differentiated state. Loss of Nerfin-1 activates Notch signaling in medulla neurons, which promotes neuron dedifferentiation. Inhibition of Notch activity largely blocks dedifferentiation caused by Nerfin-1 depletion and prevents tumorigenesis.

RESULTS

Nerfin-1 is expressed mainly in early-stage medulla neurons

It is known that the Hippo pathway plays an important role in the optic lobe (Kawamori et al., 2011; Reddy et al., 2010). As Nerfin-1 is a possible interacting partner of the Hippo effectors Scalloped and Yorkie (Feng et al., 2013; Rhee et al., 2014), we sought to determine whether Nerfin-1 functions in the optic lobe. Throughout the analysis, two cross-sections of optic lobe were analyzed (Fig. 1A,C-D′). Layer 1 is the equatorial plane of the brain hemisphere, in which NB lineages are seen distinctively and the order of neuron formation can be determined by their spatial position. Layer 2 is between the ventral surface and the equatorial plane, in which medulla neurons make up the majority of the optic lobe.

To analyze the Nerfin-1 expression pattern, antibodies against Nerfin-1 were generated and used to co-stain the optic lobe with Pros and Embryonic lethal abnormal vision (Elav), which mark the GMC and medulla neurons, respectively. Our results suggested that Nerfin-1 is mainly expressed in differentiated neurons, as its

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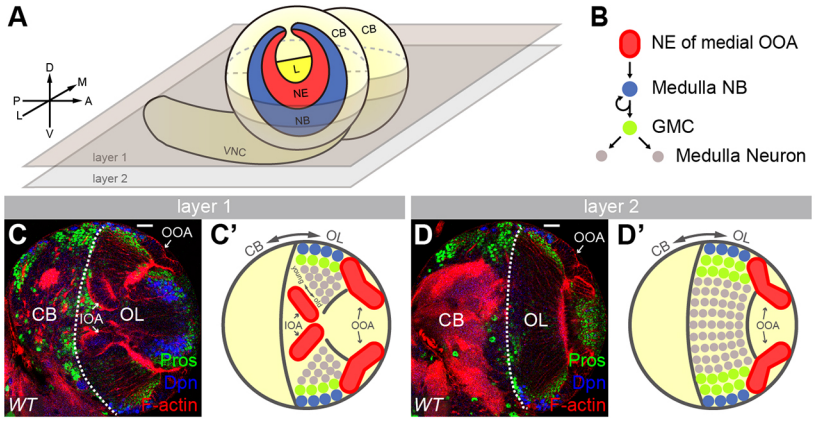

Fig. 1. *Drosophila* larval optic lobe anatomy. (A) Schematic of the larval CNS. NE cells (red), medulla NBs (blue) and lamina (L, yellow) in the optic lobe are shown. The gray planes show the cross-sections scanned in this study. CB, central brain; VNC, ventral nerve cord. (B) Schematic depicting neurogenesis in the medulla cortex. (C-D′) Confocal (C,D) and schematics (C′,D′) of two cross-sections of larval brain correspond to layer 1 (C,C′) and layer 2 (D,D′) in A. Dashed lines represent the CB-OL boundary. Pros and Dpn mark GMC and NBs, respectively. F-actin draws the outline. OL, optic lobe; IOA, inner optic anlagen; OOA, outer optic anlagen. Scale bars: 20 µm.

expression overlaps extensively with Elav, but not with Pros (Fig. 2A-A″′). This observation was further confirmed using Nerfin-1-GFP flies, which express GFP under the *nerfin-1* promoter (Kuzin et al., 2007) (Fig. 2B-B″′). Interestingly, Nerfin-1 protein levels decreased in older neurons, whereas Nerfin-1-GFP levels remained similar (Fig. 2A″,B″), implicating a post-translational regulation of *nerfin-1* during the development of medulla neurons. Collectively, our results indicate that Nerfin-1 is expressed mainly in early-stage medulla neurons.

Nerfin-1 absence leads to ectopic NBs in the optic lobe

To examine Nerfin-1 function, flip-out clones expressing Nerfin-1 RNAi transgenes were generated. Interestingly, in these clones large numbers of cells expressed Deadpan (Dpn), a neuroblast marker, despite being located where post-mitotic neurons should be (Fig. S1A-D). Efficiencies of both Nerfin-1 RNAi lines were confirmed with the Nerfin-1 antibody (Fig. S1E-F′). To validate our observation, clones of the null allele *nerfin-1*^{159} were generated using mosaic analysis with a repressible cell marker (MARCM) system (Lee and Luo, 2001) (Fig. S1G,G′). Consistent with Nerfin-1 RNAi, a great number of Dpn^{+} cells were detected in *nerfin-1* mutant clones in the medulla cortex (Fig. 3A-C). To determine whether these cells were *bone fide* ectopic NBs, additional NB markers such as Asense (Ase) and Miranda (Mira) were used to examine the optic lobe, and similar upregulation was detected (Fig. 3D-F). Furthermore, these NB-like cells were pH3 positive, exhibited proliferation potential (Fig. 3G-I) and formed brain tumors at the adult stage (Fig. 3J). Taken together, we conclude that ectopic NBs are induced in the optic lobe in the absence of Nerfin-1.

