ABSTRACT

The termination of the proliferation of Drosophila neural stem cells, also known as neuroblasts (NBs), requires a 'decommissioning' phase that is controlled in a lineage-specific manner. Most NBs, with the exception of those of the mushroom body (MB), are decommissioned by the ecdysone receptor and mediator complex, causing them to shrink during metamorphosis, followed by nuclear accumulation of Prospero and cell cycle exit. Here, we demonstrate that the levels of Imp and Syp RNA-binding proteins regulate NB decommissioning. Descending Imp and ascending Syp expression have been shown to regulate neuronal temporal fate. We show that Imp levels decline slower in the MB than in other central brain NBs. MB NBs continue to express Imp into pupation, and the presence of Imp prevents decommissioning partly by inhibiting the mediator complex. Late-larval induction of transgenic Imp prevents many non-MB NBs from decommissioning in early pupae. Moreover, the presence of abundant Syp in aged NBs permits Prospero accumulation that, in turn, promotes cell cycle exit. Together, our results reveal that progeny temporal fate and progenitor decommissioning are co-regulated in protracted neuronal lineages.

KEY WORDS: Neuroblast, RNA-binding protein, Mediator complex, Cell cycle exit, Mushroom body

INTRODUCTION

A long-lived neural stem cell shows age-dependent changes in proliferation and progeny cell fate (Li et al., 2013). In Drosophila, temporal regulation is lineage specific. Individual neuroblasts (NBs) produce a unique series of neuronal types and end neurogenesis at independent times in development (Chai et al., 2013; Homem et al., 2015; Lee et al., 1999; Lin et al., 2012; Sousa-Nunes et al., 2010; Truman and Bate, 1988; Yu et al., 2010). The timing of termination of individual NBs is likely to be co-regulated with their temporal fate, which guides birth order-dependent neuronal identities, to produce the required number and types of neurons in each lineage (Maurange et al., 2008). Such co-regulation could be achieved by a lineage-autonomous developmental clock that governs both progeny temporal fate and NB aging in each neuronal lineage.

Temporal protein gradients, including IGF-II mRNA-binding protein (Imp), Syncrpic/hnRNPQ (Syp) and Chronologically inappropriate morphogenesis (Chinmo) govern neuronal temporal fates in diverse NB lineages (Liu et al., 2015; Ren et al., 2017; Zhu et al., 2006). The RNA-binding proteins Imp and Syp are expressed in the NB, with Imp levels descending and Syp levels ascending over time. The serially derived neuronal progeny inherit the Imp and Syp levels from the NB. Imp enhances and Syp represses the translation of Chinmo, a BTB zinc-finger nuclear protein. This results in a high-to-low Chinmo protein gradient among the newborn neurons. The opposing Imp/Syp temporal gradients (as revealed by RNA-seq) extend throughout neurogenesis but exhibit distinct lineage-characteristic expression levels and progression rates (Liu et al., 2015; Ren et al., 2017). In the antennal lobe (AL) lineages, one NB can yield a sequence of more than 20 neuronal types from ~80 asymmetric cell divisions that span over 4 days of larval development (Yu et al., 2010; Lin et al., 2012). The opposing Imp/Syp temporal gradients are steep and progress rapidly in such fast changing NBs. By contrast, the NBs that make neurons of the mushroom body (MB) learning and memory center continue to divide throughout larval and pupal development but produce only three morphological classes of neurons (Lee et al., 1999). Imp and Syp are expressed in shallow, slowly progressing gradients in the long-lived MB NBs. The close correlation between the progression of Imp/Syp gradients and the progeny’s temporal fate changes argues for direct coding of diverse neuronal temporal fates by distinct levels of Imp and/or Syp.

NB termination is also temporally regulated. During active cycling, NBs regrow promptly following each asymmetric cell division (Knoblich, 2008; San-Juán and Baonza, 2011; Song and Lu, 2011). Upon exposure to a pulse of ecdysone hormone, all NBs, except the four pairs of MB NBs, undergo size reduction while cycling because of sluggish regrowth. The size-reduced NBs divide slowly and eventually divide symmetrically into two post-mitotic cells (Homem et al., 2014; Maurange et al., 2008). The stage-specific progressive termination of NBs is an actively regulated process, which we refer to as ‘decommissioning’.

