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The Fd4 transcription factor translates transient spatial cues in progenitors into long-term lineage identity

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eLife Assessment

This **important** study focuses on the molecular mechanisms underlying the generation of neuronal diversity. Taking advantage of a well-defined neuroblast lineage in *Drosophila*, the authors provide **convincing** evidence that two transcription factors of the conserved forkhead box (FOX) family provide a mechanistic link between transient spatial cues that initially specify neuroblast identity and terminal selector genes that define post-mitotic neuron identity. The findings will be of interest to developmental neurobiologists.

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Abstract

Neural diversity is required for the brain to generate complex behaviors. During development, neural progenitors are exposed to different combination of transient spatial cues for their identity specification. This identity is then interpreted by their progeny to activate terminal selector genes to become lineage-specific neurons. After spatial cues fade, it remains unclear how progenitors maintain their unique identity so that their progeny express the accurate, lineage-specific terminal selector genes. Using single cell RNA sequencing in *Drosophila*, we identified a Forkhead domain transcription factor, Fd4, that is exclusively expressed in a single neural progenitor (neuroblast) and its new-born progeny. This neuroblast (NB), named NB7-1, forms at the intersection of the transient spatial cues Vnd (columnar expression) and En (row expression). We show that Fd4 expression overlaps spatial factor expression and terminal selector gene expression, thereby making Fd4 an excellent candidate for bridging transient spatial factors to lineage-specific terminal selector genes. We show that Fd4 is required for expression of terminal selector genes that maintain neuronal identity. Conversely, Fd4 misexpression generates ectopic NB7-1 progeny at the expense of Fd4-negative progenitor lineages. We conclude that Fd4 is continuously expressed in the NB7-1 and its new-born neuronal progeny where it activates terminal selector genes to produce lineage-specific neurons. We propose that Fd4 is a pioneering member of a class of “lineage identity genes” that translate transient spatial cues into a long-term lineage identity.

Introduction

From flies to mammals, neurogenesis begins with the formation of a small population of neural stem cells that generate a large diversity of neurons necessary to generate complex behaviors. In the mammalian nervous system, Hox genes play a critical role in patterning and segmenting the rostrocaudal axis of the neural tube. Opposing gradients of morphogen Sonic hedgehog (Shh) and Wnt/BMP signaling then establish dorsoventral patterning. Within the domains shaped by the interaction of Hox genes and Shh/Wnt/BMP signals, homeobox genes including Pax, Nkx, and Dbx are expressed in a spatially restricted patterns, subdividing neural tube into distinct progenitor domains. Each of these domains subsequently expresses distinct combinations of terminal selector genes that maintain neuronal identity (Philippidou and Dasen, 2013 [DOI](#); Sagner and Briscoe, 2019 [DOI](#)).

In the *Drosophila* nervous system, Hox genes also pattern the anterior-posterior axis of the neuroepithelium (Technau et al., 2014). However, individual neuroblast identity within each segment is determined by the combinatorial action of “spatial transcription factors” (STFs) expressed in rows and columns in every segment (Skeath and Thor, 2003). The columnar genes *ventral nervous system defective* (*vnd*), *intermediate neuroblast defective* (*ind*) and *muscle segment homeobox* (*msh*; FlyBase: *Dr*) subdivide the neuroepithelium into ventral, intermediate and dorsal columns, respectively (Isshiki et al., 1997; McDonald et al., 1998; Weiss et al., 1998). Similarly, the row genes *mirror* (*mirr*), *hedgehog* (*hh*), *wingless* (*wg*), *gooseberry* (*gsb*), and *engrailed* (*en*), subdivide the neuroepithelium along the anterior-posterior axis. Together, the row and column genes subdivide neuroectoderm into a chessboard-like pattern (Skeath and Thor, 2003), with each ‘square’ of this pattern consisting of a unique STF combination. All three columnar genes function to specify neuroblast columnar identity (Isshiki et al., 1997; McDonald et al., 1998; Weiss et al., 1998); similarly, the row genes *en*, *hh*, *wg* and *gsb* specify neuroblast row 1-7 identity (Anderson et al., 2025; Bhat, 1996; Chu-LaGraff and Doe, 1993; Skeath et al., 1995; Zhang et al., 1994). Subsequently, most neuroblasts sequentially express a cascade of “temporal transcription factors” (TTFs) that diversify neurons in each neuroblast lineage (Doe, 2017; Isshiki et al., 2001; Pollington et al., 2022). The integration of STF and TTF expression activates downstream terminal selection genes, generating a diversity of neuronal types (Gabilondo et al., 2016; Stratmann and Thor, 2017; Stratmann et al., 2016).

After STFs establish the neuroblast identity, their expression fades away prior to expression of terminal selector genes, which are typically homeodomain (HD) TFs. HDTFs are widely conserved proteins that are first expressed in new-born post-mitotic neurons (Doe and Thor, 2024; Hobert, 2016; Leung et al., 2022). This raises the question: what factors act to bridge transient STF expression that specifies initial neuroblast identity to expression of lineage-specific HDTFs that consolidate and maintain neuronal identity? Here we report on the expression and function of the transcription factor Fd4 (Flybase: *fd96Ca*). We show that it (1) is expressed continuously in NB7-1 during embryonic stages, (2) is transiently expressed in new-born neurons in this lineage, (3) is necessary to specify NB7-1 progeny identity, and (4) is sufficient to induce ectopic NB7-1 identity. This places Fd4 as downstream of STFs but upstream of terminal selector genes. We propose that Fd4 acts as a *neuroblast lineage identity gene* that acts as a bridge linking transient spatial cues that specify initial neuroblast identity to the terminal selector genes that maintain neuroblast progeny identity.

Results

Fd4 is transiently co-expressed with STFs and is maintained throughout the NB7-1 lineage

FD4 is specifically expressed in NB7-1 (Anderson et al., 2025) and first detectable in NB7-1 at stage 11, where it is co-expressed with En and Vnd STFs (Figure 1A, Supp Figure 1). Expression of Fd4 in NB7-1 persists at least until third instar larval stage (data not shown). In contrast, spatial factors En and Vnd are transiently expressed in the neuroectoderm prior to NB7-1 formation (Chu et al., 1998; Jiménez et al., 1995; McDonald et al., 1998; Mellerick and Nirenberg, 1995), in NB7-1 prior to Fd4 expression, and are downregulated by stage 13 (Figure 1A-B, Supp Figure 1). Thus, En and Vnd expression precedes Fd4, followed by a window of co-expression, and then the STFs disappear and Fd4 maintains expression (Figure 1C). This leads to the following hypotheses: (i) STFs specify the neuroblast and its early-born progeny, (ii) STFs and Fd4 act redundantly to specify mid-born neurons in the lineage, and (iii) Fd4 alone maintains neuroblast identity and specifies late-born neurons in the lineage (Figure 1D).

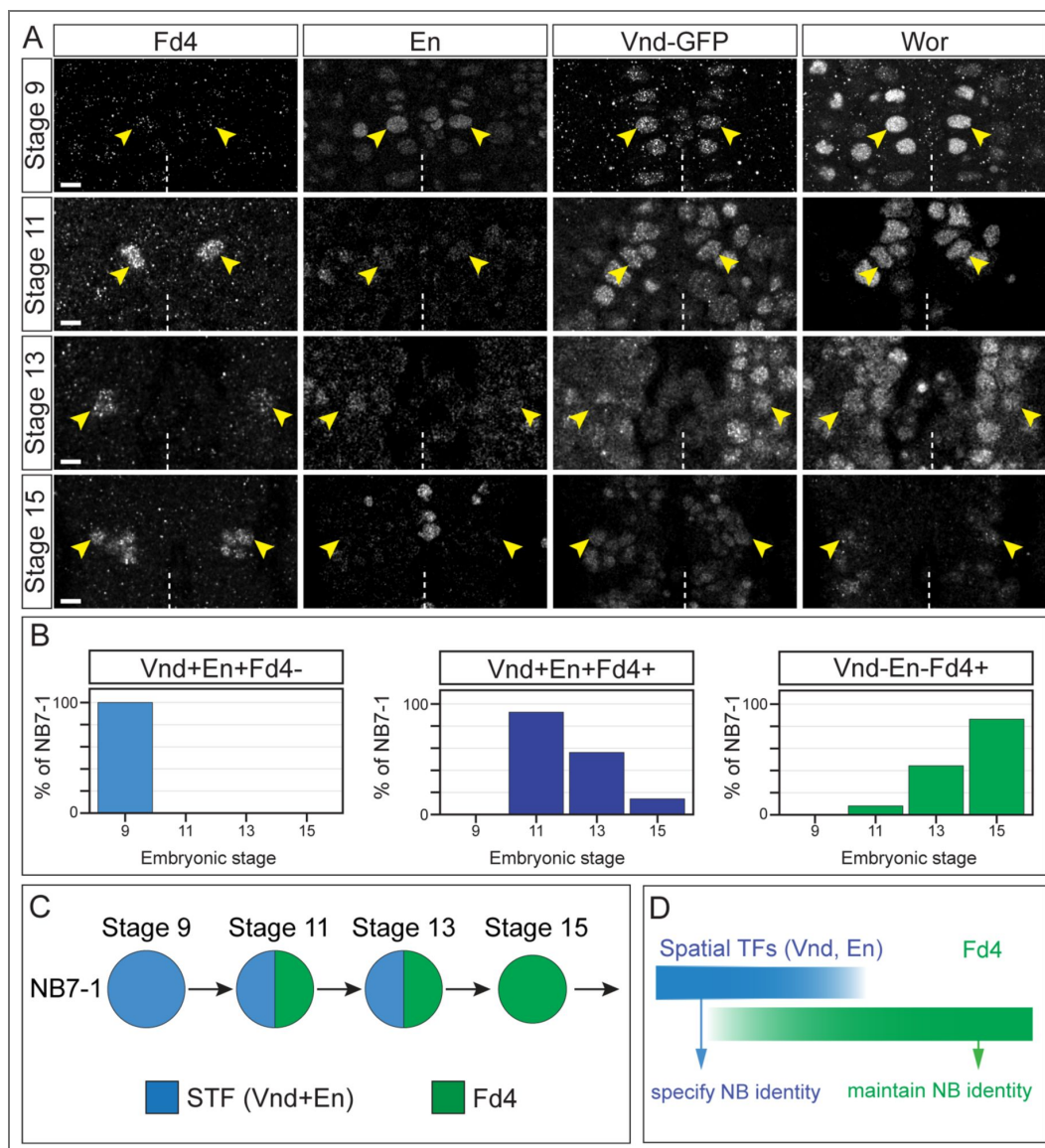


Figure 1. Fd4 is expressed in NB7-1 after the expression of spatial factors Vnd and En

(A) Expression of Fd4, En, Vnd and Wor in a segment during embryonic stage 9, 11, 13 and 15. Anterior, up. Dashed lines, ventral midline. Scale bar: 10 μ m. (B) Quantification of NB7-1 expressing various combination of En, Vnd, and Fd4. (C) Summary of expression for Fd4, and En Vnd double positive cells. STF: spatial transcription factors. (D) Proposed function of spatial transcription factors and Fd4 in neuroblast identity.

Fd4 is expressed in NBs, GMCs, and new-born neurons, but not in differentiated neurons

STFs are expressed in neuroblasts and GMCs, with little if any expression in post-mitotic neurons (Chen and Konstantinides, 2022 [↗](#)). In contrast, terminal selector genes (e.g. *Even-skipped*; *Eve*) are typically expressed in new-born post-mitotic neurons (Doe et al., 1988 [↗](#)). This raises the question: what factors act to bridge transient STF expression that specifies initial neuroblast identity to expression of lineage-specific HDTF terminal selectors that consolidate and maintain neuronal identity?

To determine the expression of Fd4 along the neuron differentiation axis (neuroblast > GMC > new-born neuron > mature neuron), we used an Fd4 antibody to examine its expression from neuroblast to neuron differentiation. We observe Fd4 protein in neuroblasts (Wor+), GMCs (Wor+Pros+), new-born neurons (Pros+Elav+ adjacent to the neuroblast), but not in mature neurons (Pros+Elav+ far from the neuroblast) (Figure 2A [↗](#)). We conclude that Fd4 expression initiates in NB7-1, is maintained in GMCs and new-born neurons, and is down-regulated in mature, differentiated neurons. This expression pattern effectively exposes all new-born neurons in the lineage to transient Fd4 at a time when they have not yet consolidated their neuronal identity.

