Figures and figure supplements

Neural stem cell-encoded temporal patterning delineates an early window of malignant susceptibility in Drosophila

Karine Narbonne-Reveau et al
**Figure 1.** A subset of dNBs induced by Pros knock-down propagates malignant tumors in adults. The scale bar in all images represents 30 μm. NBs and dNBs are always labeled using an anti-Mira antibody. Neurons are labelled using anti-Elav. (A) Schematic drawing representing a ventral view of 1d adult brain and VNCs.
Figure 1 continued

the late larval (L3) and adult Drosophila CNS. Ventral nerve cord (VNC). NBs are represented as red circles. The pox\(^n\)-GAL4 driver is active in six lateral NBs of the larval VNC (marked in green on the scheme). In pox\(^n\)-GAL4, UAS-GFP larvae (pox\(^n\)>GFP), GFP labels the six NBs (white arrows) and their recently generated progeny due to transient GAL4 and GFP perdurance. All NBs are absent in the adult VNC. (B) In pox\(^n\)-GAL4, UAS-pros\(^{RNAi}\), UAS-GFP, UAS-dcr2 larvae (pox\(^n\)>pros\(^{RNAi}\), GFP), six tumors of dNBs are generated. dNBs are represented on the scheme as green circles filled in red. A subset of dNBs persist and form small tumors in 1 day-old adult VNCs. (C) In 6 day-old adults, pox\(^n\)>pros\(^{RNAi}\), GFP tumors cover the whole VNC and invade adjacent tissues such as the brain, and halteres (D) Mean tumor volumes quantified in wt pox\(^n\)>GFP adult VNCs and in pox\(^n\)>pros\(^{RNAi}\), GFP 1 and 6 day-old adult VNCs. No tumor is observed in wt adults. 1 day-old pox\(^n\)>pros\(^{RNAi}\), GFP VNCs (n= 5 VNCs, m = 1.4x10\(^5\), SEM = 6.3x10\(^4\)) and 6 day-old pox\(^n\)>pros\(^{RNAi}\), GFP VNCs (n = 7 VNCs, m = 1.5x10\(^5\), SEM = 1.8x10\(^5\)), p-value is 2.5x10\(^{-3}\). (E) pox\(^n\)>pros\(^{RNAi}\), GFP tumors are almost exclusively composed of dNBs in late L3, and devoid of neurons (Elav). At around 20 hr after pupa formation, a brief pulse of neuronal differentiation in pox\(^n\)>pros\(^{RNAi}\), GFP tumors is seen. GFP briefly labels recently differentiated Elav\(^+\) neurons due to transient GAL4 perdurance. In adults, persisting dNBs reconstitute malignant tumors.

DOI: 10.7554/eLife.13463.003
Figure 1—figure supplement 1. Progeny-to-NSC dedifferentiation and temporal progression in the developing central nervous system of Drosophila. (A) Type-I and Type-II NBs in the larval central nervous system. Type-I NBs (large red circle) divide asymmetrically to self-renew and generate GMCs (medium red circle with nuclear Pros), which divide once to generate two neurons (small gray circles with nuclear Nerfin) or glia. Type-II NBs divide asymmetrically to self-renew and generate immature Intermediate Neural Precursors (imINPs, expressing Brat). imINPs maturate into INPs and undergo several rounds of asymmetric divisions to self-renew and generate GMCs (expressing nuclear Pros+). Each GMC divides once, giving birth to two differentiating neurons or glial cells. (B) During embryonic and larval development, each VNC NB sequentially expresses a series of transcription factors (Hunchback (Hb), Kruppel (Kr), Pdm1/2 (Pdm), Castor (Cas) and Seven-up (Svp)) inherited by the GMC. The various GMCs subsequently generate different types of neurons (differently colored circles), which identity is determined by their birth-order. NBs that are mutant for temporal factors, such as cas or svp, fail to progress further in the series and continuously generate the same type of neurons. Such NBs fail to exit the cell cycle during pupal stages and continue dividing during adulthood.
DOI: 10.7554/eLife.13463.004
**Figure 1—Figure supplement 2.** pox^{n-}\text{pros}^{\text{RNAi}}, GFP late L3

pox^{n-}\text{pros}^{\text{RNAi}} larvae possess tumors in the VNC but not in the brain. pox^{n-}\text{-Gal4} expression (GFP staining) in the larval CNS is limited to six lateral NBs and their closed progeny in the VNC (1). Thus, removing pros by RNAi using pox^{n-}\text{-Gal4} leads to the formation of six tumors in the VNC. pox^{n-}\text{-Gal4} is not active in brain NBs, although being expressed in a small subset of neurons (2 and 3). Consequently, no tumors are generated in the brain. NBs are labeled with Mira in red, and the neurons are labeled with Elav in blue.