The initiation of NB decommissioning depends on the ecdysone receptor and core components of the mediator complex, which promote key enzymes to increase oxidative phosphorylation in energy metabolism that slows down NB regrowth (Homem et al., 2014). Subsequent nuclear accumulation of Prospero then drives cell cycle exit of NBs (Maurange et al., 2008). Individual NBs exit cell cycle at characteristic times around 24 h after pupal formation (APF) (Awasaki et al., 2014). However, the mechanisms underlying such temporal differences in NB decommissioning remain unexplored. Moreover, despite global ecdysone action, the MB NBs actively regrow and cycle for ~100 more divisions at the pupal stage (Ito and Hotta, 1992; Truman and Bate, 1988). The MB NBs finally shrink but terminate by apoptosis around 96 h APF (Siegrist et al., 2010).
Given that individual lineages vary in length and produce diverse neuron types in distinct time courses, it is likely that NB decommissioning and temporal fate progression are co-regulated in time. However, it is not known which mechanisms are responsible for such co-regulation or what factors are involved. Here, we address this question by examining whether and how the temporal factors Imp and Syp guide the course of NB decommissioning. We found that Imp prevents MB NBs from shrinking in early pupae partly through inhibiting the mediator complex. Other NBs lacking Imp shrunk rapidly in early pupae, and exit the cell cycle upon Syp-dependent nuclear accumulation of Pros. Imp is dominant to Syp, as Syp can promote cell cycle exit only in Imp-negative decommissioned NBs. Taken together, NB decommissioning is an actively programmed developmental process that is temporally regulated by the levels of Imp and Syp proteins in each NB to ensure the proper completion of a neuronal lineage.

RESULTS

Imp and Syp play independent roles in NB decommissioning

In order to monitor decommissioning of central brain NBs, we expressed a cell cycle-sensitive GFP using a GAL4 driver that is active in all but optic lobe NBs (Fig. S1B). In the wild-type control, almost all NBs had disappeared by 48 h APF, except for four remaining MB NBs, previously described as actively dividing until 96 h APF (Siegrist et al., 2010). As expected, the MB NBs had disappeared by adult stage (Fig. 1A″). We followed the progressive ending of non-MB NBs (~100 per hemisphere) from wandering larva to early pupae. Their average size had decreased significantly by 8 h APF, and mostly reduced to a size comparable to that of their daughter ganglion mother cells (GMCs) within 24 h APF (Fig. 1A′,A″,E). Notably, the aged NBs did not exit the cell cycle in a synchronized wave. A few NBs (though small in size) remained at 24 h APF (Fig. 1A″), as evidenced by the expression of Miranda (Mira), an NB-specific gene (Ikeshima-Kataoka et al., 1997), and the occasional presence of phospho-Histone3 (pH3), indicating mitosis (Fig. S1A).

Those progressively ending NBs in early pupae were negative for Imp and positive for Syp (Fig. 1F,G, Fig. S2B,C). Most, if not all, NBs showed abundant Imp and minimal Syp in early larvae (Fig. S2A-C). We therefore wondered if NBs purposely locked in the initial state of Imp/Syp expression (high Imp, low to no Syp) could escape decommissioning. We tested this idea by silencing Syp with targeted RNAi, which consequently maintained detectable Imp throughout NB life (Fig. 1F, Fig. S2B). We found that NBs with persistent Imp and minimal Syp expressions escaped decommissioning (Fig. 1B). Most, if not all, NBs remained at 48 h APF (Fig. 1B-B″); a few sustained and continued to cycle at the adult stage (Fig. 1B′′, Fig. S1). Moreover, the size of Syp-depleted NBs was not reduced by 24 h APF, and those that persisted were consistently larger than GMCs (Fig. 1E). Continuously expressing transgenic Imp elicited similar phenotypes (Fig. 1C-C″). We examined the altered Imp/Syp levels by immunostaining (Fig. S2). Notably, Imp/Syp mutual inhibition is less evident with progressively ending NBs (though small in size) remaining at 24 h APF (Fig. 1A″), as evidenced by the expression of Miranda (Mira), an NB-specific gene (Ikeshima-Kataoka et al., 1997), and the occasional presence of phospho-Histone3 (pH3), indicating mitosis (Fig. S1A).

Taken together, our results indicate that prolonged Imp expression, rather than lower Syp expression, protects MB NBs from decommissioning in the early pupa.

