Mastermind Mutations Generate a Unique Constellation of Midline Cells within the Drosophila CNS

Yi Zhang*, Randi Wheatley, Eric Fulkerson, Amanda Tapp, Patricia A. Estes*
Department of Genetics, North Carolina State University, Raleigh, North Carolina, United States of America

Abstract

Background: The Notch pathway functions repeatedly during the development of the central nervous system in metazoan organisms to control cell fate and regulate cell proliferation and asymmetric cell divisions. Within the Drosophila midline cell lineage, which bisects the two symmetrical halves of the central nervous system, Notch is required for initial cell specification and subsequent differentiation of many midline lineages.

Methodology/Principal Findings: Here, we provide the first description of the role of the Notch co-factor, mastermind, in the central nervous system midline of Drosophila. Overall, zygotic mastermind mutations cause an increase in midline cell number and decrease in midline cell diversity. Compared to mutations in other components of the Notch signaling pathway, such as Notch itself and Delta, zygotic mastermind mutants cause the production of a unique constellation of midline cell types. The major difference is that midline glia form normally in zygotic mastermind mutants, but not in Notch and Delta mutants. Moreover, during late embryogenesis, extra anterior midline glia survive in zygotic mastermind mutants compared to wild type embryos.

Conclusions/Significance: This is an example of a mutation in a signaling pathway cofactor producing a distinct central nervous system phenotype compared to mutations in major components of the pathway.

Introduction

The central nervous system (CNS) of metazoan organisms consists of many different types of neurons and glia generated through the combinatorial action of intrinsic transcription factors and extrinsic signaling inputs from neighboring cells [1–3]. During CNS development and in a number of developmental contexts, the Notch pathway functions as a prominent signaling system providing positional input between cells in direct contact with one another [4,5]. Previously, several roles for Notch during the development of specific cell lineages within the CNS midline of Drosophila melanogaster embryos have been described [6]. Here, we characterize functions of the co-activator, mastermind (mam) during the development of midline lineages.

One of the most surprising findings from comparative developmental biology is the extensive conservation of signaling pathways both within multiple tissues of a given organism as well as within the same tissue across diverse organisms. The Notch signaling pathway is a salient example and is used repeatedly to construct tissues during development and maintain homeostasis in adults [4,7–9]. Notch signaling occurs between contacting cells when the Notch protein, a transmembrane receptor on the surface of one cell, binds one of its ligands, Delta (Dl) or Serrate/Jagged, on an adjacent cell. After binding one of these ligands, the Notch receptor is cleaved and its intracellular domain (NICD) transported to the nucleus where it interacts with the DNA-binding protein CSL (CBF1 in mammals, Suppressor of hairless (Su(H)) in Drosophila, and LAG-1 in C. elegans; hereafter referred to as Su(H); [10]). In cells devoid of Notch signaling, Su(H) functions as a repressor; whereas, in cells containing activated Notch, the NICD binds to both Su(H) and the co-activator Mam, resulting in a complex that activates transcription of target genes [11–14]. A striking example of the pleiotropic effects of Notch on a cell lineage can be found during CNS midline cell development in fruit flies [6]. In that study, Dl mutants were used to show that Notch promotes formation of midline glia and several midline neurons, while inhibiting the formation of other midline neurons.

The CNS is located on the ventral side of the Drosophila embryo and consists of a repeated unit found within all thoracic and abdominal segments. Midline cells of Drosophila are located in the center of the embryonic CNS (Figure 1A) and they signal to and organize axons in a manner analogous to floor plate cells within the spinal cord of vertebrates, using similar signaling molecules [15,16]. Because of its simplicity, the fly midline is used to study axon guidance as well as transcription factors and signaling pathways involved in nervous system development [17–19]. Previous studies indicate the initial specification of Drosophila midline cells depends on expression of single-minded (sim), the master regulator of this
Here, we provide the first study of mam functions in the various CNS midline lineages of Drosophila. The results indicate that both anterior and posterior midline glia (AMG and PMG) appear to form normally in mam mutant embryos, in contrast to midline glia in Notch and Dl mutants, which are completely absent. The presence of midline glia in mam mutants allows us to follow their development in late embryogenesis, when zygotic mam mutants cause an increase in the number of midline glia that survive in the mature CNS. In addition, mam and Notch mutants differ in the composition of MP1 neurons, whereas the other midline neural phenotypes observed in mam mutants are also observed in Notch and Dl mutants [6]. Further comparisons of Notch and mam mutants indicate that differences in the expression of the midline gene, sim, contribute to the observed difference in midline phenotypes. Taken together, the results demonstrate that zygotic mutations in the mam co-factor result in a midline cellular composition distinct from zygotic Notch mutations.