To further validate our observations, we examined Fd4 expression in identified neurons within the NB7-1 lineage. NB7-1 sequentially produces five *Eve*+ U motor neurons (UMNs) from first-born U1 motor neuron to fifth-born U5 motor neuron. *Eve* is a terminal selector gene, and its continuous expression is required to maintain neuronal activity and function (Heckscher et al., 2015 [↗](#)). We find that Fd4 expression first becomes detectable at embryonic stage 10 (Figure 2B [↗](#)). Notably, Fd4 is strongly expressed in newly born *Eve*+ neurons and progressively declined as *Eve*+ neurons mature (Figure 2B-C [↗](#)). Thus, unlike *Eve*, Fd4 expression is transient in postmitotic neurons and restricted to the time immediately following their birth (summarized in Figure 2D [↗](#)). Taken together, we find that Fd4 is continuously expressed in NB7-1 but transiently expressed in new-born neurons. We propose that Fd4 is required to maintain NB7-1 lineage identity to prime new-born neurons to activate lineage appropriate terminal selector genes (Figure 2E [↗](#)).

Fd4 is required to specify NB7-1 progeny

Neuroblast identity is comprised of its molecular profile; neuroblast *lineage* identity is determined by the neural progeny each neuroblast produces. We have shown that Fd4 is expressed in a highly specific pattern – just one lineage – but in all the new-born neurons in that lineage, making Fd4 an excellent candidate to specify neuroblast lineage identity. To investigate this hypothesis, we assayed the production of NB7-1 progeny in *fd4* mutant and Fd4 misexpression paradigms. Within the NB7-1 lineage, early-born progeny express the terminal selector genes: *Eve*+ motor neurons and *Dbx*+ interneuron siblings (Lacin et al., 2009 [↗](#)). In total, eight *Dbx*+ interneurons are produced in this lineage: five are produced as siblings of *Eve*+ neurons, whereas the remaining three are born later, after *Cas* is expressed in NB7-1, and are *Cas*+ (see below).

In *fd4* mutants, we observe no change in the number of *Eve*+ neurons or *Dbx*+ neurons (data not shown). However, the tandem gene *fd5* (Flybase: *fd96Cb*) is also enriched in NB7-1 lineage (Anderson et al., 2025 [↗](#)) and acts redundantly with *fd4* during leg development (Ruiz-Losada et al., 2021 [↗](#)). Furthermore, we found that *fd5* is co-expressed with NB7-1 and its progeny (Supp Figure 2 [↗](#)). To assess their roles, we aimed to remove both *fd4* and *fd5* in NB7-1 lineage. Because many *Eve*+ and *Dbx*+ cells are generated outside of NB7-1 lineage, it was essential to identify the *Eve*+ or *Dbx*+ cells within NB7-1 lineage in wild type and *fd4* mutant embryos. To achieve this, we replaced the open reading frame of *fd4* with *gal4* (called *fd4-gal4*) in the *fd5* mutant background (see Methods), thereby simultaneously knocking out both *fd4* and *fd5* (called *fd4/fd5* mutant hereafter) while labeling the NB7-1 lineage. We find that in wild type, NB7-1 generates 28.6±2.6 cells, while *fd4/fd5* mutant produces 27.0±5.5 cells (Figure 3A [↗](#); quantified in 3B). Thus, Fd4 is not required for lineage development.

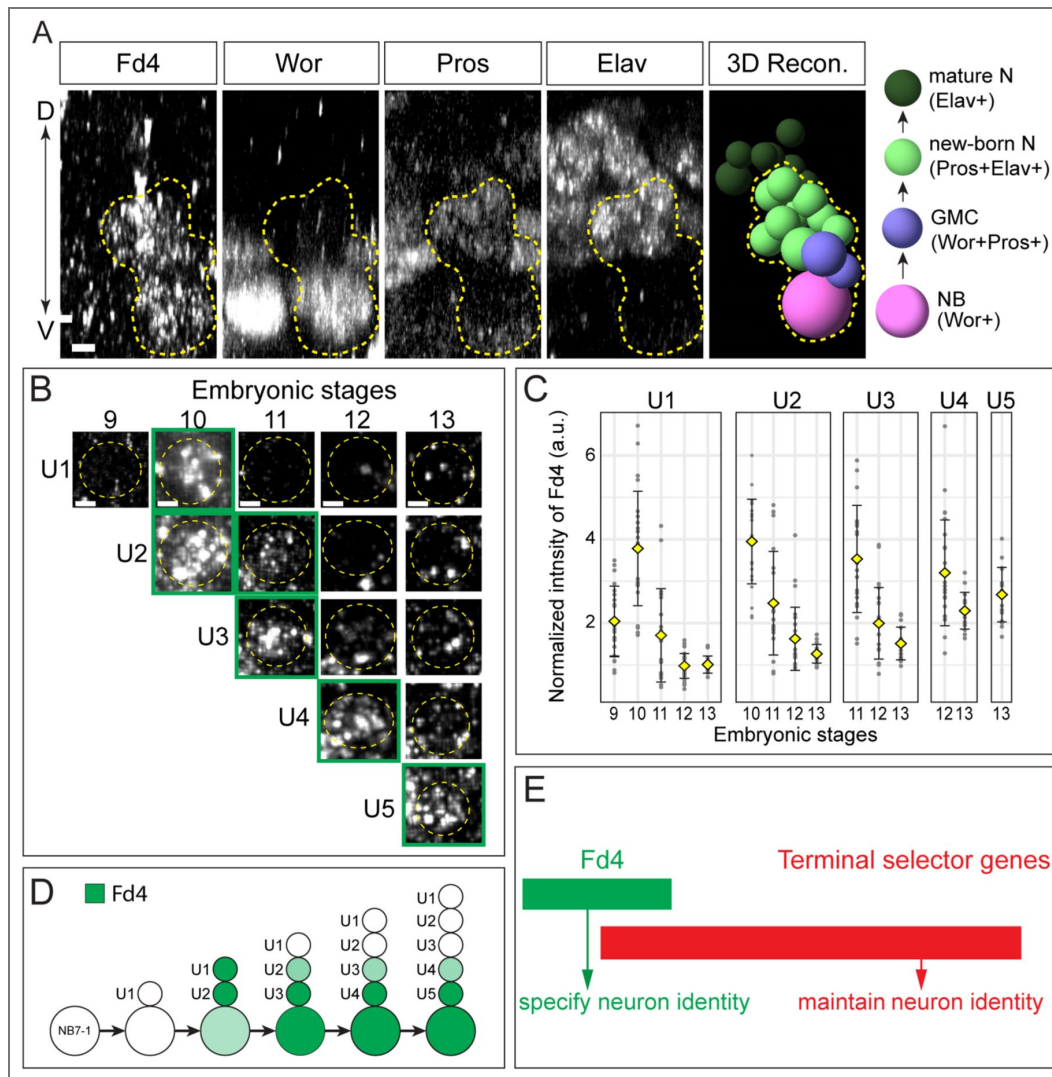


Figure 2. Fd4 is expressed in NBs, GMCs, and new-born neurons, but not in differentiated neurons

(A) Expression of Fd4, Worniu (Wor), Prospero (Pros), and Elav in a hemisegment of a stage 12 embryo. Posterior view with dorsal oriented upwards and ventral downwards. The left 4 panels show marker expression, and the fifth panel (3D Recon.) shows a 3D reconstruction of Fd4+ cells and adjacent Fd4-Elav+ cells from the left 4 panels using Imaris Spots function. The right-most image shows the expression profiles of different cell types. Yellow dashed lines outline the cells expressing Fd4. Scale bar: 2 μ m. (B) Representative images of Fd4 expression in U motor neurons (UMN) in the stage 9, 10, 11, 12, and 13 embryos. Yellow dashed lines outline the UMNs. Green squares box the UMNs that express Fd4. Scale bar: 1 μ m (C) Quantification of Fd4 intensity in UMNs. Each dot is a measurement of Fd4 fluorescence intensity in a UMN normalized to the fluorescent intensity in a Fd4-negative cell. Yellow diamond represents the average intensity, and error bars are standard deviation. a.u., artificial unit. Number of hemisegment with UMNs measured: stage 9, 21; stage 10, 24; stage 11, 20; stage 12, 26; stage 13, 18. (D) Summary of Fd4 expression in Eve+ cells during early NB7-1 lineage. The intensity of green represents the expression levels of Fd4. (E) Proposed functions of Fd4 and terminal selector genes in neuron identity.