Late-larval induction of Imp effectively protects non-MB NBs from early pupal decommissioning

It is possible that MB and non-MB NBs undergo progressive ageing at different speeds during larval development and only the non-MB NBs have aged sufficiently to undergo decommissioning around pupal formation. If this is true, overexpressing Imp from the beginning of neurogenesis could slow down the ageing of NBs and indirectly affect their responses to ecdysone. Alternatively, Imp might acutely regulate NB decommissioning by governing ecdysone signaling. To address this possibility, we tried not to perturb normal NB ageing (if any) and examined whether late larval induction of transgenic Imp could protect non-MB NBs from early pupal decommissioning.

We temporally controlled the induction of transgenic Imp in all NBs positive for dpnEE-GAL4 (Awasaki et al., 2014), using a temperature-sensitive GAL80 (McGuire, et al., 2003). Transgenic Imp was induced at a specific time around pupation with a temperature shift from 18°C to 29°C (Fig. 3A). Induction at 8 h before pupa formation (BPF) or later failed to extend the life of non-MB NBs beyond early pupal development (Fig. 3C; data not shown). Induction at 12 h and 15 h BPF, by contrast, substantially prolonged the life of 30% and 44% of non-MB NBs, respectively (Fig. 3D,E). Induction earlier than 15 h BPF only increased the percentage of sustained NBs to ~50% (Fig. 3F,G) as opposed to ~85% with continuous Imp expression (Fig. 3H). The incomplete penetrance was probably due to a mosaic inactivation of GAL80(ts), as a comparable percentage (~65%) of NBs had detectable Imp levels and expression of a UAS reporter approximately 12 h after induction (data not shown). Continuous co-induction of Imp and an anti-apoptotic p35 transgene did not extend more NBs into
the adult stage (Fig. S4). This result argues against the possibility that Imp expression selectively prolongs NBs that end by cell cycle exit rather than apoptosis. The potency of late larval Imp induction in sustaining many non-MB NBs suggests that Imp can act acutely to suppress NB decommissioning during the larval-to-pupal transition.

![Fig. 1. Imp and Syp regulate non-MB NB decommissioning.](image)

(A-D‴) Ectopic Imp or Syp-depletion prolongs NB life into the adult. Composite confocal images of control (CTRL; A-A‴), Syp RNAi (B-B‴), Imp gain of function (GOF; C-C‴), and Syp/Imp-depleted (D-D‴) fly brains at specific developmental times, immunostained for GFP (green), Mira (magenta) and phospho-Histone H3 (pH3, blue). Transgenes were driven by dpnEE-GAL4 in the NBs of central brain. Yellow arrows indicate the MB NBs. Insets show the boxed areas at higher magnification. Scale bar: 50 µm (10 µm in inset). (E) Quantification of NB size in the anterior region of the fly brain (measured by the diameter of Mira-labeled NBs, mean±s.d., n=6 brains). (F) Syp depletion prolongs NB Imp expression. Representative confocal images of 8 h APF fly brains immunostained for Imp (magenta), GFP (green) and Dpn (blue) in control and Syp depletion conditions/experiments driven by dpnEE-Gal4. Scale bar: 10 µm. (G) Imp gain of function did not affect Syp expression. Representative confocal images of 8 h APF fly brains immunostained for Syp (magenta), GFP (green) and Dpn (blue) in control and dpnEE-Gal4-driven Imp gain-of-function conditions/experiments. In F and G, NBs with a maximum diameter at the given focal plane are circled. Scale bar: 10 µm.
Imp suppresses NB decommissioning partly by inhibiting the mediator complex

The ecdysone receptor and the mediator complex have been recently implicated in NB shrinking and ultimately ending (Homem et al., 2014). The mediator complex is essential for a switch in energy metabolism that slows down NB regrowth during successive divisions and thus progressively reduces the NB cell size. NB shrinkage and termination can also be delayed by expressing Med12 (Kohtalo, Kto), the inhibitory subunit of the mediator complex (Homem et al., 2014; Malik and Roeder, 2010).

To examine the possibility that Imp prevents NB shrinkage by suppressing the mediator complex, we determined Med12 expression in the NBs following various Imp/Syp manipulations. We found elevated Med12 protein levels around pupation in non-MB NBs with Syp RNAi and Imp overexpression (Fig. 4A-B′,D,D′). The Med12 protein levels were reduced to lower than normal upon silencing both Imp and Syp using RNAi (Fig. 4C,C′,E). Imp-depleted MB NBs also showed a significant reduction in Med12 expression (Fig. 4F-H). These observations suggest that Imp promotes Med12, which could in turn suppress the mediator complex and thus prevent NB shrinkage.