**Results**

*Mam* was identified in a screen for genes that function in midline development

To identify genes involved in *Drosophila* midline development, we used EMS to introduce mutations throughout the genome of the fly and then examined midline cells using a reporter gene combination that drives GFP expression in all midline cells (*UAS-GFP, sim-GAL4*). In this way, GFP could be visualized and followed in live embryos during late embryonic and larval development; stages that are difficult to examine using routine immunostaining techniques. 1037 lines carrying lethal mutations on the second chromosome were established and embryos from each line were collected and examined for midline cell defects (Figure 2A). Of the 1037 lethal lines screened, 21 showed midline defects based on the *UAS-GFP, sim-GAL4* reporter. These mutations were mapped within the genome using complementation; first with deficiency lines and then with fly lines containing mutations in single genes. In this report, we focus on one of the mutations that disrupted midline development and mapped to the mam locus [32–34]. Mam encodes the transcriptional co-activator of canonical Notch signaling [12] and is a glutamine-rich nuclear protein with a predicted 1596 amino acid sequence [35]. The protein contains a highly conserved basic domain within the N-terminus that binds to both the NICD and Su(H); and 3 glycine-valine (GV) runs and 2 acidic clusters in the C-terminal region needed for 1) interactions with p300 and RNA polymerase and 2) stability of the NICD/Su(H) complex (Figure 2B; [32,36–39]). Sequence analysis of the mam*AC* allele isolated in our screen predicts it encodes a truncated protein lacking both the C-terminal acid cluster and the GV runs (Figure 2C) and our phenotypic analysis indicates it behaves as a strong loss of function mutation (see below). The midline of mam*AC* mutant embryos was disorganized and less compact than the midline of wild type embryos during late embryonic stages (Figure 2D and E). Numerous studies have described mam functions in CNS development [40–42], yet its role in midline development has not been reported. This, the midline phenotype of mam*AC* mutant embryos and the previously characterized roles of Notch signaling during midline cell development, led us to investigate how various midline lineages were affected in mam*AC* mutant embryos.

**AMG and PMG are present in mam*AC*, but not N*Sex11* mutant embryos**

Previous lineage analysis suggested midline glial precursors undergo multiple divisions to give rise to 2 populations of midline glia at late stages [6,30,43]. At stage 13, each segment contains about 6 AMG derived from the anterior compartment of the segment that express *runt* but not *en*; and 4 PMG cells, derived from the posterior compartment that express *en* but not *runt* (Figure 1B). Later, at stage 16, only 3 AMG survive to enwrap the axon commissures, while all of the PMG and remaining AMG are depleted by apoptosis [43–45]. Both AMG and PMG are missing in *Dl* mutants, suggesting the Notch pathway is required for development of both glial lineages [6]. To examine midline glial development in mam*AC* mutant embryos, we monitored *Wrapper*, an immunoglobulin protein required for midline glial survival, and expressed almost exclusively in the midline glia, at a high level in AMG and a lower level in PMG [43,46]. The development of
AMG can be followed using the co-localization of Wrapper and Rant, while the PMG can be identified using co-localization of Wrapper and En (Figure 3A–P). During mid and late embryogenesis, Wrapper protein was never detected in the midline of N55e11 homozygous embryos, a null allele of Notch (Figure 4F and S1A), but present at high levels in the AMG and at lower levels in the PMG of wild type and mamAC mutant embryos (Figure 3A–P). At stage 13 (mid embryogenesis), both wild type and mamAC mutants contained 6 AMG per segment (Figure 3A and I; Table 1). Wild type embryos contained 4 PMG, whereas mamAC mutants contained about 3 per segment (P = 0.0001; Figure 3A and I; Table S2). By stage 16 (late embryogenesis), wild type embryos contained just 3 AMG (Figure 3D and L; Table 1), whereas mamAC mutants contained approximately 5 AMG (P = 0.0001; Table 1; Figure 3H and P). The PMG were not detectable at stage 16 in mamAC mutants compared to wild type embryos.