DOI: 10.7554/eLife.13463.005
Figure 1—figure supplement 3. L1/L2-induced MARCM pros<sup>-/-</sup> clones generate malignant tumors in adult. (A) wt late L3 NBs express Mira and generate neurons labeled with anti-Elav. NBs are absent in adult VNCs. GFP<sup>+</sup> pros<sup>-/-</sup>
Figure 1—figure supplement 3 continued

MARCM clones induced during early larval (L1/L2) development generate dNBs that form tumors that persist in adults and cover the VNC. (B) In late L3, GFP+ pros− MARCM clones induced during L1/L2 are almost exclusively composed of dNBs (Mira+) and are devoid of neurons (Elav−). At around 20 hr after pupa formation, a large number of dNBs differentiates into Elav+ neurons. In adults, persisting dNBs reconstitute malignant tumors. Yellow asterisk indicates axonal bundles induced by earlier neuronal differentiation of dNBs during metamorphosis.

DOI: 10.7554/eLife.13463.006
Figure 2. Chinmo is ectopically expressed in tumors induced by dedifferentiation. All clones are induced in L1 (24 hr after larval hatching) using MARCM and labeled with GFP. (A) NBs in wt clones (arrows) have silenced Chinmo at late L3 stages. Note that at this stage, Chinmo remains strongly expressed in early-born neurons (empty arrowheads). In adults, NBs are absent in wt clones. Chinmo is not expressed anymore in early-born neurons in adults. (B) NBs (arrows) in svp-/- clones maintain Chinmo in late L3. Chinmo is also maintained in svp-/- NBs persisting in adults and their newly generated neurons (arrow). (C) pox+ NBs in late L3 have silenced Chinmo (arrows) while Chinmo expression is observed in early-born neurons (empty arrowheads). (D) A subset of pox>pros RNAi dNBs maintains Chinmo in late L3 and adults. (E) A subset of dNBs in VNC pros-/- MARCM clones maintains Chinmo at late L3 stages. All surrounding wt NBs have silenced Chinmo (asterisks). Aberrant Chinmo expression is maintained in a subset of dNBs in adult pros-/- clones. (F) A subset of dNBs in nerfin-/- clones maintains Chinmo in late L3 and adult VNCs. (G) A subset of dNBs induced in brat-/- clones maintains Chinmo in late L3 and adult brains. (H) During early development (from L1 to mid-L3), Chinmo (purple) is expressed in NBs and early-born neurons. It is silenced in NBs during mid-larval stages by the progression of the temporal series. In svp-/- mutant NBs, Chinmo is maintained in NBs and their progeny up to adulthood. In pros-/-, nerfin-/- or brat-/- tumors, Chinmo escapes temporal regulation in a subpopulation of dNBs and remains expressed in tumors as development progresses.

DOI: 10.7554/eLife.13463.007
The temporal factors Hb, Kr and Pdm and the Svp nuclear receptor are not expressed in larval prosRNAi tumors. The embryonic temporal factors Hb, Kr and Pdm and the Svp receptor are not expressed in pox>n>GFP wt NBs neither in pox>n>prosRNAi tumors during late larval stages. wt NBs never express Cas during late larval stages, whereas dNBs occasionally express Cas. pox>n-Gal4 is marked with GFP, NBs and dNBs are stained with Mira and delimited with dashed lines, and neurons are stained with Elav.

DOI: 10.7554/eLife.13463.008
Late L3

GFP Mira

Adult

MARCM pros−/−

MARCM cas−/−, pros−/−

Figure 2—figure supplement 2. Ectopic expression of the temporal factor Cas in early-induced pros−/− tumors does not contribute to persistence in adult. pros−/− cas−/− MARCM clones were induced in late L2. At this stage, the endogenous pulse of Cas occurring in early L2 NBs has passed. Therefore, loss of Cas does not block temporal progression in the NB from which the tumor originate, and only Cas expression in dNBs is eliminated. pros−/−, cas−/− MARCM clones are still able to generate large adult tumors, like pros−/− NB clones. Therefore, ectopic Castor in dNBs is not responsible for pros−/− tumor persistence and growth after metamorphosis.

DOI: 10.7554/eLife.13463.009
Figure 2—figure supplement 3. Chinmo is a marker of early-born neurons in type-II lineages. wt MARCM clone (GFP⁺) labeling a type-II lineage. Ase is exclusively expressed in the INP arising from Type-II NB. Images represent three sections through a type-II lineage. Deep early-born neurons express Chinmo.

DOI: 10.7554/eLife.13463.010
Figure 2—figure supplement 4. Chinmo is expressed in young but not old NBs. Chinmo is expressed in young post-embryonic NBs and their progeny from L1 to early L3 (arrows). Passed 60 hr after larval hatching (ALH) Chinmo is silenced in NBs and subsequently generated neurons (arrows). Note that Chinmo is kept expressed in early-born neurons (empty arrowheads) until early pupal stages.