To test whether the RNA-binding protein Imp directly associates with transcripts encoding Med12 and/or other mediator complex subunits, we pulled down endogenous Imp::GFP (Toledano, et al., 2012) and quantified various co-precipitated transcripts by qPCR following RNA immunoprecipitation (RIP-qPCR). Eight mediator complex components, including Med4, Med6, Med9, Med10, Med11, Med22, Med27 and Med31, have been implicated in promoting NB shrinkage following pupation (Homem et al., 2014). Among them, Med6, Med27 and Med31 transcripts were significantly enriched over the negative control rp49 (RpL32) (Fig. 4I). We confirmed the requirement of Med6 for prompt NB shrinking by RNAi. Moreover, silencing Med6 could effectively block the accelerated NB shrinkage caused by Imp RNAi, as double RNAi (like repressing Med6 alone) delayed NB shrinkage and sustained most NBs into 48 h APF (Fig. S5). By contrast, the Med12 mRNA could not be significantly enriched (Fig. 4I). In all cases, the control GFP immunoprecipitation from a wild-type strain was very clean, ranging from 0.09% to 0.54% of input (Fig. 4I). These results implicate multiple core components of the mediator complex, but not Med12, as Imp direct targets.

Although Med12 was not recovered as a direct target of Imp, an easy way to modify the mediator complex’s activity is by knocking down or overexpressing the inhibitory subunit Med12 (Malik and Roeder, 2010). To test this possibility, we examined the genetic interactions between Imp/Syp and the mediator complex by manipulating Med12. Syp-depleted NBs did not shrink normally (Fig. 1B). However, addition of Med12 RNAi, which activates the mediator complex, rescued the early pupal NB shrinking back to wild type (Fig. 5A-A″). The size-reduced NBs (positive for Mira despite a gradual loss of labeling with dpnEE-GAL4) did not exit the cell cycle; instead they persisted with evidence of cycling into adult stage (Fig. 5A″, Fig. S1A). This is reminiscent of the tiny non-MB NBs caused by repression of both Imp and Syp (Fig. 1D″).
contrast, activating the mediator complex by silencing Med12 could make Imp-overexpressing NBs not only shrink but also exit the cell cycle by 48 h APF (Fig. 5B-B″″). Further, silencing Med12 alone was also sufficient to make MB NBs shrink rapidly in early pupae, similar to non-MB NBs (Fig. 5C). These results suggest that Imp suppresses NB decommissioning by inhibiting the mediator complex.

However, overexpressing Med12 only partially blocked the rapid shrinkage of Imp-depleted MB NBs in early pupae (Fig. 5D with an average diameter of 5.6±1.5 at 48 h APF, compared with Fig. 2D′ with 4.1±0.9 in Imp RNAi and Fig. 2C′ with 8.9±1.6 in wild-type control). Furthermore, silencing Med6 failed to protect non-MB NBs beyond the mid-pupal stage (Fig. S5A-A‴). These limited effects, compared with Imp overexpression that could sustain many NBs into adult stage, argue that Imp plays additional roles in suppression of NB shrinkage. Taken together, we can conclude that Imp prevents NB decommissioning partly by inhibiting the mediator complex, which involves direct repression of mediator complex core components as well as indirect enhancement of the mediator complex inhibitor Med12.