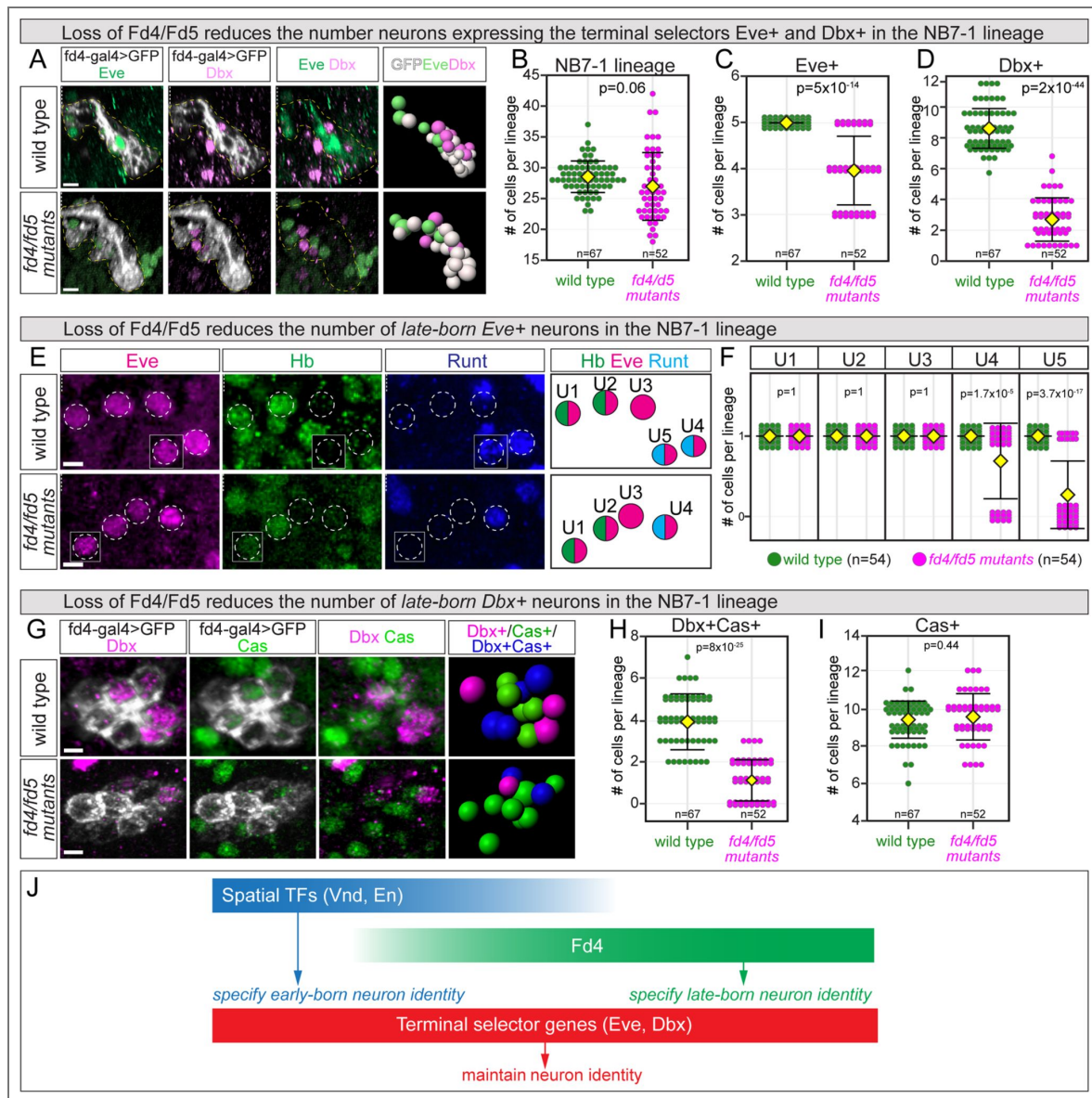


Figure 3. Fd4 is required for late-born neuron specification

(A) Eve and Dbx expression in the NB7-1 lineage of wild type and *fd4/fd5* mutant stage 17 embryos. Posterior view with dorsal oriented upwards, and ventral downwards. The NB7-1 lineage is outlined with yellow dashed line. Rightmost panels are 3D reconstruction of the NB7-1 lineage from the left three panels with Imaris Spots function. Scale bar: 2 μ m. (B-D) Quantification of total (B), Eve+ (C) and Dbx+ (D) cells in each lineage. Each dot represents an individual lineage. Yellow diamond, mean; error bars, standard deviation; n, number of lineages analyzed; p, the p-value of Student's t-test. (E) UMNs in wild type and *fd4/fd5* mutant stage 17 embryos. Identity of UMNs is determined by the expression of marker Hb or Runt, and the relative position of the cell within the hemisegment. The rightmost panels are summary cartoon from the left panels. Scale bar: 2 μ m. (F) Quantification of each individual UMNs in each lineage. Each dot represents an individual lineage. Yellow diamond, mean; error bars, standard deviation; n, number of lineages analyzed; p, the p-value of Student's t-test. (G) Dbx and Cas expression in the NB7-1 lineage of wild type and *fd4/fd5* mutant stage 17 embryos. Dorsal view with anterior oriented upwards, and posterior downwards. Rightmost panels are 3D reconstruction of the NB7-1 lineage from the left three panels with Imaris Spots function. Scale bar: 2 μ m. (H-I) Quantification. Each dot represents an individual lineage. Yellow diamond, mean; error bars, standard deviation; n, number of lineages analyzed; p, the p-value of Student's t-test. Genotypes: wild type: *fd4-gal4, fd5^{1nt}, UAS-myr-sfGFP*; *fd4/fd5* mutant: *fd4-gal4, fd5^{1nt}/Df(3R)BSC493, UAS-myr-sfGFP*. (J) Summary of functions of spatial TFs, Fd4 and terminal selector genes in neuron identity.

In contrast, we observe a significant loss of Eve⁺ and Dbx⁺ cells in *fd4/fd5* mutant embryos (Figure 3A; quantified in 3C, 3D). Further analysis shows that the missing Eve⁺ cells are later-born Runt⁺ U4-U5 neurons (Figure 3E; quantified in 3F), while the missing Dbx⁺ cells are later-born Cas⁺ interneurons, even though the overall number of Cas⁺ cells remains unchanged (Figure 3G; quantified in 3H, 3I). Taken together, we find that early U1-U3 neurons are generated independently of Fd4, whereas later-born Runt⁺ U4-U5 and Cas⁺Dbx⁺ interneurons require Fd4 for their proper specification. We conclude that Fd4 acts to maintain NB7-1 lineage identity and specify late-born neurons (Figure 3J).

Fd4 misexpression induces ectopic NB7-1-specific progeny

We have shown that Fd4 is required for proper generation of NB7-1 progeny, raising the question of whether Fd4 is sufficient to induce ectopic NB7-1 progeny in other neuroblast lineages. To determine if other lineages could be transformed into NB7-1 lineage, we misexpressed Fd4 using the *sca-gal4* driver, which is first expressed in all neuroectoderm and persists into all newly-formed neuroblasts, and assayed for Eve⁺ Dbx⁺ neurons (see above). In wild type, each abdominal hemisegment produced 18.1 ± 1.2 Eve⁺ cells and 17.2 ± 1.8 Dbx⁺ cells, including those in the NB7-1 lineage (Figure 4A,B). In contrast, pan-neuroblast expression of Fd4 resulted in widespread expression of the NB7-1 lineage markers Eve and Dbx in all regions of the hemisegment (Figure 4C,D; quantified in 4E,F). Notably, misexpression of Fd5 didn't induce any NB7-1 lineage markers (data not shown). Our results support a model in which Fd4 is sufficient to induce NB7-1 lineage identity in most, if not all, lineages.

We next asked whether widespread expansion of Eve⁺ and Dbx⁺ cells following Fd4 misexpression was due to altered spatial patterning (Supp Figure 3A) or altered temporal patterning (Supp Figure 3E). We misexpressed Fd4 in the neuroectoderm and found no change in Vnd or En expression (Supp Figure 3B, C), nor repression of Ind (Supp Figure 3B) or Wg (Supp Figure 3D). Furthermore, continuous misexpression of Fd4 in neuroblasts from the Hb to Cas temporal window did not affect the timing of early (Hb) or late (Cas) TTF expression (Supp Figure 3F). We conclude that Fd4 does not regulate STF or TTF patterning, and importantly that Fd4 activates terminal selector genes Eve and Dbx in the NB7-1 lineage.

Fd4 misexpression induces NB7-1 lineage markers and represses NB5-6 lineage markers

To more precisely explore the effects of Fd4 in individual neuroblast lineages, we selectively misexpressed Fd4 in NB5-6 (this section) and NB7-3 (next section), as we have excellent lineage markers for both neuroblasts. In wild type, NB5-6 delaminates simultaneously with NB7-1, but is located at the lateral side of the neuroblast array and is specified by spatial factors Wg (row anterior to En) and Msh (lateral column) (Chu-LaGriffa and Doe, 1993; Isshiki et al., 1997). The NB5-6 lineage and its progeny can be specifically labeled with *lbe-Gal4* (Figure 5A) (Baumgardt et al., 2009). The thoracic NB5-6 (NB5-6T) generates 20.2 ± 2.9 cells (Figure 5B) before undergoing apoptosis, and does not produce Eve⁺ U1-U5 MNs (Figure 5C). The last four cells produced by NB5-6T are specified by the LIM-HD TF Apterous (Ap) (Baumgardt et al., 2009) (Figure 5C), which is never observed in the NB7-1 lineage. When Fd4 is misexpressed in the NB5-6 lineage using *lbe-Gal4*, the number of progeny remains the same (21.1 ± 3.1) (Figure 5A,B), showing that Fd4 does not alter lineage length.

Although misexpression of Fd4 in NB5-6 does not alter lineage length, it results in a significant reduction in the number of the NB5-6 lineage marker Ap⁺ cells and a concomitant increase in the NB7-1 lineage marker Eve⁺ cells (Figure 5C). Moreover, the ectopic Eve⁺ cells express the early-born (Hb) and late-born (Runt) U1-U5 markers (Figure 5D,E). These ectopic Eve⁺ cells, like the wild type U1-U5 MNs, project axons out of CNS and target the dorsal muscles (Figure 5F,G). When we dissected newly hatched larvae and stain for the motor neuron marker phosphorylated-Mad (pMad), we find that the ectopic Eve⁺ cells are pMad⁺, consistent with an induction of the Eve⁺ U1-U5 motor neurons (Figure 5H). Interestingly, following Fd4 misexpression only seven of 18 examined NB5-6 lineages produced Dbx⁺ cells (0.4 ± 0.8), and just one of these was Cas⁺

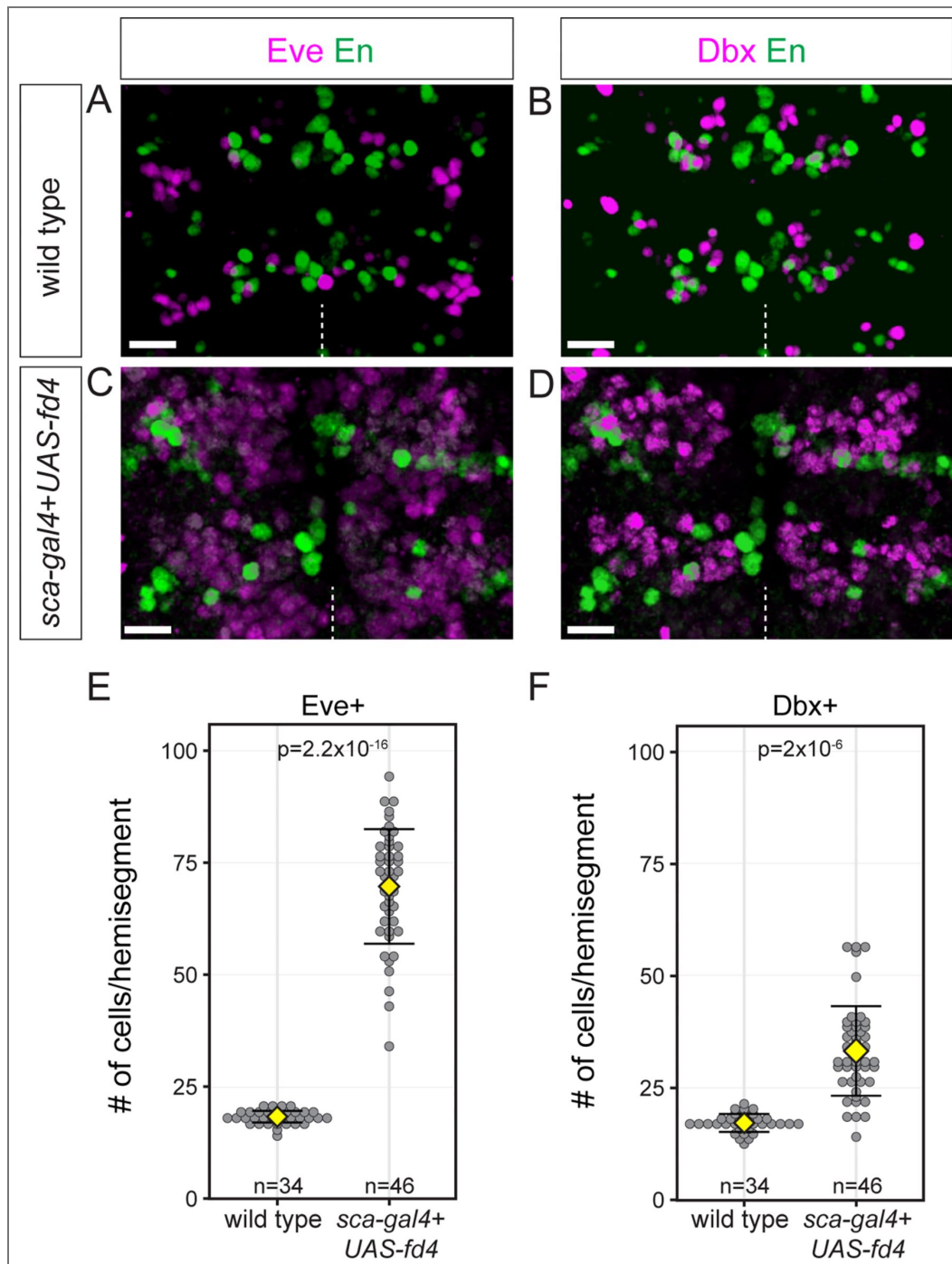


Figure 4. Wild spread of neuronal markers Eve and Dbx following ubiquitous Fd4 misexpression

(A-B) Eve and Dbx expression in wild type. En as a marker for segment boundary. Scale bars: 10 μ m. (C-D) Eve and Dbx expression following Fd4 misexpression. En as a marker for segment boundary. Scale bars: 10 μ m. (E-F) Quantification of Eve+ and Dbx+ cells in each hemisegment. Each dot represents an individual hemisegment. Yellow diamond, mean; error bars, standard deviation; n, number of lineages analyzed; p, the p-value of Student's t-test. Genotypes: wild type: *y-w*; Fd4 misexpression: *sca-gal4,UAS-fd4*.