Nerfin-1 maintains the differentiation of medulla neurons

To explore the origin of these ectopic NBs, time-course experiments were carried out using the MARCM system. As clones over 24 h old always contained multiple NB lineages (Fig. S2), quantification could only be carried out before 24 h. In *nerfin-1*^{159} clones under 24 h old, only one original NB (Dpn^{+}/Ase^{+}/Mira^{+}) was detected (n>15) (Fig. 4A, Fig. S2). In addition, Dpn expression was switched off normally in GMCs (Dpn^{-}/Ase^{+}/Mira^{+}) (Fig. 4A) and the number of GMCs remained similar (Fig. 4B). Furthermore, medulla neurons (Dpn^{-}/Ase^{-}/Mira^{-}) were generated normally at 16 h, with Ase and Mira expression suppressed (Fig. 4Ad-d″, Fig. S2). However, they began to dedifferentiate soon, as weak Dpn staining was detected in several clones (Fig. 4Ae-e″). On the other hand, ectopic Mira and Ase expression appeared 20 h later (Fig. S2). Taken together, we conclude that the absence of Nerfin-1 does not affect NB and GMC, thus neurons can be generated but they begin to dedifferentiate at a very early stage. Consistently, Elav was detected in young neurons, but not in ectopic Dpn^{+} cells that were generated from dedifferentiation of old neurons (Fig. 4C-D′). Protein level changes in *nerfin-1*^{159} lineages are summarized in Fig. 4E.

To further validate our conclusion, flip-out clones of Nerfin-1 RNAi were induced 96 h after larvae hatched (ALH). Consistent with our expectation, Dpn^{+} cells were detected in clones induced in post-mitotic neurons far below the surface (Fig. 4F,F′). Furthermore, *elav-Gal4*, a pan-neuronal Gal4, was used to express Nerfin-1 RNAi in medulla neurons. However, *elav-Gal4* is not strictly expressed in neurons only. Thus, a temperature-sensitive Gal80 protein (Gal80^{ts}) approach was used to delay the expression. If Nerfin-1 depletion does not function in neurons, those generated

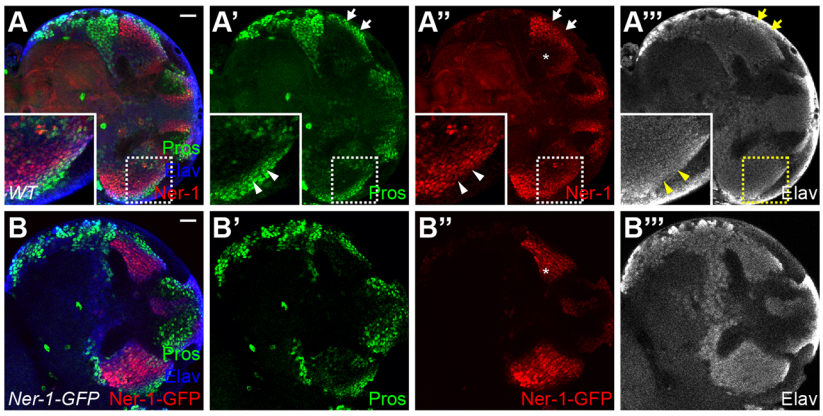

Fig. 2. *nerfin-1* expression in medulla neurons. (A-A″′) Representative larval brains showing Nerfin-1, Pros and Elav staining. Insets are magnifications of boxed regions. Arrowheads and arrows indicate the edge of neurons and GMCs, respectively. Asterisk in A″ indicates old neurons with low expression level of Nerfin-1. (B-B″′) Representative larval brains showing Nerfin-1-GFP, Pros and Elav staining. Asterisk in B″ indicates old neurons. Scale bars: 20 µm.

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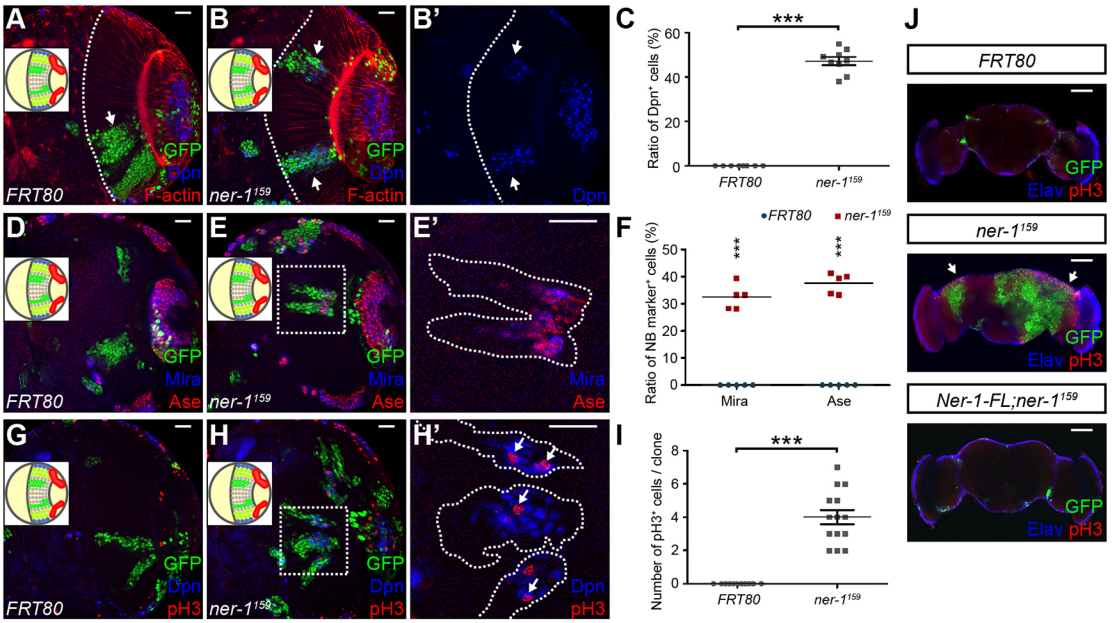