Syp promotes NB cell cycle exit by permitting nuclear Pros accumulation

Aged NBs with reduced cell size require Syp to exit the cell cycle (Fig. 1D, Fig. 5A-A‴). In wild-type NBs, nuclear accumulation of Pros promotes NB cell cycle exit (Maurange et al., 2008). Moreover, Syp protein may bind directly with pros mRNA as evidenced by a great enrichment of pros mRNA in Syp RIP (McDermott et al., 2014). These phenomena raised the possibility that Syp RNAi delays NB cell cycle exit by preventing Pros accumulation. Pros immunostaining in wild-type pupal brains revealed a drastic increase of Pros among the progeny of pupal NBs (Fig. 6B,B′ compared with 6A,A′). Knocking down Syp in NBs by targeted RNAi suppressed the enhancement of Pros in the late-born progeny (Fig. 6C,C′), apparently without affecting the basal Pros levels required to prevent dedifferentiation of GMCs. In late NBs, Syp RNAi significantly reduced the modest levels of Pros proteins and consistently excluded Pros from entering the nuclei (Fig. 6C,C′, E compared with 6B,B′). It has been shown that acute induction of transgenic Pros can promptly terminate actively cycling NBs (Li and Vaessin, 2000). We found transgenic Pros to be equally potent in the termination of otherwise long-lasting Syp-depleted NBs (Fig. 6D,D′). These results indicate that Syp enhances Pros accumulation, which in turn promotes NB cell cycle exit. Notably, Syp is required but not sufficient to increase Pros protein levels. For instance, a continuous induction of Syp from the beginning of neurogenesis failed to elicit precocious Pros accumulation in either NBs or their progeny (data not shown). Co-induction of Imp and Syp throughout neurogenesis did not accelerate or delay Pros expression among serially derived progeny, ascribing the increase of Pros in late-born neurons to temporal cues independent of Imp/Syp (Fig. 6D,D′). By contrast, ectopic Imp could effectively prevent Pros accumulation in Syp-positive NBs (Fig. 6D,D′,E,I-I″), arguing that activation of NB decommissioning is a prerequisite to nuclear accumulation of Pros. Consequently, we suggest that Syp plays a permissive role in the enhancement of Pros during late larval and early pupal neurogenesis.
In sum, Imp negatively regulates the ecdysone-induced, mediator complex-dependent decommissioning of NBs. The decommissioning triggers a timer for up-regulating *pros*; the ultimate increase in Pros protein requires Syp. This thereby allows individual NBs to have independent decommissioning programs based on temporal progression of Imp/Syp gradients.

**DISCUSSION**

Decommissioning of NBs is a temporally controlled developmental program that proceeds from NB shrinking to cell cycle exit.
This phenomenon indicates that the mediator complex is selectively involved in NB shrinkage, and that NB shrinkage does not automatically lead to cell cycle exit. Imp can suppress NB shrinkage and in turn prevent cell cycle exit (Fig. 1C–C″, Fig. 2A, A′, Fig. 3, Fig. 6). By contrast, Syp is selectively required for NB cell cycle exit (Fig. 1B–B″, Fig. 5A–A″, Fig. 6). We established their respective roles by directly controlling both Imp and Syp to mitigate the fact that they negatively regulate each other (Liu et al., 2015). Silencing Imp made the ‘forever-young’ Syp-depleted NBs shrink rapidly in early pupae but only moderately reduced the number of NBs ectopically present in adult brains (Fig. 1D–D″, Fig. S1C). This ascribes the suppression of NB shrinkage in Syp-depleted NBs to an increase in Imp rather than reduced Syp (Fig. 1B–B″, F). It further demonstrates the requirement of Syp for NB cell cycle exit.

Ectopic Imp can override the Syp-dependent cell cycle exit, as overexpressing Imp not only blocked NB shrinkage but also prevented cell cycle exit even in the presence of enhanced Syp (Fig. 1C–C″, G, Fig. 6I–I″). The dominant role of Imp effectively explains why MB NBs, which have prolonged Imp expression, can escape the global initiation of NB decommissioning by ecdysone signaling. Consistent with this idea, repressing Imp, but not overexpressing Syp, made MB NBs shrink rapidly in early pupae (Fig. 2). The MB NBs with reduced cell size showed no evidence of cycling but could survive into late pupal stage despite strong Syp expression. This is consistent with normal ending of MB NBs by apoptosis rather than through Syp-dependent, Pros-mediated cell cycle exit (Siegrist et al., 2010). Insulin signaling and the transcription factor Retinal homeobox are known to promote MB NB proliferation and survival (Kraft et al., 2016; Lin et al., 2013; Siegrist et al., 2010). Although endogenous Imp protects MB NBs from shrinking in early pupae through inhibiting the mediator complex, it is unclear how ectopic Imp can extend MB NBs into adults that might involve different mechanisms.