Additional AMG survive in mam mutant embryos

As described above, analysis of mam mutants indicated they contained additional AMG during late embryonic stages. To further investigate the AMG in mamAC mutant embryos, we investigated the interaction between mam and the EGFR signaling pathway, which is known to affect AMG survival. For these cells to survive, they must receive Spitz (Spz) from lateral CNS axons that cross the midline [44]. In AMG that die, the Head Involution Defective (HID) protein is active and stimulates apoptosis, whereas in surviving AMG, cell surface EGFR binds to Spz, leading to HID phosphorylation. Phosphorylated HID is inactive, and therefore, Spz-activated glia survive. Because Notch and EGFR signaling act antagonistically in many tissues [49–51], we wanted to determine their relationship in AMG. However, this is not possible in Notch.
mutants because they lack midline glia. Instead, we investigated interactions between mam and EGFR in AMG by overexpressing the secreted form of Spi in the midline of mamDC mutant embryos. As described above, we found approximately 6 AMG per segment in both wild type and mamDC mutant embryos during mid embryogenesis, using the co-localization of Sim and Runt (Table 1; Figure 5A and B). Embryos overexpressing spi had a significant increase in AMG (P = 0.001; Table 1 and Figure 5C) to 8 per segment at stage 13. During this stage, embryos overexpressing spi in a mamDC mutant background could not be distinguished from embryos expressing spi in a wild type background or wild type embryos (Table 1 and Figure 5D). By late embryogenesis, the number of AMG in wild type embryos decreased to approximately 3 per segment as previously reported [6]; Figure 5E). Interestingly, all 3 classes: 1) mamDC mutants, 2) embryos overexpressing spi, and 3) embryos overexpressing spi in a mamDC mutant background each had around 5 AMG per segment and each class was significantly different from wild type embryos (Table 1 and Figure 5F–H). This, together with the known neurogenic nature of mam mutations [42], suggested midline glia may be exposed to additional spi provided by the extra neurons generated in mamDC mutants. To investigate this, we compared the interaction of the midline glia with lateral axons in wild type and mamDC mutant embryos using Wrapper and the BP102 monoclonal antibody (Figure S1D–I). The results indicate that the additional AMG present in these embryos do enwrap lateral axons and have increased glial processes that stain with the wrapper antibody (see also Figure 3M–P). Moreover, the nerve cord does not retract normally in mamDC mutant embryos (data not shown), which may also be a consequence of extra neural tissue present in these embryos. These results suggest the greater number of neurons generated in mamDC mutant embryos may provide excess spi that allows additional AMG to survive.

**Notch activation expands expression of a Wrapper reporter**

Results described above as well as previous studies [6] suggest Notch signaling promotes AMG and PMG development, which are completely absent in N55e11 zygotic mutants. Because the AMG developed normally in mamAC zygotic mutants, we next compared the effect of overexpressing mam to the overexpression of other Notch signaling components. For these experiments, we examined both the presence of AMG using a Runt antibody, as well as the regulation of gene expression within AMG using a wrapper reporter gene. The reporter contains an 884 bp wrapper enhancer sufficient to drive expression of the GFP reporter gene in midline glia (Figure 6A and F; [52]). Expressing a constitutively active form of Su(H), UAS-Su(H)-VP16 [53], in all midline cells using sim-GAL4, causes a three-fold increase of midline glial cells at the expense of midline neurons [6]. Expression of the wrapper transcriptional reporter was greatly expanded when either the NCD (Figure 6B) or Su(H)-VP16 (Figure 6C) was overexpressed in the midline using the sim-GAL4 driver. Co-localization with Runt indicated the
embryos. AMG are present within (B) precursors expressing during late embryogenesis (stage 16). The stars indicate muscle compare the phenotype of AMG in different genetic backgrounds sim
gous males or (E) embryogenesis. Transheterozygous embryos were generated by NICD expansion was due to the formation of additional AMG expressing glia cannot be detected in (F) disorganized compared to (A) wild type embryos. In contrast, midline

Figure 4. Comparison of AMG in different genetic backgrounds (Figure 6A). Likewise, significantly more AMG survived until stage 16 in the NICD and Su(H)/VPI6 overexpression embryos compared to wild type embryos (Table 1; Figure 6F, G and H) as previously reported[6]. Therefore, over activation of the Notch pathway in the midline led to an increase in the number of AMG as well as activation of the wrapper reporter in the additional cells.