Fig. 3. Nerfin-1 depletion results in ectopic NBs and tumorigenesis. (A-B′) Nerfin-1 depletion induces ectopic Dpn$^+$ cells in the medulla cortex. Arrows show the clones. Dashed lines represent the CB-OL boundary. Insets show clone regions schematically. (C) Quantification of the ratio of Dpn$^+$ cells in clones from A,B (n=8,9, respectively). Data are mean±s.e.m.; ***P<0.001. (D-E′) Nerfin-1 depletion induces ectopic Mira$^+$ and Ase$^+$ cells in the medulla cortex. Insets show clone regions schematically. (E′) Magnification of boxed region in E with clone outlined. (F) Quantification of the ratio of Mira$^+$ or Ase$^+$ cells in clones from D,E (n=5 for each). Data are mean±s.e.m.; ***P<0.001. (G-H′) Ectopic Dpn$^+$ cells are able to proliferate. pH3 labels cells undergoing mitosis. Insets show clone regions schematically. (H′) Magnification of boxed region in H with clones outlined. Arrows indicate pH3$^+$/Dpn$^+$ cells. (I) Quantification of the number of pH3$^+$ cells in clones from G,H (n=11,14, respectively). Data are mean±s.e.m.; ***P<0.001. (J) Nerfin-1 depletion induces tumors in adult brain, which is rescued by expression of full-length Nerfin-1. Arrows indicate the tumors. Scale bars: 100 µm in J; 20µm in A-B′,D-E′,G-H′.

before Nerfin-1 RNAi misexpression should remain differentiated and ectopic NBs should be found only in the superficial layer. Interestingly, ectopic NBs were detected among neurons at different stages (Fig. 4G-I), suggesting that Nerfin-1 is essential for the medulla neurons to maintain their differentiation.

All three zinc fingers contribute to Nerfin-1 function

Nerfin-1 contains three C2H2-type zinc-finger domains considered to bind DNA (Fig. S3A). To test their function, we generated transgenic flies carrying Nerfin-1 truncations with a single zinc-finger deletion (Nerfin-1-dZF1/2/3). As shown in Fig. S3B-C″,G, expression of full-length Nerfin-1 dramatically decreased the number of ectopic NBs in *nerfin-1^{159}* clones. On the other hand, deletion of the first or the second zinc finger alone was sufficient to disrupt Nerfin-1 activity (Fig. S3D-E″,G). Nerfin-1-dZF3 only partially inhibited the dedifferentiation (Fig. S3F,G). Taken together, all three zinc fingers contribute to Nerfin-1 activity, whereas the first two play a more major role.

Nerfin-1 represses Notch signaling and prevents dedifferentiation

As Dpn derepression happens earlier than Ase and Mira in *nerfin-1^{159}* clones (Fig. 4A,E, Fig. S2), we speculated that mechanisms other than direct transcriptional regulation might be involved. To investigate further, activity of Hippo, JAK/STAT, EGFR and JNK signaling was tested upon losing Nerfin-1. None of these signaling activities was obviously altered (Fig. S4). However, Nerfin-1 loss caused dramatic upregulation of Notch protein level (Fig. 5A,A′) and expression of Notch reporters, E(spl)mγ-GFP and Su(H)$_{m8}$-lacZ (Fig. 5B-D), suggesting that the Notch pathway might be involved in Nerfin-1-mediated induction of ectopic NBs.

We then analyzed the effect of Notch hyperactivation in the optic lobe. We misexpressed Fringe (Fng), an enhancer of Delta-Notch signaling (Panin et al., 1997), using *elav-Gal4*. As expected, ectopic NBs were detected in medulla cortex (Fig. 5E,F). However, considering the non-specificity of *elav-Gal4* and that Notch signaling promotes NB self-renewal (Bowman et al., 2008; Wang et al., 2006), it is difficult to determine the origin of these ectopic NBs. To confirm the function of Notch hyperactivation in medulla neurons specifically, we misexpressed Notch intracellular domain (NICD), the constitutively active form of the Notch receptor, with *elav-Gal4* and used Gal80^{ts} to control the expression. Consistent with Nerfin-1 RNAi, NICD misexpression induced ectopic NBs among neurons at different stages (Fig. 5G,H). Furthermore, flip-out clones of NICD were induced 96 h ALH. Dpn$^+$ cells were found in separate clones induced in post-mitotic neurons far below the surface (Fig. 5I,I′). The same phenotype was detected when the expression of Numb, an inhibitor of Notch signaling, was inhibited (Fig. 5J,J′). Taken together, the Notch pathway potentially participates in the dedifferentiation caused by Nerfin-1 absence and promotes the neuron-to-NB reversion.

Inhibition of Notch signaling rescues Nerfin-1-mediated dedifferentiation

Compared with neurons, activity of Notch signaling is higher in NBs (arrowheads, Fig. 5A′,B′,C′), so it is unclear whether Notch pathway hyperactivation is a cause or a consequence of dedifferentiation. To examine this, we knocked down the expression of the Notch receptor or of Suppressor of Hairless [Su(H)], the transcription factor of the Notch pathway (Fortini and Artavanis-Tsakonas, 1994), in the absence of Nerfin-1. As