DOI: 10.7554/eLife.13463.011
Figure 2—figure supplement 5. Percentage of Chinmo-expressing dNBs in tumors. Percentage of Chinmo\(^+\) dNBs in *pox\(^\text{prosRNAi}\)* larval and 6-day old adult tumors. Late L3 (n = 6 VNCs, 2888 dNBs, m = 15.46, SEM = 2.13), 6-day old adults (n=8 VNCs, 5308 dNBs, m = 18.47, SEM = 1.28).
DOI: 10.7554/eLife.13463.012
Figure 3. Chinmo sustains tumor growth beyond developmental stages. All clones were induced in L1/L2. (A) Expression of prosRNAi in Flp-out clones induces malignant tumors, covering the VNC in adults. (B) Expression of both prosRNAi and chinmoRNAi in Flp-out clones induces tumors that fail to grow further in the adult VNC. (C) Mean tumor volume in Flp-out prosRNAi and prosRNAi;chinmoRNAi clones induced during early larval stages quantified in the VNC of wandering L3 (wL3), 1 day-old and 6 day-old adults. wL3: prosRNAi (n = 6 VNCs, m = 7.4x10^4, SEM=2.1x10^4), prosRNAi;chinmoRNAi (n = 7 VNCs, m = 2.3x10^5, SEM = 5.7x10^4); 1 day-old adult: prosRNAi (n = 3 VNCs, m = 4.7x10^5, SEM = 2.5x10^5), prosRNAi;chinmoRNAi (n = 5 VNCs, m = 9.5x10^4, SEM = 2.5x10^4); 6 day-old adult: prosRNAi (n = 5 VNCs, m = 1.5x10^6, SEM = 2.1x10^5), prosRNAi;chinmoRNAi (n = 6 VNCs, m = 2.4x10^6, SEM = 1.9x10^5). p-values are respectively 2.2x10^-2, 3.6x10^-2 and 8.7x10^-3. (D) Mean percentage of PH3^+ dNBs in late L3 and 1 day-old adult Flp-out prosRNAi and prosRNAi;chinmoRNAi induced during early larval stages. Late L3: prosRNAi (n = 7 VNCs, m = 11.6, SEM=0.99), prosRNAi;chinmoRNAi (n = 6 VNCs, m = 8.06, SEM = 0.92); 1 day-old adult: prosRNAi (n = 4 VNCs, m = 10.92, SEM = 0.79), prosRNAi;chinmoRNAi (n = 4 VNCs, m = 1.97, SEM = 0.50). p-values are respectively 1.4x10^-2 and 2.9x10^-2; p-value between prosRNAi;chinmoRNAi wL3 and 1-day old adults is 9.5x10^-3. (E) Tumorigenic growth after transplantation of VNCs is assessed by the presence of GFP in the abdomen of transplanted flies after 7 days (p-value is 6.0x10^-6). (F) MARCM brat^-/-, chinmo^-/- clones induced during early larval stages rapidly lead to large malignant tumors in the adult brain. However, most clones undergo complete neuronal differentiation during metamorphosis, as shown with an absence of dNBs and large ectopic axonal bundles in adult clones (inset). Occasional remaining dNBs are not able to reconstitute large tumors (inset). Below, brat^-/-, chinmo^-/- clones are represented schematically during development. (G) MARCM brat^-/- clones induced during early larval stages rapidly lead to large malignant tumors in the adult brain. (H) Mean tumor volumes in MARCM brat^-/- and brat^-/-, chinmo^-/- clones induced during early larval stages quantified in 6 day-old adult central brains. MARCM brat^-/- clones (n = 4 brains, m = 3.0x10^6, SEM = 1.0x10^6) and MARCM brat^-/-, chinmo^-/- clones (n = 13 brains, m = 7.1x10^6, SEM = 3.3x10^6). p-value is 8.4x10^-4. (I) Overexpression of chinmo in Flp-out clones induces NB amplification in larvae (yellow dotted line), giving rise to tumors composed of proliferating dNBs in adults.