Late larval induction of transgenic Imp could protect the normally ‘aged’ NBs from decommissioning in early pupae. However, a slightly later induction around pupation failed to show any protection (Fig. 3C). This assigns the critical window of Imp’s action to be at or before the prepupal ecdysone pulse. The nearness of the window to the start of decommissioning suggests that the Imp mRNA-binding protein itself (rather than the

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**Fig. 5.** The mediator complex functions downstream of Imp to induce NB shrinkage. (A–B″″) Med12 depletion blocks the inhibition of NB shrinkage caused by persistent Imp expression. Composite confocal images of Syp and Med12 joint knockdown (A) and Imp gain of function combined with Med12 knockdown (B) driven by dpnEE-Gal4. Brains were dissected at specific developmental ages and immunostained for GFP (green), Mira (magenta) and phospho-Histone H3 (pH3, blue). Insets show the boxed areas at higher magnification. Scale bar: 50 µm (10 µm in inset). (C–E) The mediator complex acts downstream of Imp to regulate MB NB shrinkage. Representative confocal images of 48 h APF fly brains immunostained for GFP (green), Dpn (magenta) and Pros (blue) in Med12 depleted (C) and combined Imp-depleted with Med12 gain-of-function conditions (D) driven by Gal4-OK107. Diameter of MB NBs at 48 h APF (mean±s.d., n=6 brains) is indicated at the top right of each panel. Scale bar: 20 µm. (E) Mean MB NB diameter (±s.d., n=6 brains) of flies at different developmental stages with transgenic manipulations driven by GAL4-OK107. *P<0.05, **P<0.01 (Student’s t-test).
accumulative effects of protracted Imp expression) suppresses the initiation of NB decommissioning by ecdysone. Being able to protect most NBs by acute Imp induction further implies that NBs do not age chronically as a result of repeated cycling.

Knocking down Med12 to enhance the mediator complex’s function could largely erase the negative effects of ectopic Imp on NB shrinkage and cell cycle exit (Fig. 5B-B″). However, overexpressing Med12 alone only partially blocks the rapid shrinkage of the Imp-depleted MB NBs (Fig. 5D). This argues that prolonged Imp expression protects MB NBs from early pupal decommissioning not only by enhancing Med12 but also by regulating other effectors to suppress the mediator complex. Consistent with this notion, we found that the mRNAs of several mediator complex core components, including Med6/27/31, can be greatly enriched by Imp RIP, implicating them as Imp direct targets (Fig. 4I). By contrast, Imp RIP failed to pull down Med12 mRNA, suggesting an indirect positive regulation of Med12 by Imp. Besides, high Imp blocked NB decommissioning to a much larger degree than did inhibiting the mediator complex, suggesting possible involvement of mediator complex-independent targets. Imp could regulate a number of targets through diverse mechanisms, including localization, stability, translation, and nuclear export (Degrauwe et al., 2016). For example, Imp can promote the survival of target transcripts by counteracting the actions of siRNA (Toledano et al., 2012). Taken together, our data indicate that Imp could regulate the mediator complex and other ecdysone downstream effectors by controlling mRNA stability and/or translation to prevent NBs from decommissioning until the production of most progeny.
With the exception of the MB, aged NBs exit the cell cycle due to nuclear accumulation of Pros. Drastic enhancement of Pros levels also occurs among the newborn progeny from late larval and early pupal NBs. Syp is required for the nuclear accumulation of Pros in aged NBs as well as the strong Pros expression in late-born neurons (Fig. 6). However, strong Syp alone is not sufficient to increase Pros protein levels, even though Syp protein might bind directly with pros mRNA to enhance its stability and/or translation (McDermott et al., 2014). These observations argue that Pros is regulated at both transcriptional and post-transcriptional levels. As to timely cell cycle exit, a Syp-independent mechanism may upregulate pros transcription at certain times following the activation of NB decompartmenting. Strong Syp levels then allow prompt increases in Pros proteins, leading to nuclear accumulation of Pros and termination of the stem cell mode of asymmetric cell division.

Imp/Syp levels in post-mitotic cells determine their birth time/order-dependent cell fates (Liu et al., 2015; Ren et al., 2017). Imp/Syp expression levels in progenitors govern their readiness for aging and cell cycle exit. This allows decompartmenting to be selectively activated in NBs when they have produced essential progeny and are ready for aging. The opposing Imp/Syp temporal gradients with distinct lineage-specific temporal dynamics could therefore tailor neurogenic programs characteristic of different neuronal lineages (Fig. 7). Notably, various stem cells across diverse species express analogous descending Imp gradients (Nishino, et al., 2013; Toledano, et al., 2012). It is possible that the mechanisms of progeny temporal fating and progenitor aging co-evolved on the pre-existing Imp gradients in neural stem cells. We propose that co-regulation of progeny temporal fate and progenitor decompartmenting achieves the complex protracted neuronal lineages required for building sophisticated brains.