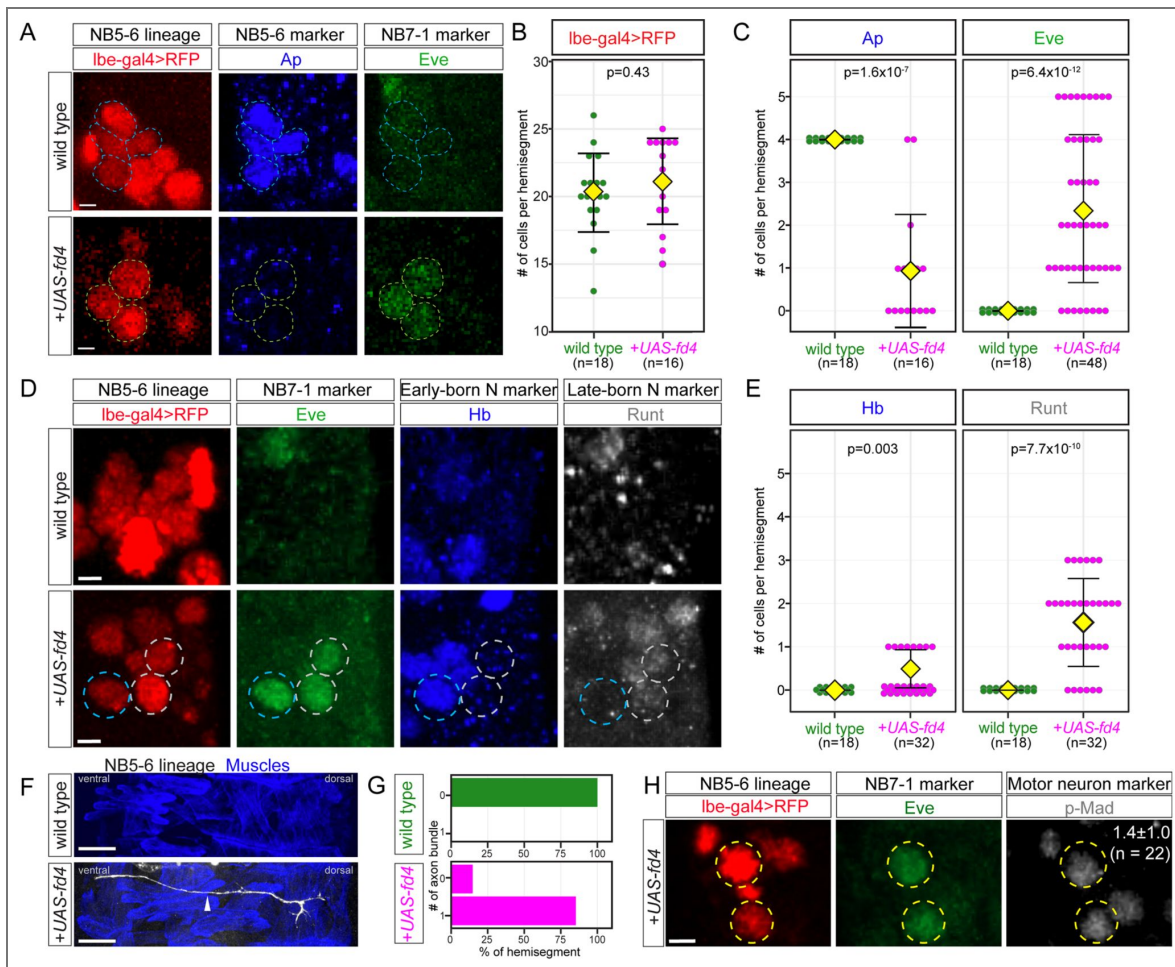


Figure 5. Fd4 misexpression in NB5-6 induces NB7-1 lineage markers and represses NB5-6 lineage markers

(A) Expression of *Ap* and *Eve* in wild type (top row) or following *Fd4* misexpression (bottom row) in NB5-6 using the NB5-6-specific *lbe-gal4* driver. Scale bar: 2 μ m (B-C) Quantification of the number of *RedStinger*+ (B), *Ap*+ (C) and *Eve*+ (C) cells. Each dot represents an individual lineage. Yellow diamond, mean; error bars, standard deviation; n, number of lineages analyzed; p, the p-value of Student's t-test. (D) Expression of NB7-1 markers (*Hb*, *Runt*, *Eve*) in wild type NB5-6 lineage and following *Fd4* misexpression in the NB5-6 lineage. Scale bar: 2 μ m (E) Quantification of *Hb*+*Eve*+ and *Runt*+*Eve*+ cells. Each dot represents an individual lineage. Yellow diamond, mean; error bars, standard deviation; n, number of lineages analyzed; p, the p-value of Student's t-test. (F) Lateral view of *lbe-gal4*+ axon projection in wild type (top panel) and *Fd4* misexpressed (bottom panel) embryos. The axons (white, arrowhead) was overlaying the body wall muscles (blue), and the muscles were labeled with antibody against Tropomyosin 1 (*Tm1*). Scale bars: 20 μ m. (G) Quantification of percent of axons projected out of CNS to the body wall muscles in wild type and *Fd4* misexpressed embryos. (H) Expression of motor neuron marker *pMad* in ectopic *Eve*+ cells in *Fd4* misexpressed NB5-6 lineage in newly hatched larvae. The number in *pMad* panel shows the average number of *pMad*+ *Eve*+ cells per hemisphere. Scale bar: 2 μ m. Genotypes: (A-E, H) wild type: *lbe-gal4,UAS-RedStinger*; *Fd4* misexpression (+*UAS-fd4*): *lbe-gal4,UAS-RedStinger,UAS-fd4*; (F-G) wild type: *10xUAS-myr-smGdP.HA,lbe-gal4*. *Fd4* misexpression (+*UAS-fd4*): *10xUAS-myr-smGdP.HA, lbe-gal4, UAS-fd4*

(0.06 ± 0.2); the reason for the difference in Eve and Dbx in response to Fd4 misexpression is unknown. Taken together, we find that expression of Fd4 in NB5-6 is sufficient to transform NB5-6 lineage into NB7-1 lineage. We conclude that Fd4 is necessary and sufficient to induce NB7-1 lineage identity within the neuroblast population as a whole and specifically in NB5-6.

Fd4 misexpression induces NB7-1 lineage markers and represses NB7-3 lineage markers

To determine if Fd4 could reprogram another neuroblast into NB7-1-like lineage, we misexpressed Fd4 in NB7-3, a neuroblast that is different from NB5-6 in many aspects. NB7-3 is one of the last neuroblasts to form (Broadus et al., 1995), has a relatively small size, generates a short 3 division lineage, generates a ventral-muscle targeting motor neuron (GW), two serotonergic neurons (EW1, EW2), and one Corazonin+ cell (EW3) (Karcavich and Doe, 2005; Novotny et al., 2002), which are never observed in the NB7-1 lineage. The NB7-3 lineage can be labeled with *eagle-Gal4* and its progeny can be identified with antibody staining against Serotonin (EW1, EW2) or Corazonin (EW3) (Figure 6A). When Fd4 was misexpressed in NB7-3 with *eagle-Gal4*, there was no change in NB7-3 lineage length producing an average of 4 neurons in control and misexpression experiments (Figure 6B, quantified in 6C). Despite the unchanged neuron numbers, we observed a significant reduction of Serotonin+ and Corazonin+ neurons and a corresponding increase in Eve+ neurons (Figure 6C). Serotonin/Corazonin and Eve+ neurons are mutually exclusive; we never observe cells coexpressing both markers, ruling out a mixed lineage identity. We conclude that Fd4 is sufficient to repress NB7-3 specific markers and activate NB7-1-specific markers.

To gain a deeper understanding of the role of Fd4 in specifying lineage identity, we assayed for changes in motor neuron projections in the NB7-1 and NB7-3 lineages. In wild type, the NB7-3 generates a GW motor neuron that co-expresses the ventral muscle motor neuron transcription factor Nkx6 (Flybase: HGTX), and the pan-motor neuron marker pMad (Figure 7A; quantified in 7B). In contrast, NB7-1 generates the Eve+pMad+ U1-U5 motor neurons. All five UMNs project to the dorsal body wall muscles (Landgraf et al., 1997; Schmid et al., 1999). When Fd4 is misexpressed in NB7-3 lineage, we observed an increase in ectopic Eve+ motor neurons and a reduction of Nkx6+ motor neurons (Figure 7A; quantified in 7B), indicating a transformation from NB7-3 to NB7-1 lineage identity. Interestingly, Fd4 misexpression in NB7-3 generate ectopic U1-U5 motor neurons that did not always fasciculate together when exiting the CNS (Figure 7C; quantified in 7D); this indicates either incomplete U neuron specification or a difference in the timing of ectopic U neuron outgrowth. Furthermore, in wild type, the NB7-3 derived Nkx6+ motor neuron innervates a ventral body wall muscle, whereas NB7-1 derived Eve+ neurons innervate more dorsal body wall muscles (Figure 7E; quantified in 7G) (Schmid et al., 1999). In contrast, Fd4 misexpression in the NB7-3 lineage generated motor neurons that projected dorsally beyond their normal ventral muscle target (Figure 7F; quantified in 7G; summarized in 7H). We observed that these transformed neurons did not innervate the dorsal muscles, perhaps their late birth did not give them time to extend to the most distant dorsal muscles. We conclude that Fd4 is sufficient to induce NB7-1 lineage identity at the expense of NB7-3 identity.

Discussion

Fd4 maintains neuroblast identity established by transient spatial factors En and Vnd

We identified Fd4 as a NB7-1 specific transcription factor which is continuously expressed in NB7-1 and its new-born neurons into larval stages. NB7-1 expresses En and Vnd and loss of these spatial factors leads to the loss of NB7-1 (McDonald et al., 1998), loss of Fd4 (Anderson et al., 2025), and loss of NB7-1 lineage markers (McDonald et al., 1998). Importantly, loss of *fd4* results in the loss of NB7-1 identity based on failure to generate the NB7-1-specific U motor neurons, whereas misexpression of Fd4 transforms most or all lineages towards a NB7-1 lineage.

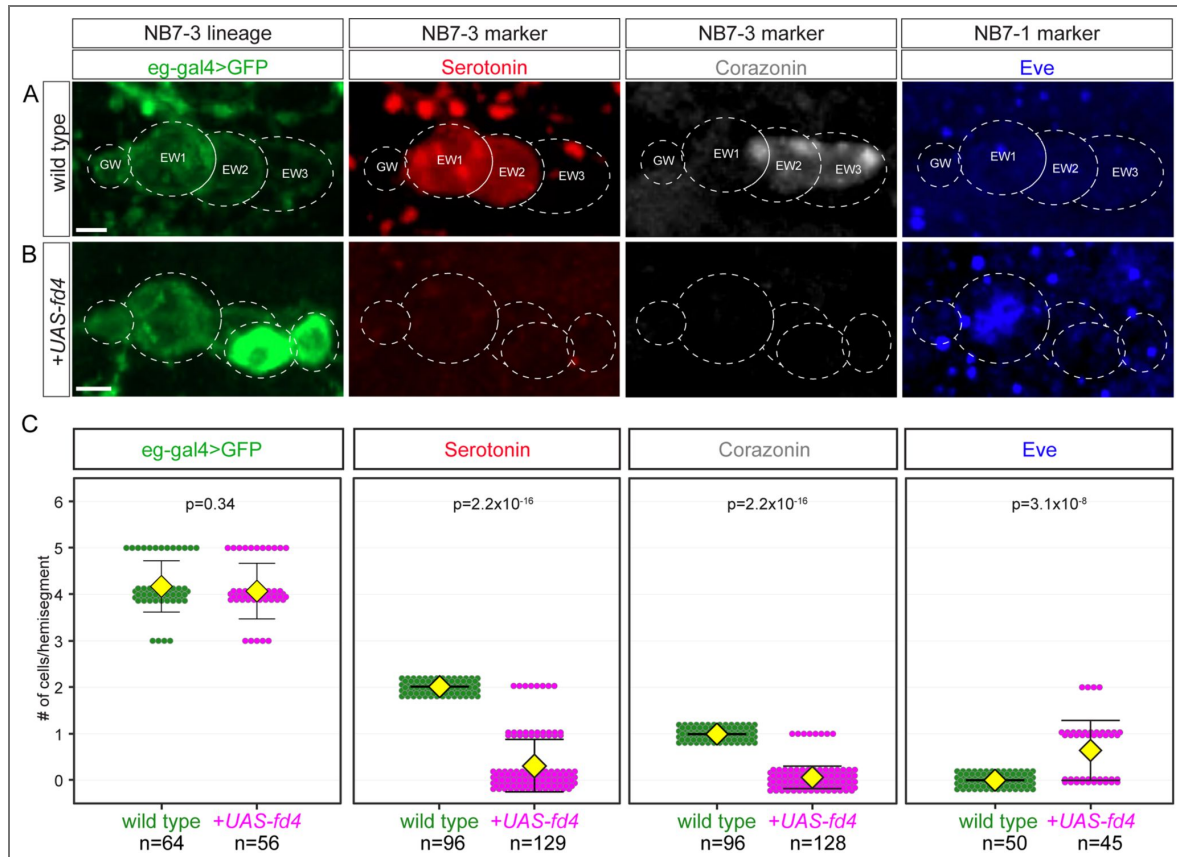


Figure 6. Fd4 misexpression in NB7-3 induces NB7-1 lineage markers and represses NB7-3 lineage markers

(A-B) Expression of serotonin (5-HT), Corazonin (Crz) and Eve in wild type (A) and following Fd4 misexpression in the NB7-3 lineage (B). Scale bar: 2 μ m. (C) Quantification. Each dot represents an individual lineage. Yellow diamond, mean; error bars, standard deviation; n, number of lineages analyzed; p, the p-value of Student's t-test. Genotypes: wild type: *eg-gal4,UAS-myr-sfGFP*; Fd4 misexpression (+*UAS-fd4*): *eg-gal4,UAS-myr-sfGFP,UAS-fd4*.