DOI: 10.7554/eLife.13463.013
Figure 3—figure supplement 1. Chinmo knock-down leads to reduced tumor growth and increased differentiation. (A) chinmoRNAi expression in prosRNAi tumors using nab-GAL4 efficiently knocks down Chinmo expression (remaining Chinmo+ cells are neurons in which chinmoRNAi is not expressed). (B) When prosRNAi is expressed in all larval NBs using nab-GAL4, VNCs becomes very large in late L3 and are mainly composed of dNBs at the expense of neurons. In pharate adults, some dNBs have differentiated in neurons, but many dNBs continue proliferating giving rise to large and deformed VNCs. nab>prosRNAi; chinmoRNAi also induces dNBs but VNCs are smaller. In pharate adults, many dNBs have differentiated into neurons, leading to tumor regression in VNCs. Pharate adult pictures are projections of several confocal sections. (C) prosRNAi and prosRNAi; chinmoRNAi respectively expressed in a restricted subset of NBs using eg-GAL4. eg>prosRNAi induces larger NB amplification than eg>prosRNAi; chinmoRNAi (dashed line) in late L3. (D) Mean larval tumor volume in nab>prosRNAi (n = 16 brains, m = 42753198, SEM = 3323616), nab>prosRNAi; chinmoRNAi (n = 12 brains, m = 17419207, SEM = 1127497) and nab>prosRNAi; chinmoRNAi (n = 21 brains, m = 60341998, SEM = 2099849), p-values are respectively 2.6x10^-7 and 7.9x10^-5. (E) dNB cell diameter is decreased when chinmo expression is knocked down (prosRNAi; chinmoRNAi), and is increased when Chinmo is overexpressed (prosRNAi; chinmo). prosRNAi (n = 4 VNCs, m = 5.00, SEM = 0.05), prosRNAi; chinmoRNAi (n = 4 VNCs, 550 dNBs, m = 4.08, SEM = 0.05), prosRNAi; chinmo (n = 4 VNCs, 319 dNBs, m = 7.22, SEM = 0.11). p-values are respectively 1.8x10^-5 and 3.3x10^-5. (F) The number of dNBs per larval tumor is reduced by Chinmo knockdown in eg>prosRNAi. ProsRNAi (n = 14, m = 298.5, SEM = 31.3), prosRNAi; chinmoRNAi (n = 4 VNCs, 550 dNBs, m = 4.08, SEM = 0.05), prosRNAi; chinmo (n = 4 VNCs, 319 dNBs, m = 7.22, SEM = 0.11). p-values are respectively 1.8x10^-5 and 3.3x10^-5. Figure 3—figure supplement 1 continued on next page.
Figure 3—figure supplement 1 continued

- 12, m = 80.7, SEM = 6.5), prosRNAi; chinmoRNAi2 (n = 26, m = 61.9, SEM = 3.3), p-values are respectively 1.7x10^{-5} and 2.6x10^{-7}. (G) Mean percentage of PH3+ dNBs in late L3 nab>prosRNAi (n = 6 VNCs, m = 7.75, SEM = 0.53) and nab>prosRNAi; chinmoRNAi (n = 6 VNCs, m = 4.77, SEM = 0.52), p-value is 2.2x10^{-3}. (H) In nab>prosRNAi, chinmoRNAi pharates the proportion of dNBs (Volume of Mira labeling/ Total volume of the VNC) is decreased compared to nab>prosRNAi pharates, while the proportion of neurons (Volume of Elav labeling/ Total volume of the VNC) is increased. Vmira/Vtotal for prosRNAi (n = 5 VNCs, m = 0.764, SEM = 0.030), Vmira/Vtotal for prosRNAi; chinmoRNAi (n = 4 VNCs, m = 0.310, SEM = 0.055), p-value=1.6x10^{-2}. Velav/Vtotal for prosRNAi (n = 5 VNCs, m = 0.236, SEM = 0.030), Velav/Vtotal for prosRNAi; chinmoRNAi (n = 4 VNCs, m = 0.690, SEM = 0.055), p-value=1.6x10^{-2}. DOI: 10.7554/eLife.13463.014
Figure 3—figure supplement 2. pros\textsuperscript{RNAi}; chinmo\textsuperscript{RNAi} tumors fail to become malignant in adults. (A) Adults with pros\textsuperscript{RNAi} Flp-out (FO) clones induced in L1/L2 more frequently contain tumors than adults with pros\textsuperscript{RNAi}; chinmo\textsuperscript{RNAi} clones. In the brain of 1 day-old adults, 79% of animals with pros\textsuperscript{RNAi} clones (n = 24 VNCs and brains) and 53% of animals with pros\textsuperscript{RNAi}; chinmo\textsuperscript{RNAi} clones (n = 17 VNCs and brains) contain tumors, p-value is 0.048. In the brain of 6 day-old adults, 78% of animals with pros\textsuperscript{RNAi} clones and 33% of animals with pros\textsuperscript{RNAi}; chinmo\textsuperscript{RNAi} clones contain tumors (n = 18). In the VNC of 6 day-old adults, 80% of animals with pros\textsuperscript{RNAi} clones (n = 18 VNCs and brains) and 25% of animals with pros\textsuperscript{RNAi}; chinmo\textsuperscript{RNAi} clones (n = 18 VNCs and brains) contain tumors. p-value is 0.018. Results are provided by at least 2 independent experiments. (B) Mean tumor volume in Flp-out pros\textsuperscript{RNAi} and pros\textsuperscript{RNAi}; chinmo\textsuperscript{RNAi} induced in L1/L2 and quantified in the brain of late larvae, 1 day-old and 6 day-old adults. Late larval brain pros\textsuperscript{RNAi} (n = 7 brains, m = 3.3\times10^4, SEM = 4.2\times10^3), pros\textsuperscript{RNAi}; chinmo\textsuperscript{RNAi} (n = 5 brains, m = 1.0\times10^5, SEM = 1.2\times10^5); 1 day-old adult brain pros\textsuperscript{RNAi} (n = 8 brains, m = 8.5\times10^5, SEM = 3.0\times10^5), pros\textsuperscript{RNAi}; chinmo\textsuperscript{RNAi} (n = 5 brains, m = 6.5\times10^4, SEM = 2.4\times10^4); 6 day-old adult brain pros\textsuperscript{RNAi} (n = 8 brains, m = 1.7\times10^6, SEM = 3.0\times10^6), pros\textsuperscript{RNAi}; chinmo\textsuperscript{RNAi} (n = 8 brains, m = 6.3\times10^5, SEM = 1.4\times10^5), p-values are respectively 2.5\times10^{-3}, 1.6\times10^{-3} and 1.6\times10^{-4}. (C) In 6 day-old adult Flp-out pros\textsuperscript{RNAi}; chinmo\textsuperscript{RNAi} clones, remaining dNBs sometimes appear quiescent (characterized by cytoplasmic extensions). DOI: 10.7554/eLife.13463.015
Chinmo+ dNBs exhibit a higher mitotic index than Chinmo- dNBs within proRNAi tumors. (A) Dividing Chinmo+ and Chinmo- (red) dNBs marked with anti-PH3 (blue) in late L3 and adults pro>proRNAi tumors. (B) Quantification of the mitotic index of wt NBs and pro>proRNAi dNBs in late L3 and adults. Chinmo+ dNBs possess a higher mitotic index than Chinmo- dNBs both in larval and adult tumors. Late L3 wt (n = 3 VNCs, 464 NBs, m = 21.11, SEM = 0.15), late L3 pro>proRNAi Chinmo+ cells (n = 11 VNCs, 812 dNBs, m = 11.47, SEM = 0.09), late L3 pro>proRNAi Chinmo- cells (n = 11 VNCs, 4997 dNBs, m = 7.63, SEM = 0.01), adult pro>proRNAi Chinmo+ cells (n = 12 VNCs, 303 dNBs, m = 12.48, SEM = 0.14), adult pro>proRNAi Chinmo- cells (n = 12 VNCs, 1673 dNBs, m = 6.42, SEM = 0.05), p-values are respectively 1.1x10^-5 and 8.0x10^-5.