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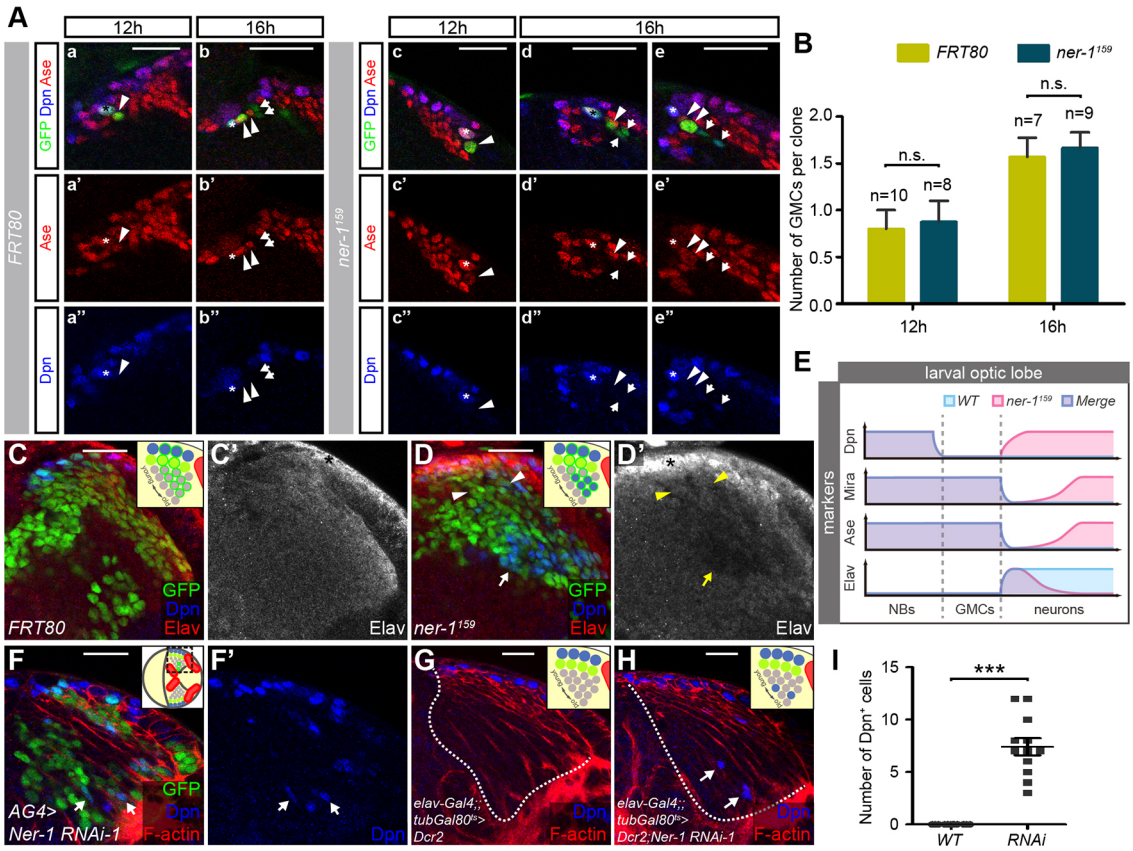

Fig. 4. Nerfin-1 absence results in dedifferentiation of medulla neurons. (A) Time-course experiment of control and *nerfin-1*^{159}. Representative NB lineages labeled by GFP are shown. NBs, GMCs and neurons are indicated by asterisks, arrowheads and arrows, respectively. (B) Quantification of the number of GMCs in clones from A. Data are mean±s.e.m.; n.s., not significant. (C-D′) Representative clones of control (C,C′) and *nerfin-1*^{159} (D,D′), showing Dpn and Elav staining. Asterisks in C′,D′ indicate nonspecific signals of Elav antibody. Arrows indicate Elav^{-} cells. Arrowheads indicate Elav^{+} cells. Insets show clone regions schematically. (E) The temporal expression of Dpn, Mira, Ase and Elav during medulla neuronal differentiation. (F,F′) Ectopic NBs are detected in flip-out clones of Nerfin-1 RNAi. Arrows indicate the clones. Insets show clone regions schematically. (G,H) Nerfin-1 knockdown leads to ectopic NBs in the medulla cortex. Dicer2 (Dcr2) is used to enhance the function of RNAi. Dashed lines represent the edge of medulla neurons. Insets show the medulla cortex schematically. Arrows in H show ectopic NBs among neurons of late stage. (I) Quantification of the number of Dpn^{+} cells according to G,H (*n*=12 for each). Data are mean±s.e.m.; ***P<0.001. Scale bars: 20 µm.

shown in Fig. 6A-H and Fig. S5A-D, knockdown of Notch or Su(H) significantly reduced the number of ectopic NBs induced by Nerfin-1 depletion. At the adult stage, Notch knockdown prevented the formation of *nerfin-1*^{159} tumors extensively (Fig. 6I-J′). To confirm these results, we carried out immunostaining analysis and found that upregulation of Notch receptor and E(spl)mγ-GFP appeared more penetrant than Dpn in *nerfin-1*^{159} clones (Fig. 6K-L″, Fig. S5E-E″). These results suggested that hyperactivation of Notch signaling is a cause rather than a consequence of dedifferentiation.

As Nerfin-1 is mainly expressed in differentiated cells, Notch signaling occurs earlier than Nerfin-1 both spatially and temporally. To examine this, we tested whether Notch inhibition affects the generation of NB lineage or Nerfin-1 expression. As shown in Fig. 6F′, Nerfin-1 exhibited a normal expression level when Notch was knocked down. Next, a time-course experiment was performed (Fig. S6A). In Notch RNAi clones under 24 h old (n>5 for each), only one original NB was detected and the number of GMCs remained similar compared with the control (Fig. S6B), indicating that NB lineage is generated normally upon Notch depletion. Taken together, Nerfin-1 loss of function induces hyperactivation of Notch signaling, which promotes the dedifferentiation of post-mitotic medulla neurons.