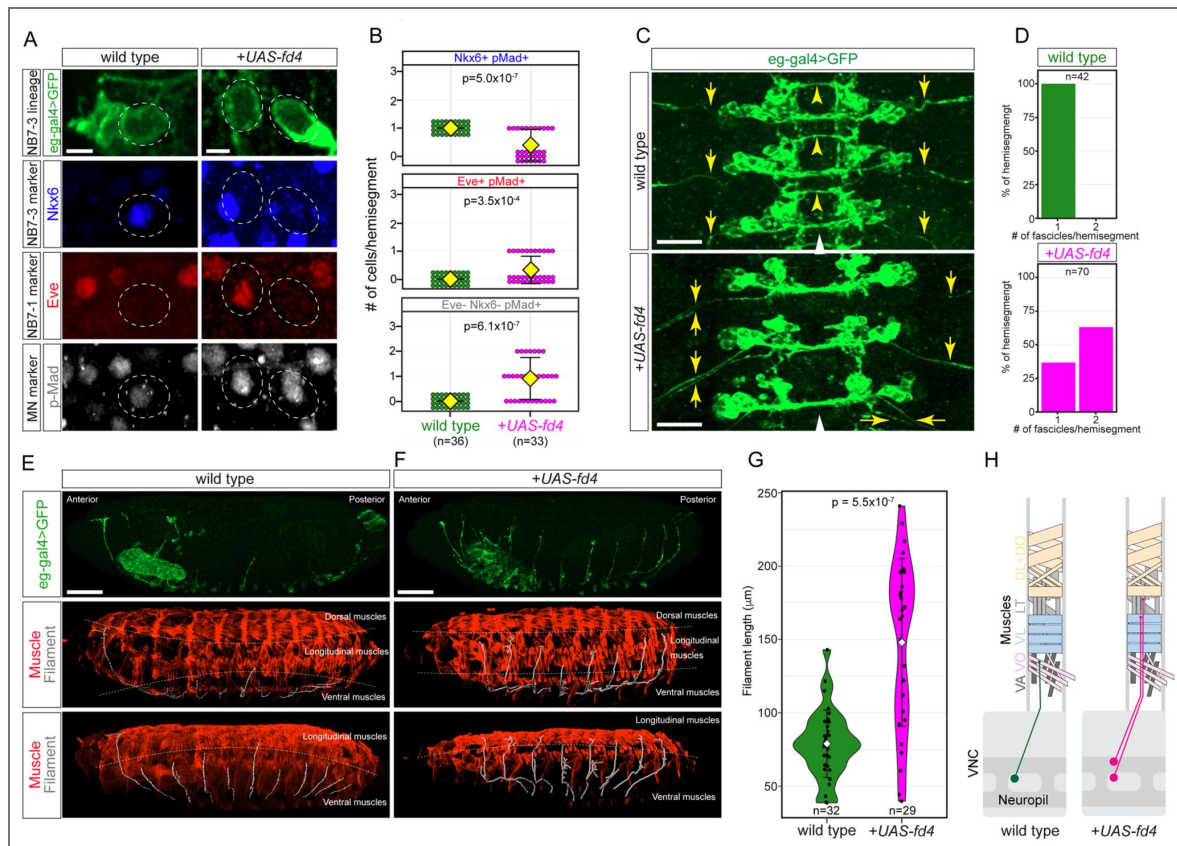


Figure 7. Fd4 misexpression in NB7-3 lineage dorsalizes motor neuron projections

(A) Expression of motor neuron markers Nkx6, Eve, and pMad in wild type (left column) and Fd4 misexpressed (right column) motor neuron in newly hatched larvae. Scale bars: 2 μ m. (B) Quantification. Each dot represents an individual lineage. Yellow diamond, mean; error bars, standard deviation; n, number of lineages analyzed; p, the p-value of Student's t-test. (C) Dorsal view of three segments of wild type (top panel) and Fd4 misexpressed (bottom panel) neuronal projections. Yellow arrows indicate the fascicles projecting out from the neuropil. White arrowheads, ventral midline. Scale bars: 20 μ m. (D) Quantification of fascicles exiting nervous system. Wild type (1 ± 0 ; top panel). Fd4 misexpression (1.6 ± 0.5 ; bottom panel). (E, F) Lateral view of eg-gal4+ motor neuron axon projection in wild type (E; left panels) and Fd4 misexpressed (F; right panels) embryos. Top two panels are the maximum projections of confocal image stacks of eg-Gal4+ neurons. Middle and bottom panels are eg-Gal4+ neuron axons reconstructed with Imaris (white), overlaying the body wall muscles (red). The muscles are labeled with antibody against Tropomyosin 1 (Tm1). The dashed lines indicate the boundary between dorsal and longitudinal (middle panels), and longitudinal and ventral muscles (middle and bottom panels). Scale bars: 50 μ m. (G) Quantification of motor neuron axon lengths. Each black dot represents the length of an axon measured from the VNC. White diamonds indicate the average length. The error bars are standard deviation. (H) Summary. Genotypes: wild type: *eg-gal4,UAS-myr-sfGFP*; Fd4 misexpression (+UAS-fd4): *eg-gal4,UAS-myr-sfGFP,UAS-fd4*.

Thus, Fd4 is necessary and sufficient to specify lineage identity. We propose a model where transient expression of spatial factors En and Vnd activates Fd4 and establishes NB7-1 identity, with Fd4 translating transient spatial cues into a long-term lineage identity (Figure 8 [↗](#)).

How does Fd4 sustain the positional identity established by spatial factors? In *Drosophila*, spatial factors regulate chromatin status, allowing temporal factors bind to lineage-specific open chromatin and produce lineage-specific progeny (Sen et al., 2019 [↗](#)). Thus, Fd4 may act downstream of En/Vnd to maintain chromatin status necessary for NB7-1 specific neuronal identity (e.g. Eve expression). Interestingly, Fd4 is also sufficient to activate NB7-1 lineage markers in other NB lineages, suggesting Fd4 alone may be sufficient to change chromatin status, similar to other Forkhead domain proteins (Guo et al., 2024 [↗](#); Jin et al., 2020 [↗](#); Sanese et al., 2019 [↗](#)). In mammals, other mechanisms for stabilizing lineage identity have been reported, including morphogens, transcriptional feedback mechanisms, non-coding RNAs, and chromatin regulators (reviewed in Delgado and Lim 2017 [↗](#)), and it remains an open question whether Fd4 uses any of these mechanisms in sustaining neuroblast identity.

We found that the *fd4/fd5* mutant resulted in loss of the NB7-1-specific U4 and U5 neurons (born after U1-U3) and late-born Dbx⁺ cells. Why does loss of Fd4/Fd5 cause only a loss of late-born neurons? We suggest that the U1-U3 identities are specified by the STFs Vnd and En, which are expressed in the NB7-1 lineage during the time U1-U3 are produced (Figure 1 [↗](#), Supp Figure 1 [↗](#)). We propose that the STFs specify the early-born U1-U3 neurons, followed by Fd4/Fd5 taking over the role of specifying the later-born U4/U5 neurons and maintaining all aspects of the lineage. This model would also explain the evolutionary advantage of genes like Fd4/Fd5. Specifically, if the overlapping expression of En and Vnd specifies NB7-1 identity, why the need for Fd4/Fd5? Given the transient nature of spatial cues, Fd4/Fd5 likely serve as a molecular ‘memory’ that preserves neuroblast identity, allowing late-born progeny to inherit lineage-specific transcriptional programs even after the original spatial cues have faded.

Fd4 transforms lineage identity, but not lineage length

We found that Fd4 is sufficient to induce NB7-1 identity in NB7-3, but not sufficient to change the length of its lineage. NB7-3 is a late-forming neuroblast that has a small size and makes a short three division lineage. Misexpression of Fd4 in NB7-3 was sufficient to activate NB7-1 specific lineage markers and repress NB7-3 specific lineage markers, but the NB7-3 lineage remained short. Thus, Fd4 can transform some aspects of neuroblast identity (molecular markers) but not all aspects (lineage length). It is likely that the smaller size of NB7-3 limits its number of divisions, or alternatively, unknown spatial factors may determine neuroblast lineage length.

Fd4, Fd5 redundancy

Redundancy of closely related genes is fairly common in *Drosophila* (Bhat and Schedl, 1997 [↗](#); Grosskortenhaus et al., 2006 [↗](#); Kohwi et al., 2011 [↗](#); Yeo et al., 1995 [↗](#)). In our studies, we found that *fd4/fd5* double mutant lacks the late-born U4-U5 MNs (Figure 4 [↗](#)); single mutants have no phenotype. Our misexpression experiments show that Fd4 alone is sufficient to promote NB7-1 identity (Figure 5 [↗](#)-8 [↗](#)). Fd5 alone has no ability to activate Fd4 or generate ectopic NB7-1 derived neurons (data not shown), indicating that the two genes are not fully redundant; it is not clear why Fd4 plays the major role in transforming neuroblast identity. Based on the partial co-expression of *fd4* and *fd5* during embryonic stages, it is possible that Fd4 and Fd5 have partially redundant roles in specifying U4-U5 motor neurons, similar to the mammalian FOXP protein in GABAergic spiny neuron specification (Ahmed et al., 2024 [↗](#)). We hypothesize that the highly conserved Forkhead DNA-binding domain of Fd4 and Fd5 is required to activate Eve expression, but less well conserved domains may regulate Fd4-specific and Fd5-specific function.

How many ‘neuroblast identity’ genes exist?

Neuroblast lineages can be labeled by specific gal4 or split-gal4 drivers (Lacin and Truman, 2016 [↗](#); Soffers et al., 2025 [↗](#)), documenting the potential lineage-specific gene expression. However, few genes have been identified that are expressed in single neuroblast lineages. In the *Drosophila*

	TF class	Expression	Function
A	Spatial TFs (e.g. Vnd, En)	Column or Row <i>transient</i>	Specify NB identity
B	NB identity TFs (e.g. Fd4)	Single NB and its newborn neurons <i>length of lineage</i>	Maintain NB identity
C	Terminal selector TFs (e.g. Eve, Dbx)	Post-mitotic neurons <i>permanent</i>	Maintain neuron identity

Figure 8. Model We propose a three-step model for the specification and maintenance of neuroblast and neuron identity.

(A) Spatial transcription factors (e.g. Vnd, En) are expressed transiently in rows and columns of neuroectoderm where they act combinatorially to specify neuroblast identity. (B) neuroblast identity transcription factors (e.g. Fd4 or Lbe) are expressed in single neuroblasts and their new-born progeny throughout their lineage and act downstream of spatial factors to maintain neuroblast identity and upstream of terminal selector genes to specify late-born progeny. (C) Terminal selector genes (e.g. Eve, Dbx) are expressed in neurons where they permanently maintain neuron identity and functions.

brain, the homeodomain transcription factor Orthodenticle (Otd; FlyBase: Oc) is expressed in a single neuroblast (LalV1) that generates central complex neurons, and loss of Otd transforms the neuroblast into a different neuroblast (ALad1) that generates olfactory projection neurons (Sen et al., 2014). Thus, Otd can be considered a neuroblast identity gene. Similarly, the NB5-6 lineage is the only neuroblast labeled by the HDTF Lbe, and misexpression of Lbe ubiquitously in other lineages also leads to the ectopic production of NB5-6 specific peptidergic lineage marker neurons (Baumgardt et al., 2009; Gabilondo et al., 2016). Thus, Otd and Lbe may join Fd4 as neuroblast identity genes that perform the same function: translating transient spatial cues that specify single neuroblasts into the permanent expression of lineage-specific terminal selector genes. Our findings raise the possibility that every neuroblast lineage may express its own neuroblast identity gene; alternatively, early forming neuroblasts like NB7-1 and NB5-6 have the longest lineages, and may require lineage identity genes to maintain neuroblast identity over the length of these lineages. Advances in single cell RNA-seq may reveal additional lineage-specific neuroblast identity genes.