DOI: 10.7554/eLife.13463.016

Figure 3—figure supplement 3.
Figure 4. Chinmo promotes Imp and Lin-28 expression. (A) prosRNAi is expressed in all larval NBs using nab-GAL4. RNA-seq indicated 214 genes to be commonly up-regulated, and 388 genes were found to be commonly downregulated, when comparing nab>prosRNAi vs. nab>prosRNAi, chinmoRNAi and...
Figure 4 continued

nab>pros\textsuperscript{RNAi}, chinmo vs. nab>pros\textsuperscript{RNAi} (adj p-value < 0.05). (B) Graphical representation of the log2 fold change as a function of the base mean expression of Chinmo targets, comparing nab>pros\textsuperscript{RNAi}, chinmo to nab>pros\textsuperscript{RNAi}, chinmo\textsuperscript{RNAi}. lin-28, Imp and chinmo are highlighted in red. (C) Lin-28, Imp (cytoplasmic) and Chinmo (nuclear) are co-expressed in the same subset of dNBs in pox\textsuperscript{>pros\textsuperscript{RNAi}} larval and adult tumors (delineated with the yellow dashed lines). (D) Clonal mis-expression of Chinmo in GFP\textsuperscript+ Flp-out clones induced in L1, delimited by yellow dashed lines, induces Imp and Lin-28 co-expression in dNBs.

DOI: 10.7554/eLife.13463.017

The following source data is available for figure 4:

Source data 1. Differentially expressed genes and enriched GO terms and KEGG pathways between pros\textsuperscript{RNAi} tumors expressing various levels of Chinmo.

DOI: 10.7554/eLife.13463.018
Figure 4—figure supplement 1. Transcriptional analysis summary. (A) GO enrichment for down-regulated genes (negative Chinmo targets). (B) GO pathway enrichment for up-regulated genes (positive Chinmo targets). (C) KEGG pathway enrichment for up-regulated genes (positive Chinmo targets). (D) Top 12 up-regulated Chinmo targets when comparing pox^+>pros^RNAi, chinmo with pox^+>pros^RNAi, chinmo^RNAi. See Figure 4—source data 1 for lists of differentially expressed genes.