MATERIALS AND METHODS
Fly strains and DNA constructs
The fly strains used in this study include: (1) dpnEE-Gal4 (Awasaki et al., 2014; Yang et al., 2016); (2) UAS-Syp-RNAi [stock #33011 and 33012, Vienna Drosophila Resource Center (VDRC, www.vdrc.at)]; (3) UAS-Imp-RNAi (stock #34977 and 55645, Bloomington Drosophila Stock Center); (4) UAS-Imp-RM-Flag (Liu et al., 2015); (5) UAS-Syp-RB-HA (Liu et al., 2015); (6) UAS-Med12-RNAi [stock #34588, Bloomington Drosophila Stock Center]; (7) UAS-pros.S [stock#32245, Bloomington Drosophila Stock Center]; (8) UAS-Med6-RNAi [stock #33743, Bloomington Drosophila Stock Center]; (9) lexop-Syp-mRNA (Ren et al., 2017); (10) UAS-Kto.J [stock #63801, Bloomington Drosophila Stock Center]; (11) FRTG13, UAS-mCD8::GFP; GAL4-OK107 (Lee et al., 1999); (12) Imp [CB04573] (gift from D. L. Jones, The Salk Institute for Biological Studies, CA, USA; Toledano et al., 2012); and (13) UAS-p53 (Hay et al., 1994).

The following strains were generated in this study: (14) UAS-GFP::Sec; (15) dpn-LexA::P65; and (16) act5C-GAL80ts-insulated spacer-tub-GAL80ts. For UAS-GFP::Sec, the GFP fragment was inserted in a NoI site in the UAS-Imp-RM-Flag/UAS-GFP::Sec plasmid (Awasaki et al., 2014), then the N-terminal 1-50 amino acids of securin was inserted in-frame at the C terminus of GFP in an EcoRI site.

For dpn-LexA::P65, a previously characterized dnp NB enhancer (Emery and Bier, 1995) and a Drosohila synthetic core promoter (Pfeiffer et al., 2008) were inserted in front of LexA::P65 in pBPLexA::P65w (Pfeiffer et al., 2010) by gateway cloning (Invitrogen).

For act5C-GAL80ts-insulated spacer-tub-GAL80ts, a pGAL80ts plasmid was created by removing the HindIII/BglII fragment in pFRC-MUH (Pfeiffer et al., 2010) then inserting the GLA80ts fragment from pmK-RQ (KanR)-GAL80ts into a KpnI/XbaI site in the tubulin promoter was inserted in front of GAL80ts in pGAL80ts through gateway cloning. The Actin 5C promoter was inserted in front of GAL80ts in pGAL80ts by gateway cloning. A synthetic insulated spacer cassette (Pfeiffer et al., 2010) was inserted at a FseI site in pAct5C-GAL80ts, and the EcoRI fragment of pTub-GAL80ts was then inserted in the EcoRI site after the insulated spacer cassette.

Temporal induction of Pros
Flyes with genotype UAS-pros.S: act5C-GAL80ts-insulated spacer-tub-GAL80ts/dpn-LexA::P65; 13XactAsp-Syp-mRNA/dpnEE-Gal4 were cultured at 18°C. The GAL80 and non-temperature-sensitive LexA::P65 allowed Syp knockdown continuously. For the control experiment, collected white pupae were cultured at 18°C for 24 h before dissection. To induce ectopic Pros in NBs, collected white pupae were transferred to 29°C to inactivate the temperature sensitive GAL80 for 16 h before dissection and thus de-repress dpnEE-Gal4 for misexpression of Pros.

Temporal induction of Imp
Embryos with genotype act5C-GAL80ts-insulated spacer-tub-GAL80ts/dpnEE-Gal4; UAS-Imp-RM-Flag/UAS-GFP::Sec were collected for 24 h at 18°C and then cultured at 18°C for 5 days. The larvae were heat shocked at 37°C for 15 min and incubated at 29°C to inactivate the temperature-sensitive GAL80. White pupae were then collected at several time points (0, 8, 12, 15, 18 and 24 h) after heat shock, which resulted in the induction of Imp expression at different developmental time points (0, 8, 12, 15, 18 and 24 h) before pupal formation (BPF). Collected white pupae were cultured at 29°C and dissected at 38 h APF.