Methods

Fly genetics

eg-gal4 (RRID:BDSC_8758); *en-gal4* (Schmid et al., 1999); *lbe(K)-gal4* (Baumgardt et al., 2009); *sca-gal4* (Doe lab); *UAS-IVS-myr::GFP* (RRID:BDSC_32198); *UAS-myr::sfGFP* (RRID:BDSC_62127); *UAS-IVS-myr::smGdP-HA* (RRID:BDSC_62145); *UAS-RedStinger* (RRID:BDSC_8547); *vnd-GFP-FPTB* (RRID:BDSC_93583). *fd4* and *fd5* alleles: *Df(3R)BSC493/TM6C* (*fd4* and *fd5* deficiency) (RRID:BDSC_24997); *fd4^{5nt}/TM6B*, *fd5^{1nt}/TM6B*, and *UAS-fd4* were gifts from C. Estella (Universidad Autónoma de Madrid, Madrid, Spain).

Generation of *fd4/fd5* mutant

We used CRISPR to generate *fd4-gal4* by replacing *fd4* open reading frame (ORF) with *gal4* with the pHD-DsRed (Addgene plasmid #51434; <http://n2t.net/addgene:51434>; RRID:Addgene_51434) (Gratz et al., 2014) which also contained 1Kb of homologous arms up- and down-stream of ORF for homology directed repair (HDR). Two gRNA (AACATTGTGTAATAATGCC and TAGGATTCTCGAGGGCCG) were used to remove *fd4* ORF and cloned into pCFD4-U6:1_U6:3tandemgRNAs (Addgene plasmid #49411; <http://n2t.net/addgene:49411>; RRID:Addgene_49411) (Port et al., 2014). The gRNA and HDR constructs were co-injected into the recombined *Actin5C-Cas9.P; fd5^{1nt}/TM6B* flies by Rainbow Transgenic Flies, Inc (Camarillo, CA, USA). Two independent transformants were obtained.

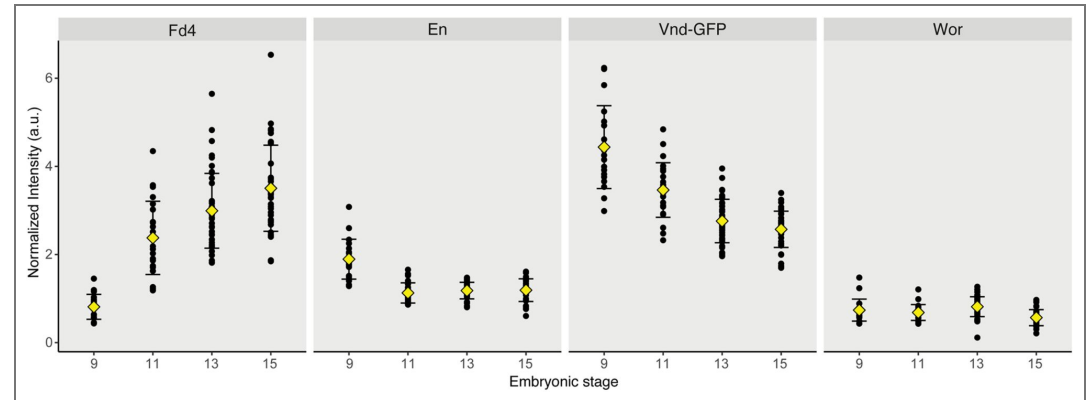
Antibody staining and imaging

Embryos were fixed and stained as previously described (Grosskortenhaus et al., 2005). Primary antibodies used were: rabbit anti-Cas (Mellerick et al., 1992), 1:1000 (Doe lab); rabbit anti-Corazonin, 1:2000 (Isshiki et al., 2001); guinea pig anti-Dbx, 1:200 (Doe lab); rat anti-Elav, 1:100 (DSHB, RRID:AB_528218); mouse anti-En, 5µg/mL (DSHB, RRID:AB_528224); guinea pig anti-Eve, 1:200 (Desplan lab, NYU, New York, NY, USA); mouse anti-Eve[2B8], 5µg/mL, (DSHB, RRID_AB:528230); rabbit anti-Eve, 1:250 (Doe lab); guinea pig anti-Fd4, 5µg/mL (Doe lab); chicken anti-GFP, 1:1000 (Aves Labs, RRID:AB_2734732); mouse anti-Hb [F18-1G10.2], 1:200 (Abcam, Waltham, MA, USA); rabbit anti-Hb, 1:200 (Tran and Doe, 2008); rat anti-Ind, 1:100 (Weiss et al., 1998); rat anti-Nkx6, 1:500 (Broihier et al., 2004); rabbit anti-pMad [EP823Y], 1:300 (Abcam, Waltham, MA, USA); mouse anti-Prospero monoclonal purified IgG, 1:1000 (Doe lab); guinea pig anti-Runt, 1:1000 (Sullivan et al., 2019); rat anti-Serotonin [YC5/45], 1:100, (Accurate Chemical & Scientific Corporation, Carle Place, NY, USA); rat anti-Tm1[MAC141], 1:500 (Abcam, Waltham, MA, USA); mouse anti-Wg, 5µg/mL (DSHB, RRID_AB:528512); and rabbit anti-Worniu, 1:1000 (Doe lab). Secondary antibodies used were: DyLight 405, AlexaFluor 488, AlexaFluor 555, rhodamine RedTM-X(RRX), or Alexa Fluor 647-conjugated AffiniPureTM donkey anti-IgG (Jackson ImmunoResearch, West Grove, PA, USA). The samples were mounted in 90% glycerol with Vectashield (Vector

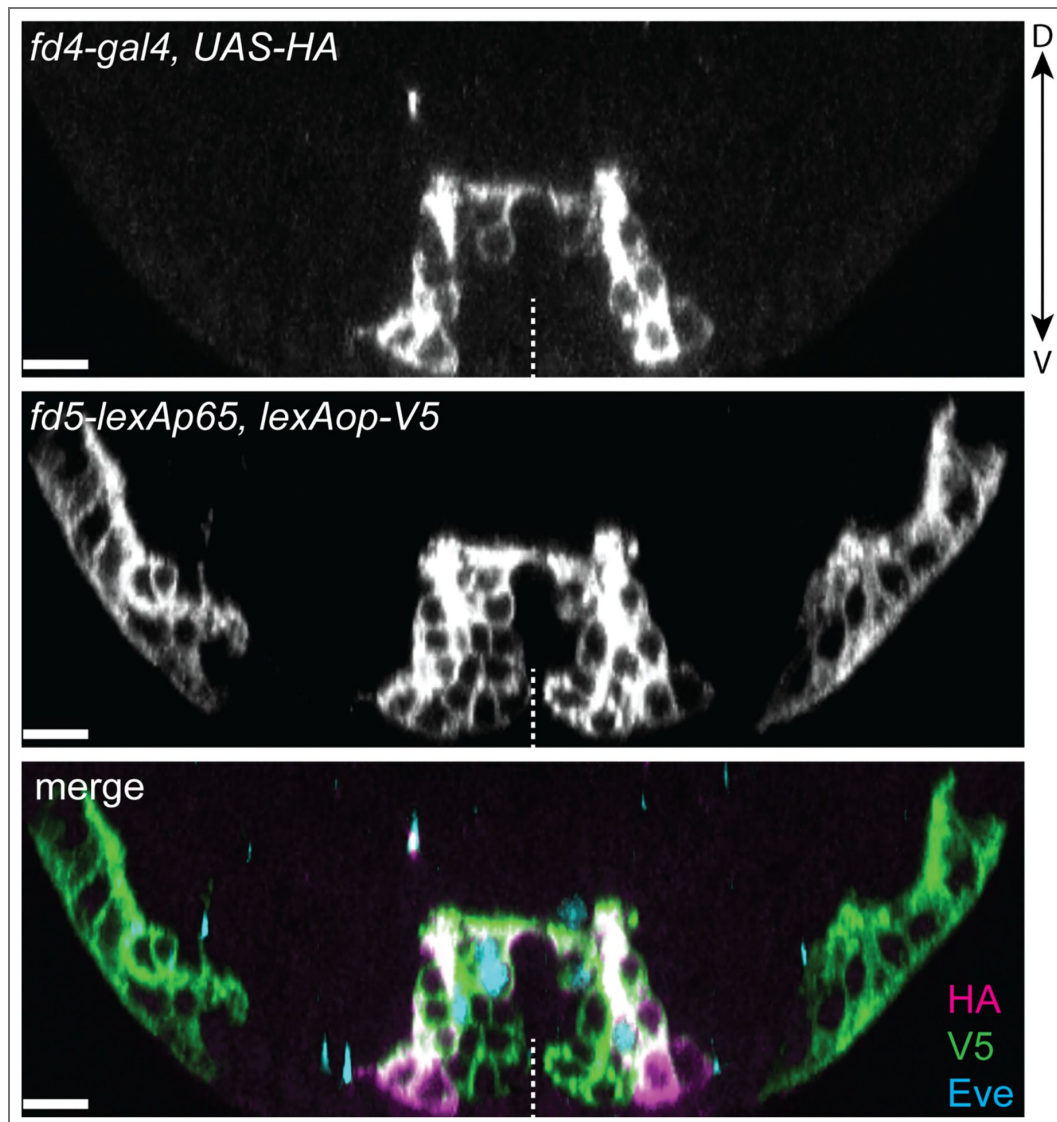
Laboratories, Burlingame, CA). Images were captured with a Zeiss LSM 800 confocal microscope with a z-resolution of 0.5 μm and processed using Imaris (Oxford Instruments plc, UK). Figures were assembled in Adobe Illustrator (Adobe, San Jose, CA).

Data availability

We will submit expression and phenotype data to FlyBase, a public repository.

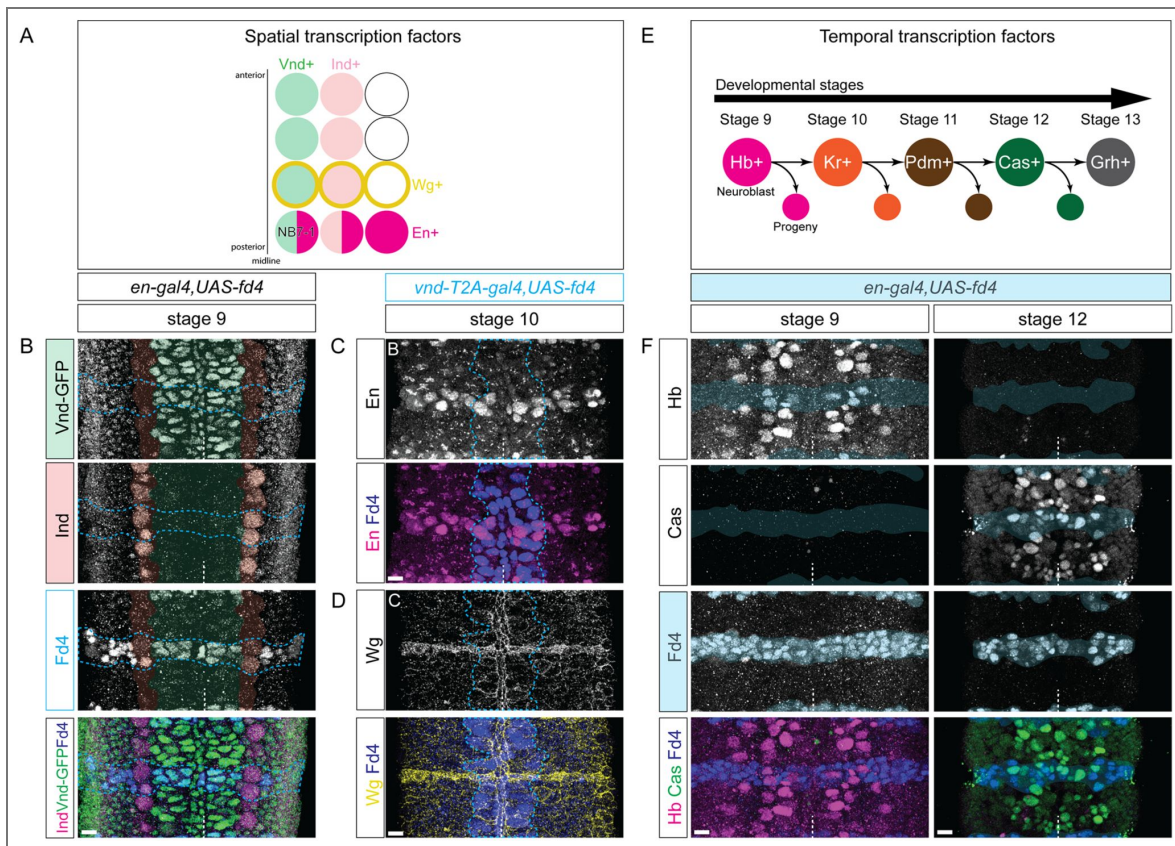


Supplemental Figure 1. Fd4 expression follows the expression of spatial factors En and Vnd Quantification of Fd4, En, Vnd-GFP and Wor fluorescence intensity in NB7-1. Each dot is a measurement of protein fluorescence intensity in a NB7-1 normalized to the fluorescent intensity in a Fd4, En, and Vnd-GFP triple negative neuroblast (Wor+). Yellow diamond represents the average intensity, and error bars are standard deviation. a.u., artificial unit. Number of NB7-1 measured: stage 9, 20; stage 11, 22; stage 13, 30; stage 15, 34.