DOI: 10.7554/eLife.13463.019
Figure 4—figure supplement 2. Chinmo is necessary for Imp expression in tumors. (A,B) Imp is expressed in prosRNAi and brat−/− tumors induced in L1/L2. Imp is absent from prosRNAi, chinmoRNAi, and brat−/− chinmo−/− tumors.
DOI: 10.7554/eLife.13463.020
Figure 5. Imp sustains Chinmo expression in tumors. (A) Chinmo is expressed in a subset of dNBs in both larval (dashed yellow lines) and adult pox\textsuperscript{n}>pros\textsuperscript{RNAi} tumors (see enlargement). (B) Chinmo is still expressed in a subset of dNBs in larval pox\textsuperscript{n}>pros\textsuperscript{RNAi}, Imp\textsuperscript{RNAi} tumors but is progressively lost in adult tumors that remain small (see enlargement). pox\textsuperscript{n}>pros\textsuperscript{RNAi}, Imp tumors tend to have an increased number of Chinmo\textsuperscript{+} cells (not quantified). (C) Mean tumor volume of 6 day-old adults in pox\textsuperscript{n}>pros\textsuperscript{RNAi}, pox\textsuperscript{n}>pros\textsuperscript{RNAi}; Imp\textsuperscript{RNAi} and pox\textsuperscript{n}>pros\textsuperscript{RNAi}; Imp. pox\textsuperscript{n}>pros\textsuperscript{RNAi} (n = 6 VNCs, m = 1.1x10^6, SEM = 1.3x10^5), pox\textsuperscript{n}>pros\textsuperscript{RNAi}; Imp\textsuperscript{RNAi} (n = 8 VNCs, m = 9.5x10^4, SEM = 2.4x10^4), pox\textsuperscript{n}>pros\textsuperscript{RNAi}; Imp (n = 6 VNCs, m = 1.0x10^6, SEM = 1.3x10^5). p-values are respectively 6.7x10^{-4} and 0.82.

DOI: 10.7554/eLife.13463.021
Figure 5—figure supplement 1. Imp mis-expression is not sufficient to initiate tumors. Clonal mis-expression of Imp in larval GFP+ Flip-out clones induced in L1/L2 does not trigger supernumerary NB, neither ectopic expression of Chinmo in NBs.

DOI: 10.7554/eLife.13463.022
**Figure 5—figure supplement 2.** Imp knock-down decreases tumor growth. (A) Imp RNAi efficiently knocks down Imp in larval dNBs induced by pox^{n}>pros^{RNAi}, Imp^{RNAi1,2}. Note that a subset of dNBs continues expressing Chinmo. (B) Mean tumor volume per VNC in pox^{n}>pros^{RNAi} and pox^{n}>pros^{RNAi}; Imp^{RNAi1,2} in 5 day-old L3 reared on normal food. pros^{RNAi} (n = 6 VNCs, m = 482720, SEM = 56988), pros^{RNAi}; Imp^{RNAi1,2} (n = 5 VNCs, m = 217124, SEM = 33046), p-value=4.3x10^{-3}.

DOI: 10.7554/eLife.13463.023
Figure 5—figure supplement 3. Imp is necessary to sustain tumor growth and Chinmo expression. After 10 days in the sterol-free food, $\text{pox}^\text{n}>\text{prosRNAi}$ larval tumors become very large, invade the central brain and optic lobes (yellow arrows) and maintain Chinmo expression (see enlargement). In contrast, tumor growth is strongly reduced and Chinmo is mostly absent (see enlargement) in $\text{pox}^\text{n}>\text{prosRNAi}, \text{ImpRNAi1;2}$ tumors. (B) Mean tumor volume per VNC in $\text{pox}^\text{n}>\text{prosRNAi}$ and $\text{pox}^\text{n}>\text{prosRNAi}, \text{ImpRNAi1;2}$ in 10 day-old larvae reared on sterol-free food. $\text{prosRNAi}$ (n = 5 VNCs, m = 40859191, SEM = 15192219), $\text{prosRNAi}; \text{ImpRNAi1;2}$ (n = 6 VNCs, m = 6012717, SEM = 822265), p-value=4.3x10^{-7}. DOI: 10.7554/eLife.13463.024
Figure 6. Chinmo, Imp and Lin-28 form an oncogenic loop. (A) Chinmo expression in 5 day-old adult VNCs color-coded relative to staining intensity. All transgenes are expressed with the pox-GAL4 driver. Humanized tumors mis-express human LIN28A or LIN28B. (B) Ratio representing the volume of Chinmo+ cells over the total tumor volume in 5 day-old adult VNCs. pox>prosRNAi (n = 4 VNCs, m = 0.188, SEM = 0.017), pox>prosRNAi, dlin-28 (n = 6 VNCs, m = 0.583, SEM = 0.072) and pox>prosRNAi, LIN28A (n = 8 VNCs, m = 0.474, SEM = 0.060). p-values are respectively 9.5x10^-3 and 8.0x10^-3. (C) Mean Chinmo intensity in Chinmo+ cells. pox>prosRNAi (n = 7 tumors, m = 962, SEM = 37), pox>prosRNAi, dlin-28 (n = 8 tumors, m = 1413, SEM = 125) and pox>prosRNAi, UAS-LIN28A (n = 9 tumors, m = 1236, SEM = 110). Each sample is the mean of 3 different focal sections of the same tumor. p-values are respectively 5.9x10^-3 and 7.1x10^-2. (D) Representation of the observed cross-regulatory interactions composing the oncogenic module.