Immunohistochemistry and confocal imaging
Brain tissues at specific developmental stages were dissected and immunostained as described previously (Lin et al., 2012). The following primary antibodies were used: chicken anti-GFP (1:1000, A10262, Life Technologies), rat anti-Mira (1:200, ab197788, abcam; shows non-specific signals in central complex neuropil), rat anti-Dpn (1:200, ab195172, abcam), rabbit anti-pH3 (1:500, #9701, Cell Signaling), mouse anti-Pros (1:200, Developmental Studies Hybridoma Bank), guinea pig anti-Mira (1:200, gift from J. Treisman, NYU School of Medicine, NY, USA; Janody, et al., 2003), rabbit anti-Imp (1:600, gift from P. Macdonald, University of Texas at Austin, TX, USA; Geng and Macdonald, 2006) and guinea pig anti-Syp (1:500; McDermott et al., 2014). All corresponding fluorescent secondary antibodies (1:500) were purchased from Life Technologies and confocal images of whole-mount fly brains were collected on a Zeiss LSM 710 confocal microscope.
Image analysis
To measure Imp signal intensity in the MB (Fig. 2), all NBs were labeled with dptEE->GFP::Sec and MB NBs were determined based on high Imp expression in the progenies. We selectively analyzed those NBs (circled) with a maximum diameter at the chosen focal planes compared with neighboring focal planes. Confocal images were exported to Adobe Photoshop, a hand-drawn mask was created for the cytoplasmic (for Imp/Syp) or nuclear (for Med12 and Pros) region of each gene to be calculated to assess immunoprecipitation efficiency. The ratio of averaged grayscale value in central brain to that of optic lobe was used to normalize the intensity across different sections/samples.

To compare Med12 (Fig. 4), Pros (Fig. 6) and Imp/Syp (Fig. S2) levels across various genotypes, images were taken using the same confocal setting (pinhole size, gain, laser power, etc.) and an image of selected focal plane was exported to Adobe Photoshop. A hand-drawn mask was created for the cytoplasmic (for Imp/Syp) or nuclear (for Med12 and Pros) region of the cell of interest with a maximum diameter at selected focal plane. The averaged grayscale value for each pre-defined region was calculated using the ‘Histogram’ algorithm in Photoshop. The grayscale value in the nuclear region was used to normalize the intensity across different sections/samples.

Immunoprecipitation
Third instar larval brains dissected in Schneider medium were homogenized in 150 μl immunoprecipitation (IP) buffer [50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% NP-40, 10% glycerol, complete EDTA-free protease inhibitor and RNase inhibitor (RNAsin PluS RNase Inhibitor, Promega)]. The lysate was precleared with 45 μl of washed Pierce Control Agarose Resin (Thermo Fisher Scientific). For each reaction, 50 μl of pre-cleared lysate was taken as a 50% input sample directly to RNA extraction. Next, 100 μl of pre-cleared lysate was incubated with 25 μl of GFP-TRAP agarose beads (Chromotek) for 2 h at 4°C with rotation. The beads were washed four times briefly each with 200 μl cold IP buffer at 4°C. After the final wash, beads were re-suspended in 100 μl extraction buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA and 1.3% SDS, 1:100 RNAsin) and incubated at 65°C, 1000 rpm (mixing frequency) for 30 min in a thermomixer. The elution step was repeated and the supernatants were pooled. RNA was extracted from inputs and immunoprecipitates with the RNA Spin RNA Isolation kit (GE Healthcare) and eluted in 40 μl of nuclease-free H2O. Reverse transcription was performed using RevertAid Premium Reverse Transcriptase (Thermo Fisher Scientific) using a real-time PCR detection system [CFX96 Touch Real-Time qPCR System (Bio-Rad)].

Real-time quantitative PCR (RT-qPCR)
RT-qPCR was performed with SYBRGreen Mastermix (Thermo Fisher Scientific) using a real-time PCR detection system [CFX96 TouchTM Real-Time PCR Detection System (Bio-Rad)]. Cycle threshold (Ct) value was calculated by the Bio-Rad CFX software using a second differential maximum method. A dilution series of the input sample allowed the percentage input of each gene to be calculated to assess immunoprecipitation efficiency. The forward and reverse qPCR primer sequences are listed in Table S1.

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Competing interests
The authors declare no competing or financial interests.

Author contributions

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Supplementary information
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