In contrast, UAS-mam sim-GAL4 embryos at both embryonic stages 13 and 16 appear normal and showed no increase in AMG at stage 16 (Figure 6D and I). Finally, embryos in which the NICD was overexpressed in the midline of mamAC mutant embryos also contained extra AMG (Figure 6J), similar to embryos overexpressing the NICD in a wild type background (Figure 6G). These results suggest AMG can form in the absence of zygotic mam function.

AMG do not form in mamAC gemline clones

Midline glia may form in zygotic mamAC mutant embryos because maternal mam transcripts are stable and produce sufficient Mam protein to function during Notch signaling when glia differentiate. To determine if AMG can form in embryos lacking maternal mam transcripts, we generated mamAC gemline clones using the FRT, hslp system [54] and examined wrapper expression. Both Notch [55] and mam [32] are maternally deposited and gemline clones of either gene exhibit a strong neurogenic phenotype [42]. We observed variable phenotypes in mamAC gemline clones and many embryos had gross developmental defects. Most embryos did not express wrapper, although some did express this gene at low and variable levels and often in only limited regions of the embryo (Figure S1B and C). Embryos containing either one or no copies of mam had the same phenotypes, suggesting that it was the maternal and not zygotic mam activity that caused the reduction in wrapper expression.

Because zygotic mamAC mutants expressed wrapper at high levels, while mamAC gemline clones did not, we compared midline development in embryos lacking either maternal or zygotic mam at earlier developmental stages. For these experiments, we examined sim expression, which is first activated at the blastoderm stage in the mesectoderm. Mesectodermal cells are located between the mesoderm and ectoderm on both sides of the embryo (Figure 7A) and Notch is needed in these cells for initial sim activation [22,26,56]. We determined if mam functions together with Notch to activate sim by examining mamAC gemline clones. Wild type embryos express sim in the mesectoderm throughout the length of the embryo (Figure 7A) at the blastoderm stage. In contrast, most embryos derived from homozygous mamAC mutant mothers contained gaps in sim expression, and many embryos expressed sim in only a few cells (Figure 7B and C). The observed variation in sim expression is similar to that observed in embryos derived from Notch gemline clones [26,56]. As development progresses, the mesoderm invaginates at gastrulation and mesectodermal cells move toward and meet at the ventral midline. After this, sim was expressed at high levels in both midline and muscle precursors of wild type embryos (Figure 7D), whereas sim expression was low or undetectable in the midline, and expanded in muscle precursor cells of embryos derived from mamAC gemline clones (Figure 7E and F). These results indicate that maternal mam, similar to maternal Notch, is required to activate sim during early Drosophila development.

sim maintenance is disrupted in N5Se11, but not mamAC zygotic mutants

Because germline clones of either mam or Notch lack sim expression early in development, midline cells do not develop [22] and the various midline lineages cannot be examined in these embryos. Therefore, to examine zygotic roles for mam and Notch on sim expression, we used our mamAC allele and the N5Se11 allele. We first determined if early sim activation was affected in mamAC zygotic mutants produced by wild type mothers (mamAC heterozygotes) and compared the results to zygotic mamAC mutants. Sim

Table 1. Comparison of AMG in wild type, mamAC and N5Se11 mutant embryos and embryos overexpressing Notch and EGF signaling components during mid and late embryogenesis.

| Genotype       | stage 13 | stage 16 |
|----------------|----------|----------|
| wild type      | 6.3 ± 0.27 a (18) | 2.9 ± 0.16 a (14) |
| mamAC          | 6.4 ± 0.41 a (9) | 4.7 ± 0.40 bc (10) |
| mamAC NICD     | 0.0 ± 0.00 b (11) | 0.0 ± 0.00 e (9) |
| UAS-NICD       | 16 ± 0.43 c (15) | 6.1 ± 0.27 bd (17) |
| UAS-Su(H)      | 16 ± 0.37 c (11) | 6.7 ± 0.19 d (12) |
| UAS-mam        | 6.6 ± 0.29 a (21) | 2.2 ± 0.94 a (15) |
| UAS-sspi       | 9.5 ± 0.61 d (11) | 4.9 ± 0.22 bc (14) |
| mamAC UAS-sspi | 7.7 ± 1.01 ad (9) | 4.5 ± 0.62 c (13) |