DOI: 10.7554/eLife.13463.025
Figure 6—figure supplement 1. Lin-28 is dispensable for tumor growth. (A) prosRNAi, Δlin28 MARCM clones induced in L1/L2 generate tumors that continue growing in adults and keep expressing Chinmo and Imp. (B) Mean tumor volumes in the brain of 6d adult Δlin28 homozygous flies containing bratΔ/Δ MARCM clones compared to MARCM bratΔ/Δ MARCM flies (n = 5 brains, m = 6.2x10^6, SEM = 9.4x10^5) and MARCM bratΔ/Δ MARCM flies in Δlin28 flies (n = 5 brains, m = 6.1x10^6, SEM = 8.5x10^5). p-value is 0.82.

DOI: 10.7554/eLife.13463.026
Figure 6—figure supplement 2. Lin-28 mis-expression is not sufficient to initiate tumors. Efficient clonal mis-expression of Lin-28 in larval GFP+ Flip-out clones does not induce NB amplification, neither ectopic expression of Chinmo and Imp in NBs.
DOI: 10.7554/eLife.13463.027
Figure 6—figure supplement 3. Lin-28 positively regulates chinmo and Imp in tumors. Overexpression of lin-28 in pox>prosRNAi tumors leads in adults to an increase in the number of Chinmo+ cells, all of which also express Imp.

DOI: 10.7554/eLife.13463.028
Figure 6—figure supplement 4. Schematic conclusions. Scheme depicting the conclusions from the experiments in Figure 6 and Figure 6-figure supplements. Chinmo, Imp and Lin-28 are coexpressed in a subset of dNBs in both larval and adult tumors. Imp is necessary for long-term maintenance of Chinmo and persistent tumor growth. Lin-28 over-expression can increase the proportion of Chinmo⁺/Imp⁺ dNBs in the tumor.

DOI: 10.7554/eLife.13463.029
Figure 7. The temporal series silences chinmo, Imp and lin-28 in NBs for their timely termination during development. (A) Chinmo, Lin-28 and Imp are coexpressed in wt VNC NBs in L2, and are silenced in NBs in late L3. Note that in late L3, surrounding early-born neurons keep expressing Imp and Chinmo whereas Lin-28 is down-regulated in all cells. (B) Imp is maintained in L1-induced MARCM svp^{-} NBs (GFP^{+}) in late L3. Lin-28 and Imp are maintained in MARCM svp^{-} NBs (GFP^{+}) that persist in adult. (C) wt and chinmo^{-} MARCM clones induced during embryogenesis. (D) Number of cells per clone in late L3 in VNC wt and chinmo^{-} MARCM clones induced during embryogenesis. wt MARCM 40A (n = 16 clones, m = 82, SEM = 4.7); chinmo^{-} MARCM clones (n=17 clones, m = 83, SEM = 4.8). p-values is 0.88. (E) Mean NB area in late L3 VNC wt and chinmo^{-} MARCM clones induced during embryogenesis. wt MARCM 40A (n = 45 NBs, m = 76.7, SEM = 3.2), chinmo^{-} MARCM clones (n = 46 NBs, m = 76.3, SEM = 2.9). p-value is 0.88. (F) Mean percentage of PH3^{+} NBs in late L3 VNC MARCM wt and chinmo^{-} clones induced during embryogenesis. wt clones (n = 4 VNCs, m = 20.6, SEM = 1.43), chinmo^{-} clones (n = 4 VNCs, m = 20.8, SEM = 2.23), p-value is 0.88. (G) NBs persist in adult MARCM svp^{-}, chinmoRNAi clones induced in L1. NBs are smaller (Figure 7—figure supplement 1A) and maintain Imp expression. Removing both Chinmo and Imp (chinmoRNAi, ImpRNAi) in a svp^{-} MARCM clone is sufficient to restore NB elimination before the end of development. (H) Schematic recapitulation of the above experiments.

DOI: 10.7554/eLife.13463.030
Lin-28 and Chinmo are respectively silenced in the CNS prior to and during metamorphosis, whereas Imp remains expressed in a subset of adult neurons. (A) Imp is expressed in a subset of wt late L3 Elav⁺ neurons, but is absent from wt L3 NBs. (B) Imp and Chinmo Figure 7—figure supplement 1 continued on next page
Figure 7—figure supplement 1 continued

are expressed in early-born neurons in late L3, but not in NBs. In contrast, Lin-28 is silenced in all neurons from late L3 (note that the Lin-28::Venus construction is expressed at very low levels in a subset of late L3 neurons, see Figure 4C). (C) Chinmo and Lin-28 are silenced in the the adult CNS while Imp remains expressed in a subset of neurons.