Medulla neurons are both the donor and acceptor of the Notch signal

To explore whether Notch signaling is constitutively activated when Nerfin-1 is absent, expression of Delta, a ligand of Notch signaling, was knocked down in *nerfin-1*^{159} clones. Interestingly, dedifferentiation caused by Nerfin-1 loss was dramatically suppressed (Fig. 7), indicating that Notch signaling is not constitutively activated, and requires a ligand for its activation. In addition, medulla neurons are both the donor and acceptor of Notch signal.

Medulla neuron dedifferentiation caused by Nerfin-1 loss is independent of Myc or Tor

A recent study suggests that Nerfin-1 maintains neuron differentiation in both central brains and VNCs (Froldi et al., 2015). In that study, Myc and Target of rapamycin (Tor) were reported to be necessary for the dedifferentiation caused by Nerfin-1 loss. In our present study, however, neither Myc knockdown nor Tor-DN (dominant negative) misexpression inhibited dedifferentiation of medulla neurons in the optic lobe (Fig. S7), indicating the presence of differential regulatory mechanisms between the optic lobe and the rest of the CNS.

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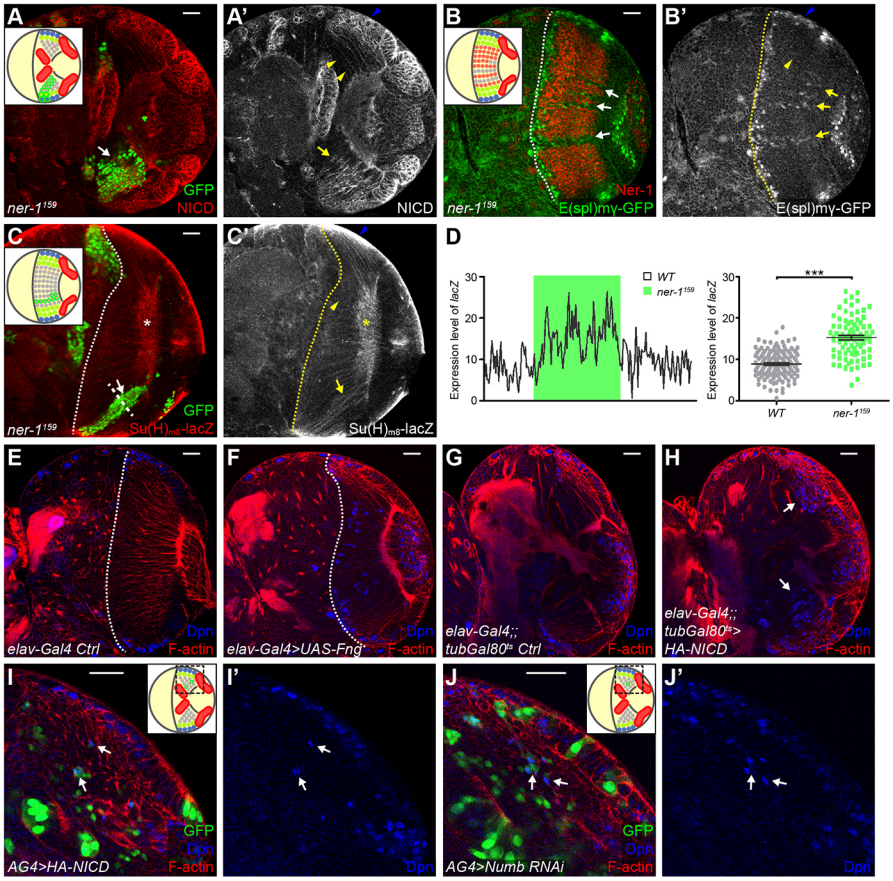

Fig. 5. Notch activity is upregulated in *nerfin-1*^{159} clones and hyperactivation of Notch signaling leads to ectopic NBs. (A-C′) Expression levels of Notch receptor (A,A′), E(spl)mγ-GFP (B,B′) and Su(H)$_{m8}$-lacZ (C,C′) are upregulated in *nerfin-1*^{159} clones. Arrows indicate *nerfin-1*^{159} clones. Blue and yellow arrowheads indicate normal NBs and neurons, respectively. Dashed lines represent the CB-OL boundary. Insets show clone regions schematically. Clones in B are labeled by loss of Nerfin-1. Asterisks in C,C′ indicate nonspecific signals of β-galactosidase antibody.
(D) Quantification of *lacZ* expression in the region marked by a thick dashed line in C. Data are mean± s.e.m.; ***P<0.001. (E-H) Misexpression of Fringe (E,F) and NICD (G,H) leads to ectopic NBs in the medulla cortex. Dashed lines represent the CB-OL boundary. Arrows in H show ectopic NBs among neurons of late stage. (I-J′) Ectopic NBs are detected in flip-out clones of NICD (I,I′) and Numb RNAi (J,J′). Clones were induced at 96 h ALH. Arrows indicate the clones. Insets show clone regions schematically. Scale bars: 20 µm.

DISCUSSION

Nerfin-1 exhibits conserved function in maintaining neuron differentiation in *Drosophila* larval CNS

Stem cells generate progeny that undergo terminal differentiation. In *Drosophila* CNS, the balance of self-renewal and differentiation of neural stem and progenitor cells is a central issue during development. On the other hand, the maintenance of differentiated status of post-mitotic neurons is also crucial for tissue function and homeostasis. It is obvious that mechanisms must exist to prevent the cells from dedifferentiation. Although proteins that function to keep differentiation have been well studied in other cell types (Bello et al., 2006; Betschinger et al., 2006; Eroglu et al., 2014; Koe et al., 2014; Lee et al., 2006; Wang et al., 2007; Weng et al. 2010; Zhang et al., 2016), few have been implicated in post-mitotic neuronal maintenance. In the central brain, loss of Midlife crisis (Mdlc), a CCCH zinc-finger protein, results in a decrease in Pros, thus derepressing NB genes in neurons (Carney et al., 2013). However, it is insufficient to make neurons revert to proliferating NBs. Furthermore, as Pros is not expressed in medulla neurons, it is unclear whether Mdlc has the same function in the optic lobe. On the other hand, absence of Lola leads to neuron-to-NB reversion and tumorigenesis (Southall et al., 2014), but it is crucial for neuronal maintenance only in the optic lobe. Recently, a paper reported that Nerfin-1 loss induces neuron dedifferentiation in both central brain and VNC (Froldi et al., 2015). In the present study, we demonstrate a conserved function for Nerfin-1 in medulla neurons in the optic lobe. Our findings indicate that Nerfin-1 is expressed mainly in early-stage medulla neurons and functions to maintain their differentiated state.