The number of AMG found in a single CNS segment of wild type, mamAC and N5Se11 mutants and embryos overexpressing the NICD (UAS-NICD sim-GAL4), Su(H) (UAS-Su(H) sim-GAL4), mam (UAS-mam sim-GAL4) or spi (UAS-sspi sim-GAL4) in the midline and embryos overexpressing sim in the midline of mam mutants (mamAC UAS-mamAC sim-GAL4) at stages 13 and 16 is shown. Results are shown as means ± SEM and the sample size is indicated in parentheses. Stage 13 ANOVA: F8,195 = 140.18, P = 0.0001 and stage 16 ANOVA: F8,220 = 147.99, P = 0.0001. Within a column, treatments with different letters are significantly different (Tukey-Kramer HSD, P < 0.05).

doi:10.1371/journal.pone.0026197.t001

Therefore, over activation of the Notch pathway in the midline led to an increase in the number of AMG as well as activation of the wrapper reporter in the additional cells.

In contrast, UAS-mam sim-GAL4 embryos at both embryonic stages 13 and 16 appear normal and showed no increase in AMG at stage 16 (Figure 6D and I). Finally, embryos in which the NICD was overexpressed in the midline of mamAC mutant embryos also contained extra AMG (Figure 6J), similar to embryos overexpressing the NICD in a wild type background (Figure 6G). These results suggest AMG can form in the absence of zygotic mam function.

AMG do not form in mamAC gemline clones

Midline glia may form in zygotic mamAC mutant embryos because maternal mam transcripts are stable and produce sufficient Mam protein to function during Notch signaling when glia differentiate. To determine if AMG can form in embryos lacking maternal mam transcripts, we generated mamAC gemline clones using the FRT, hslp system [54] and examined wrapper expression. Both Notch [55] and mam [32] are maternally deposited and gemline clones of either gene exhibit a strong neurogenic phenotype [42]. We observed variable phenotypes in mamAC gemline clones and many embryos had gross developmental defects. Most embryos did not express wrapper, although some did express this gene at low and variable levels and often in only limited regions of the embryo (Figure S1B and C). Embryos containing either one or no copies of mam had the same phenotypes, suggesting that it was the maternal and not zygotic mam activity that caused the reduction in wrapper expression.

Because zygotic mamAC mutants expressed wrapper at high levels, while mamAC gemline clones did not, we compared midline development in embryos lacking either maternal or zygotic mam at earlier developmental stages. For these experiments, we examined sim expression, which is first activated at the blastoderm stage in the mesectoderm. Mesectodermal cells are located between the mesoderm and ectoderm on both sides of the embryo (Figure 7A) and Notch is needed in these cells for initial sim activation [22,26,56]. We determined if mam functions together with Notch to activate sim by examining mamAC gemline clones. Wild type embryos express sim in the mesectoderm throughout the length of the embryo (Figure 7A) at the blastoderm stage. In contrast, most embryos derived from homozygous mamAC mutant mothers contained gaps in sim expression, and many embryos expressed sim in only a few cells (Figure 7B and C). The observed variation in sim expression is similar to that observed in embryos derived from Notch gemline clones [26,56]. As development progresses, the mesoderm invaginates at gastrulation and mesectodermal cells move toward and meet at the ventral midline. After this, sim was expressed at high levels in both midline and muscle precursors of wild type embryos (Figure 7D), whereas sim expression was low or undetectable in the midline, and expanded in muscle precursor cells of embryos derived from mamAC gemline clones (Figure 7E and F). These results indicate that maternal mam, similar to maternal Notch, is required to activate sim during early Drosophila development.

sim maintenance is disrupted in N5Se11, but not mamAC zygotic mutants

Because germline clones of either mam or Notch lack sim expression early in development, midline cells do not develop [22] and the various midline lineages cannot be examined in these embryos. Therefore, to examine zygotic roles for mam and Notch on sim expression, we used our mamAC allele and the N5Se11 allele. We first determined if early sim activation was affected in mamAC zygotic mutants produced by wild type mothers (mamAC heterozygotes) and compared the results to zygotic mamAC mutants. Sim

sim maintenance is disrupted in N5Se11, but not mamAC zygotic mutants

Because germline clones of either mam or Notch lack sim expression early in development, midline cells do not develop [22] and the various midline lineages cannot be examined in these embryos. Therefore, to examine zygotic roles for mam and Notch on sim expression, we used our mamAC allele and the N5Se11 allele. We first determined if early sim activation was affected in mamAC zygotic mutants produced by wild type mothers (mamAC heterozygotes) and compared the results to zygotic mamAC mutants. Sim
expression is normal until stage 10 in zygotic mam\textsuperscript{DC} mutant embryos (Figure 7H) and persists in subsequent stages, although at a reduced level (Figure 7K). Sim expression in the midline of N55e11 mutant embryos also appeared normal at stage 10 (Figure 7I), but completely disappeared by stage 13 (Figure 7L). These results indicate that, unlike maternal mutations in mam\textsuperscript{DC} and N55e11, zygotic mutations in these genes do not affect early sim expression prior to stage 10 and can therefore, be used to study their functions.