Supplemental Figure 2. *fd4* and *fd5* coexpress in the ventral nerve cord during embryogenesis

Expression of *fd4* and *fd5* in a stage 13 embryo. Posterior view with dorsal (D) oriented upwards, and ventral (V) downwards. White dashed lines indicate the segment midline. Scale bar: 10 μ m.



Supplemental Figure 3. Fd4 does not regulate spatial patterning genes or temporal transcription factors

(A) Schematic of spatial factor expression in one hemisegment of ventral nerve cord. (B) Fd4 misexpression in En⁺ cells in a stage 9 embryo. Overlaid green color shows Vnd expression domain, and overlaid red color shows Ind expression domain. Blue dash-lines outline the region where Fd4 is misexpressed by *en-gal4*. White dashed lines, ventral midline. Genotype: *en-gal4,UAS-fd4,vnd-GFP-FPTB*. (C-D) Fd4 misexpression in Vnd⁺ cells in a stage 9 embryo. Blue dash-lines outline the region where Fd4 is misexpressed by *vnd-T2A-gal4*. White dashed lines, ventral midline. Genotype: *vnd-T2A-gal4,UAS-fd4*. (E) Schematic of temporal transcription factor expression from stage 9 to stage 12. (F) Fd4 misexpression in En⁺ cells in a stage 9 (left) and a stage 12 (right) embryo. Overlaid blue color shows the cells with Fd4 misexpressed with *en-gal4*. White dashed lines, ventral midline. Genotype: *en-gal4,UAS-fd4*. Scale bar: 5 μm for all panels.

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Peer reviews

Reviewer #1 (Public review):

Summary:

Lai and Doe address the integration of spatial information with temporal patterning and genes that specify cell fate. They identify the Forkhead transcription factor Fd4 as a lineage-restricted cell fate regulator that bridges transient spatial transcription factors to terminal selector genes in the developing *Drosophila* ventral nerve cord. The experimental evidence convincingly demonstrates that Fd4 is both necessary for late-born NB7-1 neurons, but also sufficient to transform other neural stem cell lineages toward the NB7-1 identity. This work addresses an important question that will be of interest to developmental neurobiologists: How can cell identities defined by initial transient developmental cues be maintained in the progeny cells, even if the molecular mechanism remains to be investigated? In addition, the study proposes a broader concept of lineage identity genes that could be utilized in other lineages and regions in the *Drosophila* nervous system and in other species.

Strengths:

While the spatial factors patterning the neuroepithelium to define the neuroblast lineages in the *Drosophila* ventral nerve cord are known, these factors are sometimes absent or not required during neurogenesis. In the current work, Lai and Doe identified Fd4 in the NB7-1 lineage that bridges this gap and explains how NB7-1 neurons are specified after Engrailed (En) and Vnd cease their expression. They show that Fd4 is transiently co-expressed with En and Vnd and is present in all nascent NB7-1 progenies. They further demonstrate that Fd4 is

required for later-born NB7-1 progenies and sufficient for the induction of NB7-1 markers (Eve and Dbx) while repressing markers of other lineages when force-expressed in neural progenitors, e.g., in the NB5-6 lineage and in the NB7-3 lineage. They also demonstrate that, when Fd4 is ectopically expressed in NB7-3 and NB5-6 lineages, this leads to the ectopic generation of dorsal muscle-innervating neurons. The inclusion of functional validation using axon projections demonstrates that the transformed neurons acquire appropriate NB7-1 characteristics beyond just molecular markers. Quantitative analyses are thorough and well-presented for all experiments.

Weaknesses:

- (1) While Fd4 is required and sufficient for several later-born NB7-1 progeny features, a comparison between early-born (Hb/Eve) and later-born (Run/Eve) appears missing for pan-progenitor gain of Fd4 (with sca-Gal4; Figure 4) and for the NB7-3 lineage (Figure 6). Having a quantification for both could make it clearer whether Fd4 preferentially induces later-born neurons or is sufficient for NB7-1 features without temporal restriction.
- (2) Fd4 and Fd5 are shown to be partially redundant, as Fd4 loss of function alone does not alter the number of Eve⁺ and Dbx⁺ neurons. This information is critical and should be included in Figure 3.
- (3) Several observations suggest that lineage identity maintenance involves both Fd4-dependent and Fd4-independent mechanisms. In particular, the fact that fd4-Gal4 reporter remains active in fd4/fd5 mutants even after Vnd and En disappear indicates that Fd4's own expression, a key feature of NB7-1 identity, is maintained independently of Fd4 protein. This raises questions about what proportion of lineage identity features require Fd4 versus other maintenance mechanisms, which deserves discussion.
- (4) Similarly, while gain of Fd4 induces NB7-1 lineage markers and dorsal muscle innervation in NB5-6 and NB7-3 lineages, drivers for the two lineages remain active despite the loss of molecular markers, indicating some regulatory elements retain activity consistent with their original lineage identity. It is therefore important to understand the degree of functional conversion in the gain-of-function experiments. Sparse labeling of Fd4 overexpressing NB5-6 and NB7-3 progenies, as was done in Seroka and Doe (2019), would be an option.
- (5) The less-penetrant induction of Dbx⁺ neurons in NB5-6 with Fd4-overexpression is interesting. It might be worth the authors discussing whether it is an Fd4 feature or an NB5-6 feature by examining Dbx⁺ neuron number in NB7-3 with Fd4-overexpression.
- (6) It is logical to hypothesize that spatial factors specify early-born neurons directly, so only late-born neurons require Fd4, but it was not tested. The model would be strengthened by examining whether Fd4-Gal4-driven Vnd rescues the generation of later-born neurons in fd4/fd5 mutants.
- (7) It is mentioned that Fd5 is not sufficient for the NB7-1 lineage identity. The observation is intriguing in how similar regulators serve distinct roles, but the data are not shown. The analysis in Figure 4 should be performed for Fd5 as supplemental information.

<https://doi.org/10.7554/eLife.109188.1.sa3>

Reviewer #2 (Public review):

Summary:

Via a detailed expression analysis, they find that Fd4 is selectively expressed in embryonic NB7-1 and newly born neurons within this lineage. They also undertake a comprehensive

genetic analysis to provide evidence that *fd4* is necessary and sufficient for the identity of NB7-1 progeny.

Strengths:

The analysis is both careful and rigorous, and the findings are of interest to developmental neurobiologists interested in molecular mechanisms underlying the generation of neuronal diversity. Great care was taken to make the figures clear and accessible. This work takes great advantage of years of painstaking descriptive work that has mapped embryonic neuroblast lineages in *Drosophila*.

Weaknesses:

The argument that *Fd4* is necessary for NB7-1 lineage identity is based on a *Fd4/Fd5* double mutant. Loss of *fd4* alone did not alter the number of NB7-1-derived *Eve+* or *Dbx+* neurons. The authors clearly demonstrate redundancy between *fd4* and *fd5*, and the fact that the LOF analysis is based on a double mutant should be better woven through the text. The authors generated an *Fd5* mutant. I assume that *Fd5* single mutants do not display NB7-1 lineage defects, but this is not stated. The focus on *Fd4* over *Fd5* is based on its highly specific expression profile and the dramatic misexpression phenotypes. But the LOF analysis demonstrates redundancy, and the conclusions in the abstract and through the results should reflect the existence of *Fd5* in the conclusions of this manuscript.

It is notable that *Fd4* overexpression can rewire motor circuits. This analysis adds another dimension to the changes in transcription factor expression and, importantly, demonstrates functional consequences. Could the authors test whether U4 and U5 motor axon targeting changes in the *fd4/fd5* double mutant? To strengthen claims regarding the importance of *fd4/fd5* for lineage identity, it would help to address terminal features of U motorneuron identity in the LOF condition.

<https://doi.org/10.7554/eLife.109188.1.sa2>

Reviewer #3 (Public review):

The goal of the work is to establish the linkage between the spatial transcription factors (STFs) that function transiently to establish the identities of the individual NBs and the terminal selector genes (typically homeodomain genes) that appear in the newborn post-mitotic neurons. How is the identity of the NB maintained and carried forward after the spatial genes have faded away? Focusing on a single neuroblast (NB 7-1), the authors present evidence that the fork-head transcription factor, *fd4*, provides a bridge linking the transient spatial cues that initially specified neuroblast identity with the terminal selector genes that establish and maintain the identity of the stem cell's progeny.

The study is systematic, concise, and takes full advantage of 40+ years of work on the molecular players that establish neuronal identities in the *Drosophila* CNS. In the embryonic VNC, *fd4* is expressed only in the NB 7-1 and its lineage. They show that *Fd4* appears in the NB while the latter is still expressing the Spatial Transcription Factors and continues after the expression of the latter fades out. *Fd4* is maintained through the early life of the neuronal progeny but then declines as the neurons turn on their terminal selector genes. Hence, *fd4* expression is compatible with it being a bridging factor between the two sets of genes.

Experimental support for the "bridging" role of *Fd4* comes from a set of loss-of-function and gain-of-function manipulations. The loss of function of *Fd4*, and the partially redundant gene *Fd5*, from lineage 7-1 does not affect the size of the lineage, but terminal markers of late-born neuronal phenotypes, like *Eve* and *Dbx*, are reduced or missing. By contrast, ectopic

expression of fd4, but not fd5, results in ectopic expression of the terminal markers eve and Dbx throughout diverse VNC lineages.

A detailed test of fd4's expression was then carried out using lineages 7-3 and 5-6, two well-characterized lineages in *Drosophila*. Lineage 7-3 is much smaller than 7-1 and continues to be so when subjected to fd4 misexpression. However, under the influence of ectopic Fd4 expression, the lineage 7-3 neurons lost their expected serotonin and corazonin expression and showed Eve expression as well as motoneuron phenotypes that partially mimic the U motoneurons of lineage 7-1.

Ectopic expression of Fd4 also produced changes in the 5-6 lineage. Expression of apterous, a feature of lineage 5-6, was suppressed, and expression of the 7-1 marker, Eve, was evident. Dbx expression was also evident in the transformed 5-6 lineages, but extremely restricted as compared to a normal 7-1 lineage. Considering the partial redundancy of fd4 and fd5, it would have been interesting to express both genes in the 5-6 lineage. The anatomical changes that are exhibited by motoneurons in response to Fd4 expression confirm that these cells do, indeed, show a shift in their cellular identity.

<https://doi.org/10.7554/eLife.109188.1.sa1>

Author response:

Reviewer #1 (Public Review):

Lai and Doe address the integration of spatial information with temporal patterning and genes that specify cell fate. They identify the Forkhead transcription factor Fd4 as a lineage-restricted cell fate regulator that bridges transient spatial transcription factors to terminal selector genes in the developing Drosophila ventral nerve cord. The experimental evidence convincingly demonstrates that Fd4 is both necessary for lateborn NB7-1 neurons, but also sufficient to transform other neural stem cell lineages toward the NB7-1 identity. This work addresses an important question that will be of interest to developmental neurobiologists: How can cell identities defined by initial transient developmental cues be maintained in the progeny cells, even if the molecular mechanism remains to be investigated? In addition, the study proposes a broader concept of lineage identity genes that could be utilized in other lineages and regions in the Drosophila nervous system and in other species.