Differential mechanisms of neuronal maintenance between the optic lobe and the rest of the CNS

Interestingly, we noticed that ectopic NB induced by Nerfin-1 depletion in the optic lobe appeared much earlier than that in the central brain. Considering that Lola loss causes dedifferentiation just in the optic lobe (Southall et al., 2014), we speculate that the differentiated state of medulla neurons is less stable, possibly owing to absence of Pros. Furthermore, different from the mechanism in the central brain, the function of Nerfin-1 in the optic lobe requires the silencing of Notch signaling. Neither Myc knockdown nor Tor-DN misexpression inhibits dedifferentiation caused by Nerfin-1 loss in the medulla neurons (Fig. S7). Thus, our findings identify a distinct regulatory mechanism in medulla neurons and validate different regulatory modes between the optic lobe and the rest of the CNS.

Cyclin E expression is not affected directly by Nerfin-1 to maintain medulla neuron differentiation

On the other hand, cell cycle genes play important roles in cell differentiation. Among them, Cyclin E (CycE) is reported to be regulated directly by Lola-N (Southall et al., 2014) and is involved in the neuron dedifferentiation caused by loss of Mdlc (Carney et al., 2013). Thus, we also examined whether CycE is regulated directly by Nerfin-1 and controls cell differentiation independently of Notch and neuroblast genes. Interestingly, CycE expression levels were upregulated dramatically in *nerfin-1*^{159} clones, but such upregulation was mostly blocked by Notch repression (data not shown). These results suggest that CycE is not a direct target of Nerfin-1 for maintaining medulla neuron differentiation. CycE acts

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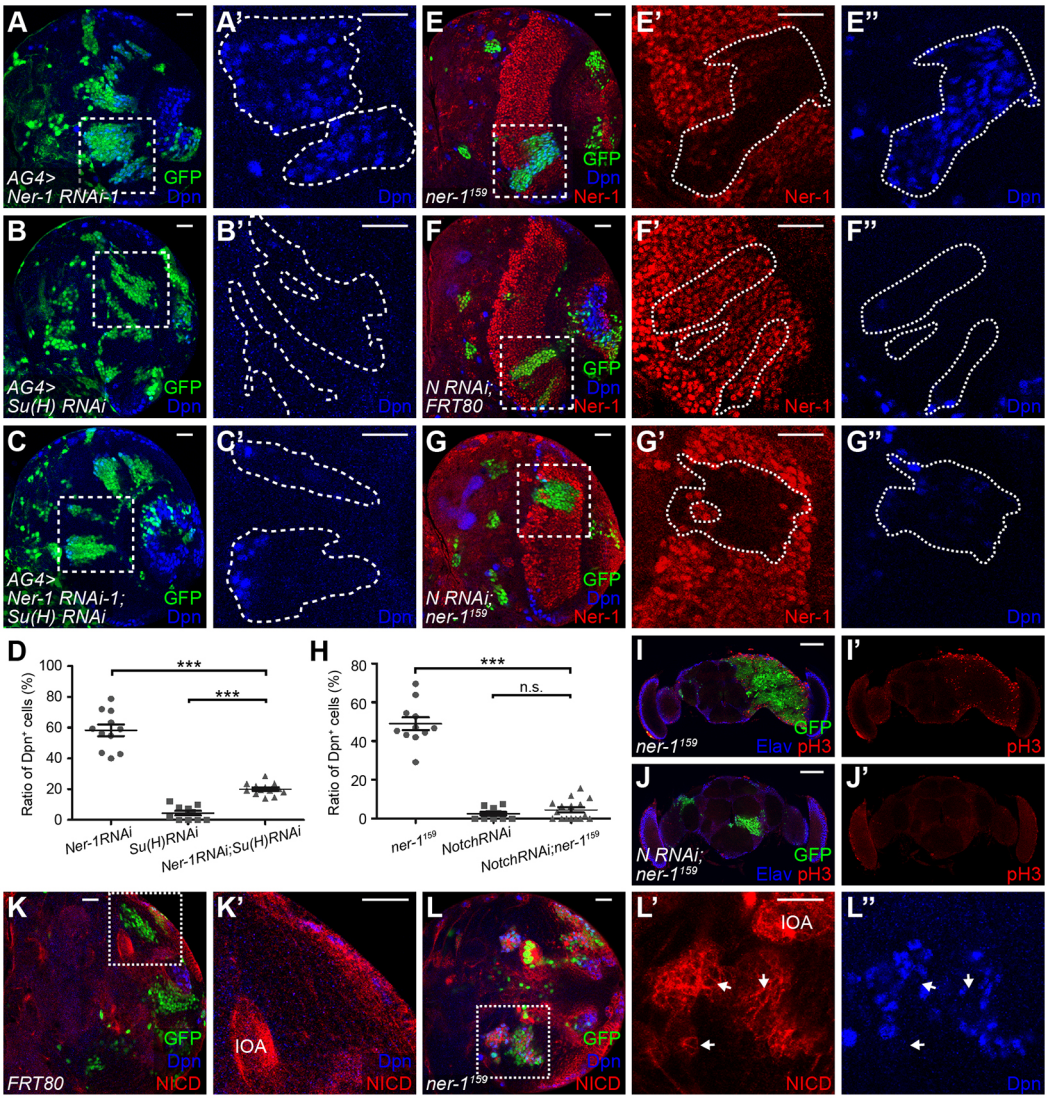