DOI: 10.7554/eLife.13463.031
Figure 7—figure supplement 2. Chinmo promotes the long-term growth of NBs stalled in an early temporal identity but is not required for Imp expression. (A) Mean NB area in MARCM svp^{-/-} and svp^{-/-}, chinmo^RNAi adults. svp^{-/-} (n = 15 NBs, m = 58.8, SEM = 3.18), svp^{-/-}, chinmo^RNAi (n = 11 NBs, m = 29.9, SEM = 2.51). p-value is 5.2x10^{-7}. (B) chinmo^RNAi efficiently knocks down Chinmo in temporally blocked svp^{-/-}, chinmo^RNAi larval NBs. Note that Chinmo is silenced in surrounding wt late L3 NBs (asterisk). Larval MARCM svp^{-/-}, chinmo^RNAi clones maintain Imp expression. Note that Imp is silenced in surrounding wt late L3 NBs (asterisks).

DOI: 10.7554/eLife.13463.032
Figure 8. pros−/− tumors induced in the VNC of midL3 larvae do not express Chinmo and do not grow in adults. (A) One day after early (L1/L2), induction most dNBs from pros−/− clones retain Chinmo expression. In contrast, Chinmo is absent from dNBs 1 day after late (midL3) clonal induction.

Figure 8 continued on next page
Figure 8 continued

(B) Three days after L1/L2-induction in larvae raised on the sterol-free diet, pros−/− clones contain Chinmo+ dNBs (54 out of 59). In contrast, 3 days after mid-L3 induction on the sterol-free diet, pros−/− clones do not contain Chinmo+ dNBs (26 out of 27). (C) Adult VNC containing L1/L2-induced pros−/− MARCM clones are covered by tumors. In contrast, midL3-induced pros−/− MARCM clones are rare and remain small in adult VNCs. (D) On the sterol-free diet, 7 days after L1/L2-induction, pros−/− clones keep proliferating and generate large tumors of GFP+ dNBs (Mira shown in red) that cover the whole CNS. In contrast, pros−/− clones, 7 days after midL3-induction, rapidly stop growing and dNBs exhibit quiescence markers such as the loss of GFP and cytoplasmic extensions (arrow emphasizes cytoplasmic extensions from dNBs, arrowhead emphasizes a cytoplasmic extension from a wt NB).

DOI: 10.7554/eLife.13463.033
Figure 8—figure supplement 1. Schematic conclusion. Schematic representation recapitulating the conclusions from experiments in Figure 8. When metamorphosis is prevented with a sterol-free diet, NBs and Chinmo\textsuperscript{-dNBs} fail to terminally differentiate but exhibit a limited proliferation potential and enter quiescence (marked by cytoplasmic extensions).

DOI: 10.7554/eLife.13463.034
Figure 8—figure supplement 2. The proliferation potential of normal NBs decreases over time while dNBs continue proliferating. (A) Mean % of PH3+ dNBs in L1/L2- and midL3-induced tumors after 10 days on the sterol-free diet. Early induction (n = 5 VNCs, 5 clones, m = 5.7, SEM = 1.30), late induction (n = 6 VNCs, 96 clones, m = 1.9, SEM = 0.05), p-value is 3.1x10^{-3}. (B) Larvae reared on the sterol-free diet do not pupariate. In 4 day-old larvae, wt NBs are large, highly active and Mira is cytoplasmic. In 10 day-old larvae, most wt NBs are small with nuclear Mira, possess cytoplasmic extensions (inset, yellow arrowhead). Pictures are projections of several confocal sections. (C) Mean percentage of PH3+ wt NBs. 4 day-old larvae (n = 4 VNCs, 319 NBs, m = 26.59, SEM = 0.41), 10 day-old larvae (n = 7 VNCs, 701 NBs, m = 5.19, SEM = 0.05), p-value is 6.1x10^{-3}. DOI: 10.7554/eLife.13463.035
Figure 9. The temporal series regulates the malignant susceptibility of neural cells born during development. (A) Larvae were raised at 18°C and heat-shocked in L1 to induce svp⁻/⁻ MARCM clones. Controls were kept at 18°C, to prevent prosRNAi expression, leading to the persistence of a single NB.
per clone in adults. In contrast, if larvae are switched to 29°C from late L3, prosRNAi is expressed leading to large tumors in adults, exclusively composed of dNBs expressing Chinmo, Imp and Lin-28. (B) Schematic recapitulation of the above experiments. Blocking temporal progression in NBs extends the window of malignant susceptibility.

DOI: 10.7554/eLife.13463.036