Thanks for the accurate summary and positive comments!

While the spatial factors patterning the neuroepithelium to define the neuroblast lineages in the Drosophila ventral nerve cord are known, these factors are sometimes absent or not required during neurogenesis. In the current work, Lai and Doe identified Fd4 in the NB7-1 lineage that bridges this gap and explains how NB7-1 neurons are specified after Engrailed (En) and Vnd cease their expression. They show that Fd4 is transiently co-expressed with En and Vnd and is present in all nascent NB7-1 progenies. They further demonstrate that Fd4 is required for later-born NB7-1 progenies and sufficient for the induction of NB7-1 markers (Eve and Dbx) while repressing markers of other lineages when force-expressed in neural progenitors, e.g., in the NB56 lineage and in the NB7-3 lineage. They also demonstrate that, when Fd4 is ectopically expressed in NB7-3 and NB5-6 lineages, this leads to the ectopic generation of dorsal muscle-innervating neurons. The inclusion of functional validation using axon projections demonstrates that the transformed neurons acquire appropriate NB7-1 characteristics beyond just molecular markers. Quantitative analyses are thorough and well-presented for all experiments.

Thanks for the positive comments!

*(1) While Fd4 is required and sufficient for several later-born NB7-1 progeny features, a comparison between early-born (Hb/Eve) and later-born (Run/Eve) appears missing for pan-progenitor gain of Fd4 (with *sca-Gal4*; Figure 4) and for the NB7-3 lineage (Figure 6). Having a quantification for both could make it clearer whether Fd4 preferentially induces later-born neurons or is sufficient for NB7-1 features without temporal restriction.*

We quantified the percentage of Hb+ and Runt+ cells among Eve+ cells with *sca-gal4*, and the results are shown in Figure 4-figure supplement 1. We found that the proportion of early-born cells is slightly reduced but the proportion of later-born cells remain similar. Interestingly, we also found a subset of Eve+ cells with a mixed fate (Hb+Runt+) but the reason remains unclear.

(2) Fd4 and Fd5 are shown to be partially redundant, as Fd4 loss of function alone does not alter the number of Eve+ and Dbx+ neurons. This information is critical and should be included in Figure 3.

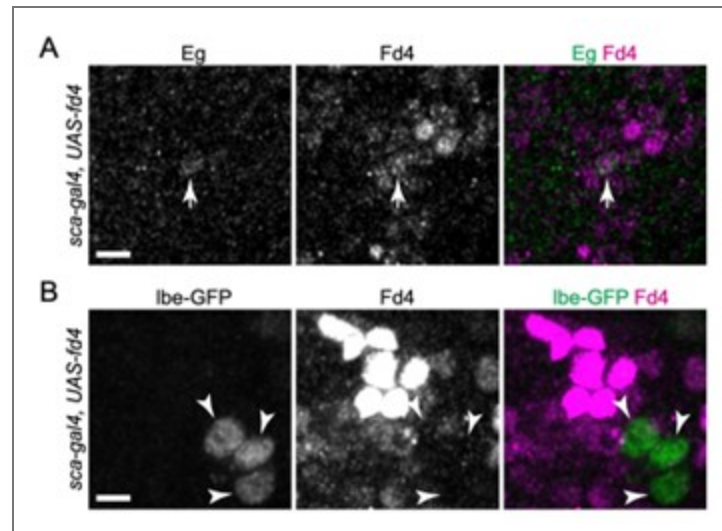
Because every hemisegment in an *fd4* single mutant is normal, we just added it as the following text: “In *fd4* mutants, we observe no change in the number of Eve+ neurons or Dbx+ neurons (n=40 hemisegments).”

*(3) Several observations suggest that lineage identity maintenance involves both Fd4-dependent and Fd4-independent mechanisms. In particular, the fact that *fd4-Gal4* reporter remains active in *fd4/fd5* mutants even after Vnd and En disappear indicates that Fd4's own expression, a key feature of NB7-1 identity, is maintained independently of Fd4 protein. This raises questions about what proportion of lineage identity features require Fd4 versus other maintenance mechanisms, which deserves discussion.*

We agree, thanks for raising this point. We add the following text to the Discussion. “Interestingly, the *fd4 fd5* mutant maintains expression of *fd4:gal4*, suggesting that the *fd4/fd5* locus may have established a chromatin state that allows “permanent” expression in the absence of Vnd, En, and Fd4/Fd5 proteins.”

(4) Similarly, while gain of Fd4 induces NB7-1 lineage markers and dorsal muscle innervation in NB5-6 and NB7-3 lineages, drivers for the two lineages remain active despite the loss of molecular markers, indicating some regulatory elements retain activity consistent with their original lineage identity. It is therefore important to understand the degree of functional conversion in the gain-of-function experiments. Sparse labeling of Fd4 overexpressing NB5-6 and NB7-3 progenies, as was done in Seroka and Doe (2019), would be an option.

We agree it is interesting that the NB7-3 and NB5-6 drivers remain on following Fd4 misexpression. To explore this, we used *sca-gal4* to overexpress Fd4 and observed that Lbe expression persisted while Eg was largely repressed (see Author response image 1 below). The results show that Lbe and Eg respond differently to Fd4. A non-mutually exclusive possibility is that the continued expression of *lbe-Gal4* UAS-GFP or *eg-Gal4* UAS-GFP may be due to the lengthy perdurance of both Gal4 and GFP.



Author response image 1.

(5) The less-penetrant induction of *Dbx*⁺ neurons in NB5-6 with *Fd4*-overexpression is interesting. It might be worth the authors discussing whether it is an *Fd4* feature or an NB56 feature by examining *Dbx*⁺ neuron number in NB7-3 with *Fd4*-overexpression.

In the NB7-3 lineages misexpressing *Fd4*, only 5 lineages generated *Dbx*⁺ cells (0.1 ± 0.4 , $n=64$ hemisegments), suggesting that the low penetrance of *Dbx*⁺ induction is an intrinsic feature of *Fd4* rather than lineage context. We have added this information in the results section.

(6) It is logical to hypothesize that spatial factors specify early-born neurons directly, so only late-born neurons require *Fd4*, but it was not tested. The model would be strengthened by examining whether *Fd4*-*Gal4*-driven *Vnd* rescues the generation of laterborn neurons in *fd4/fd5* mutants.

When we used *en-gal4* driver to express *UAS-vnd* in the *fd4/fd5* mutant background, we found an average 7.4 ± 2.2 *Eve*⁺ cells per hemisegment ($n=36$), significantly higher than *fd4/fd5* mutant alone (3.9 ± 0.8 cells, $n=52$, $p=2.6 \times 10^{-11}$) (Figure 3J). In addition, 0.2 ± 0.5 *Eve*⁺ cells were ectopic *Hb*⁺ (excluding U1/U2), indicating that *Vnd*-*En* integration is sufficient to generate both early-born and late-born *Eve*⁺ cells in the *fd4/fd5* mutants. We have added the results to the text.

(7) It is mentioned that *Fd5* is not sufficient for the NB7-1 lineage identity. The observation is intriguing in how similar regulators serve distinct roles, but the data are not shown. The analysis in Figure 4 should be performed for *Fd5* as supplemental information.

Thanks for the suggestion. Because the results are exactly the same as the wild type, we don't think it is necessary to provide an additional images or analysis as supplemental information.

Reviewer #2 (Public review):

Via a detailed expression analysis, they find that *Fd4* is selectively expressed in embryonic NB7-1 and newly born neurons within this lineage. They also undertake a comprehensive genetic analysis to provide evidence that *fd4* is necessary and sufficient for the identity of NB7-1 progeny.

Thanks for the accurate summary!

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Thanks for the positive comments!

The argument that Fd4 is necessary for NB7-1 lineage identity is based on a Fd4/Fd5 double mutant. Loss of fd4 alone did not alter the number of NB7-1-derived Eve+ or Dbx+ neurons. The authors clearly demonstrate redundancy between fd4 and fd5, and the fact that the LOF analysis is based on a double mutant should be better woven through the text.

The authors generated an Fd5 mutant. I assume that Fd5 single mutants do not display NB7-1 lineage defects, but this is not stated. The focus on Fd4 over Fd5 is based on its highly specific expression profile and the dramatic misexpression phenotypes. But the LOF analysis demonstrates redundancy, and the conclusions in the abstract and through the results should reflect the existence of Fd5 in the conclusions of this manuscript.

We agree, and have added new text to clarify the single mutant phenotypes (there are none) and the double mutant phenotype (loss of NB7-1 molecular and morphological features. The following text is added to the manuscript: “Not surprisingly, we found that *fd4* single mutants or *fd5* single mutants had no phenotype (Eve+ neurons were all normal). Thus, to assess their roles, we generated a *fd4* and *fd5* double mutant. Because many Eve+ and Dbx+ cells are generated outside of NB7-1 lineage, it was also essential to identify the Eve+ or Dbx+ cells within NB7-1 lineage in wild type and *fd4* mutant embryos. To achieve this, we replaced the open reading frame of *fd4* with *gal4* (called *fd4-gal4*) (see Methods); this stock simultaneously knocked out both *fd4* and *fd5* (called *fd4/fd5* mutant hereafter) while specifically labeling the NB7-1 lineage. For the remainder of this paper we use the *fd4/fd5* double mutant to assay for loss of function phenotypes.”

It is notable that Fd4 overexpression can rewire motor circuits. This analysis adds another dimension to the changes in transcription factor expression and, importantly, demonstrates functional consequences. Could the authors test whether U4 and U5 motor axon targeting changes in the fd4/fd5 double mutant? To strengthen claims regarding the importance of fd4/fd5 for lineage identity, it would help to address terminal features of U motoneuron identity in the LOF condition.

Thanks for raising this important point. We examined the axon targeting on body wall muscles in both wild type and in *fd4/fd5* mutant background and added the results in Figure 3-figure supplement 2. We found that the axon targeting in the late-born neuron region (LL1) is significantly reduced, suggesting that the loss of late-born neurons in *fd4/fd5* mutant leads to the absence of innervation of corresponding muscle targets.

Reviewer #3 (Public review):

The goal of the work is to establish the linkage between the spatial transcription factors (STFs) that function transiently to establish the identities of the individual NBs and the terminal selector genes (typically homeodomain genes) that appear in the newborn postmitotic neurons. How is the identity of the NB maintained and carried forward after the spatial genes have faded away? Focusing on a single neuroblast (NB 7-1), the authors present evidence that the fork-head transcription factor, fd4, provides a bridge linking the transient spatial cues that initially specified neuroblast identity with the terminal selector genes that establish and maintain the identity of the stem cell's progeny.

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Thanks for the accurate summary!

Experimental support for the "bridging" role of Fd4 comes from a set of loss-of-function and gain-of-function manipulations. The loss of function of Fd4, and the partially redundant gene Fd5, from lineage 7-1 does not affect the size of the lineage, but terminal markers of late-born neuronal phenotypes, like Eve and Dbx, are reduced or missing. By contrast, ectopic expression of fd4, but not fd5, results in ectopic expression of the terminal markers eve and Dbx throughout diverse VNC lineages.

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A detailed test of fd4's expression was then carried out using lineages 7-3 and 5-6, two well-characterized lineages in Drosophila. Lineage 7-3 is much smaller than 7-1 and continues to be so when subjected to fd4 misexpression. However, under the influence of ectopic Fd4 expression, the lineage 7-3 neurons lost their expected serotonin and corazonin expression and showed Eve expression as well as motoneuron phenotypes that partially mimic the U motoneurons of lineage 7-1.

Thanks for the positive comments!

Ectopic expression of Fd4 also produced changes in the 5-6 lineage. Expression of apterous, a feature of lineage 5-6, was suppressed, and expression of the 7-1 marker, Eve, was evident. Dbx expression was also evident in the transformed 5-6 lineages, but extremely restricted as compared to a normal 7-1 lineage. Considering the partial redundancy of fd4 and fd5, it would have been interesting to express both genes in the 5-6 lineage. The anatomical changes that are exhibited by motoneurons in response to Fd4 expression confirm that these cells do, indeed, show a shift in their cellular identity.

We appreciate the positive comments. We agree double misexpression of Fd4 and Fd5 might give a stronger phenotype (as the reviewer says) but the lack of this experiment does not change the conclusions that Fd4 can promote NB7-1 molecular and morphological aspects at the expense of NB5-6 molecular markers.

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