Fig. 6. Dedifferentiation caused by Nerfin-1 depletion is rescued by repression of Notch signaling. (A-C′) Knockdown of Su(H) significantly reduces Dpn$^+$ cells in Nerfin-1 RNAi clones. Magnification of boxed regions in A-C is shown in A′-C′, respectively, with clones outlined. (D) Quantification of the ratio of Dpn$^+$ cells in clones from A-C (n=11,10,13, respectively). Data are mean±s.e.m.; ***P<0.001. (E-G″) Notch knockdown does not affect Nerfin-1 expression but reduces the number of Dpn$^+$ cells in *nerfin-1^{159}* clones. Magnification of boxed regions in E-G is shown in E′-G″ with clones outlined. (H) Quantification of the ratio of Dpn$^+$ cells in clones from E-G (n=11,10,16, respectively). Data are mean±s.e.m.; n.s., not significant; ***P<0.001. (I-J′) Notch knockdown significantly rescues *nerfin-1^{159}* tumors at the adult stage. (K-L″) Upregulation of Notch expression is more extensive than Dpn in *nerfin-1^{159}* clones. Magnification of boxed regions in K and L is shown in K′ and L′,L″, respectively. Arrows indicate the NICD$^+$/Dpn$^-$ cells. Scale bars: 100 µm in I-J′; 20µm in A-G″,K-L″.

downstream of Notch signaling or it is subsequently upregulated after cell type change.

Notch signaling is inhibited by a suppression of Notch receptor expression

As Notch signaling is hyper-activated in *nerfin-1* mutant clones, we are interested in how it is regulated. One possibility is that Notch signaling becomes constitutively activated without the inhibition by Nerfin-1. To investigate this, we knocked down Delta upon Nerfin-1 loss and found that dedifferentiation was suppressed (Fig. 7). These results indicate that Notch signaling is not constitutively activated and that it needs a ligand. Furthermore, Notch signal is both produced and received by medulla neurons. At the same time, our results show that Nerfin-1 loss induces dramatic upregulation of the expression level of Notch receptor (Fig. 5A,A′). Thus, we hypothesize that Nerfin-1 suppresses the expression of the Notch receptor in normal medulla neurons and inhibits Notch pathway activity. When Nerfin-1 is absent, expression levels of the Notch receptor increase strikingly. The receptors then bind to Delta from the adjacent cells and activate Notch signaling in its own. However, it is still unclear whether Notch receptor is a direct target of Nerfin-1. Therefore, subsequent studies on Nerfin-1 may help us to clarify the underlying mechanisms and provide better understanding about neuronal maintenance.

MATERIALS AND METHODS

Fly strains

Flies were raised on standard yeast/molasses medium at 25°C unless otherwise stated. The full-length Nerfin-1 DNA fragment (1410 bp) was amplified from *Drosophila* cDNA. Nerfin-1-dZF1, -dZF2 and -dZF3 are truncations of Nerfin-1 with 748-810 bp, 832-900 bp and 1000-1068 bp deleted, respectively. Fragments were inserted into *pUAST-attb* vector and

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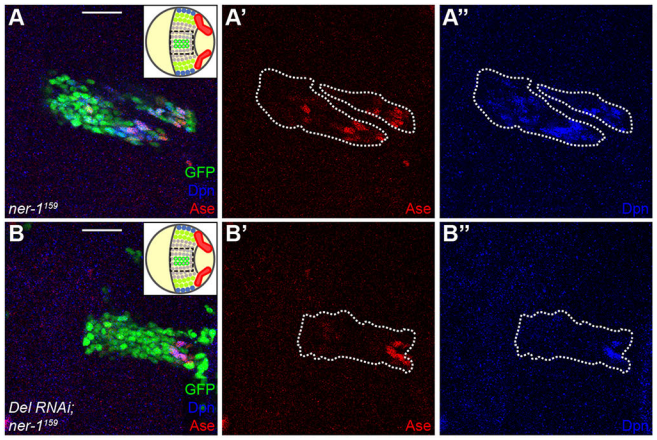

Fig. 7. Dedifferentiation caused by Nerfin-1 absence is suppressed by Delta knockdown. (A-B″) Representative *nerfin-1*^{159} clones with and without Delta RNAi showing Dpn and Ase staining. Insets show clone regions schematically. Dashed lines outline the clone region. Scale bars: 20 µm. (C) Quantification of the ratio of Dpn$^+$ or Ase$^+$ cells in clones from A and B (n=6 for each). Data are mean±s.e.m.; *P<0.05; ***P<0.001.

verified by DNA sequencing. Nerfin-1 full-length and -dZF1/2/3 transgenic flies were generated by site-specific integration into the fly genome at the 25C6 attp locus.

Ectopic expression clones were created by Flip-out using: *hsflp[122] act>CD2>Gal4; UAS-Dicer2 UAS-GFP* (referred to as AG4), *Nerfin-1 RNAi-1* (VDRC #101631), *Nerfin-1 RNAi-2* (Bloomington #28324), *yw;; HA-NICD* (Han et al., 2016), *Numb RNAi* (Bloomington #35045), *Myc RNAi* (VDRC #106066) and *Su(H) RNAi* (Bloomington #28900). Mutant clones were created by FLP-FRT-mediated recombination using: *hsflp [122]; tub-Gal4 UAS-GFP; FRT80 tub-Gal80*, *hsflp[122];; FRT80* (gifts from Jiang Jin, University of Texas Southwestern Medical Center at Dallas, TX, USA) and *Df(3L)FRT80 nerfin-1*^{159}*/TM6B* (a gift from W. F. Odenwald, Neural Cell-Fate Determinants Section, NINDS, NIH, Bethesda, MD, USA) (Kuzin et al., 2005). Markers of gene expression and activity were: *ex-lacZ* (Hamaratoglu et al., 2006), *puc-lacZ* (*puc*^{E69}, DGRC #109029), *Su(H)*$_{m8}$*-lacZ* (a gift from Rongwen Xi, National Institute of Biological Sciences, Beijing, China, and Sarah Bray, Department of Physiology, Development and Neuroscience, University of Cambridge, England, UK) (Furriols and Bray, 2001; Lin et al., 2010) and *E(spl)mγ-GFP* (a gift from Hongyan Wang, Neuroscience and Behavioral Disorders Program, Duke-National University of Singapore Graduate Medical School, Singapore). Other drivers and fly strains used were: *elav-Gal4[C155]* (Lin and Goodman, 1994), *tubGal80*^{ts} (Jiang Jin), *P[nerfin-1.GFP-NLS.SV-40] iA* (referred to as Ner-1-GFP, a gift from A. Kuzin, Neural Cell-Fate Determinants Section, NINDS, NIH, Bethesda, MD, USA) (Kuzin et al., 2007), *UAS-Tor-DN* (Bloomington #7013), *Notch RNAi* (VDRC #100002), *Delta RNAi* (VDRC #36784), *w; UAS-Fng* (Bloomington #8553) and *UAS-Dicer2* (gift from Jiang Jin).

Immunostaining

Tissues were dissected in PBS and fixed in 4% formaldehyde in PBS for 15-20 min at room temperature. Wash solution was PBS with 0.1% (larval tissues) or 0.3% (adult brains) Triton X-100.

Primary antibodies used in this study were: mouse anti-Pros (1:100, DSHB), rabbit anti-Dpn (1:200, a gift from Hongyan Wang), guinea pig anti-Dpn (1:1000, a gift from Xiaohang Yang, College of Medicine, Zhejiang University, Hangzhou, China), rat anti-Elav (1:100, DSHB), mouse anti-Mira (1:50, a gift from Hongyan Wang), guinea pig anti-Ase (1:500, a gift from Hongyan Wang), rabbit anti-pH3 (1:100, Millipore, 06-570), mouse anti-NICD (1:100, DSHB), rabbit anti-β-galactosidase (1:500, Invitrogen, A-11132), rabbit anti-pSTAT (1:500, a gift from Xinhua Lin, Institute of Zoology, Chinese Academy of Sciences, Beijing, China), rabbit anti-pErk (1:100, Cell Signaling, 4370P) and rabbit anti-Myc (1:100, Santa Cruz Biotechnology, SC-28207). F-actin was stained with phalloidin (1:20,000, Thermo Fisher Scientific) together with the secondary antibodies. Rabbit anti-Nerfin-1 (1-240 amino acids) was made by ABclonal and used at 1:100. Secondary goat or donkey antibodies (Jackson) were used at 1:1500. Samples were mounted with Vectashield mounting media (Vector Laboratories).

Clonal analysis and temperature-shift experiments

To induce flip-out clones, larvae were heat shocked 24 h after hatching (24 h ALH) for 15 min at 37°C and dissected 96 h later unless otherwise stated. To induce NICD clones, larvae were heat shocked at 96 h ALH for 7 min at 37°C and dissected 24 h later.

To induce *nerfin-1*^{159} clones, larvae were heat shocked at 24 h ALH for 30 min at 37°C and dissected 96 h later unless otherwise stated. For time-course assay, embryos were collected for 4 h. Larvae were heat shocked at 108, 104, 96, 84, 72 and 60 h ALH and dissected at 120 h ALH. MARCM clones in the adult brains were induced by heat shocking larvae at 24 h ALH for 30 min at 37°C and flies were dissected 7 days after eclosion.

Gal80^{ts} is a temperature-sensitive Gal80 protein that is functional at 18°C and non-functional at 29°C. To delay the expression of transgenes, larvae were raised at 18°C for 9-10 days and then transferred to 29°C. Dissection was carried out 24 h later.

Microscopy image acquisition and statistics

Fluorescent microscopy was performed on a Leica LAS SP8 confocal microscope; confocal images were obtained using the Leica AF Lite system. Larval tissue images were taken using a 40× objective and adult brain images were taken using a 20× objective. All the data are expressed as the mean±standard error of the mean (s.e.m.) and were analyzed using Student's t-test. The results were considered statistically significant if P<0.05.

To quantify the expression of Su(H)$_{m8}$-lacZ in Fig. 5C, the intensity of the *lacZ* and GFP signal in the region indicated by the thick dashed line was analyzed using the Leica AF Lite system. Two-hundred and ten pixels were analyzed, 124 (GFP$^-$) of which were quantified as wild type and 86 (GFP$^+$) were quantified as *nerfin-1*^{159}.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

J.X. and L.Z. conceived and designed the experiments; J.X., X.H., Y.L., Y.J. and J.X. performed the experiments; J.X., X.H. and L.Z. analyzed the data; L.G., W.W. and Y.Z. contributed regents/materials; J.X., M.-X.Y., M.H., Y.Y. and L.Z. wrote and edited the paper.

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Supplementary information

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