Figure 5. During late embryogenesis, the number of AMG that survived was similar within both mam\textsuperscript{DC} mutant embryos and embryos overexpressing Spi. Co-localization of sim (red) and runt (green) was used to compare the number of AMG in different genetic backgrounds during mid and late embryogenesis. At mid embryogenesis, (A) wild type embryos contain a compact cluster of approximately 6 AMG and (B) mam\textsuperscript{DC} mutant embryos have approximately the same number of AMG, but are less compact (arrow). During mid embryogenesis, (C) overexpression of Spi causes an increase in AMG to approximately 9 AMG, while (D) overexpression of Spi in a mam\textsuperscript{DC} background resulted in a number of AMG indistinguishable from both wild type and Spi overexpression embryos. During late embryogenesis, (E) 3 AMG are present in wild type embryos, while (F) mam\textsuperscript{DC} mutant embryos, (G) embryos overexpressing spi and (H) embryos overexpressing spi in a mam\textsuperscript{DC} mutant background, all contain about 5 AMG. Statistical comparisons of AMG cell counts are shown in Table 1. Lateral views of (A–D) stage 13 and (E–H) 16 embryos are shown and white lines indicate individual CNS midline segments.

doi:10.1371/journal.pone.0026197.g005

Figure 6. Overexpression of either the NICD or Su(H).VP16, but not mam, causes an increase in the number of AMG. (A–J) Analysis of a wrapper\textsuperscript{GFP} reporter gene indicates GFP expression is higher in the AMG (arrow) than in the PMG (arrowhead). During mid embryogenesis, the number of AMG increased when (B) the NICD in a wild type background or (E) the NICD in a mam\textsuperscript{DC} mutant background, or (C) Su(H).VP16 was overexpressed in the midline, whereas AMG number was unchanged compared to (A) wild type when (D) mam was overexpressed in the midline. During late embryogenesis, more AMG survived in embryos overexpressing (G) the NICD in a wild type background or (J) the NICD in a mam\textsuperscript{DC} mutant background or (H) Su(H).VP16 in the midline, whereas embryos overexpressing (I) mam in the midline contained about the same number of AMG as (F) wild type embryos. Shown are whole-mount (A and F) wild type, (B and G) UAS-NICD sim-GAL4, (C and H) UAS-Su(H).VP16 sim-GAL4 (D and I) UAS-mam sim-GAL4 and (E and J) UAS-NICD sim-GAL4 in a mam\textsuperscript{DC} mutant background. Statistical comparisons of AMG cell counts are shown in Table 1. Shown are lateral views of embryos labeled with anti-GFP (green) and anti-Runt (red) antibodies; anterior is toward the top, left corner.

doi:10.1371/journal.pone.0026197.g006
during subsequent stages of midline development. Moreover, the results indicate maternal mam·DC and N55e11 mutations have similar effects on sim expression during early development, whereas sim expression is maintained in zygotic mam·DC, but not N55e11 mutants during mid and late embryogenesis.

The formation of certain midline neurons requires both Notch and mam

Next, we examined the effects of the mam·DC mutation on the development of midline neurons. During embryonic stage 11, midline precursors (MPs) delaminate and divide to produce 6 neuronal subtypes [6,28]. The MPs (1–6) are named based on their anteroposterior position within the segments of the CNS and each midline neural cell type (Figure 1B–D) expresses a unique gene combination that can be used to follow them during development [31]. We selected tractable markers for the various midline lineages to examine their fate in mam·DC mutants.

We first examined the MP1 neural lineage, located within the anterior most region of each midline segment, using an Odd-skipped (Odd) antibody [31]. Odd labels 2 MP1 cells and 2 nearby MP2 cells in each CNS segment of wild type embryos (Figure 8A). To distinguish the MP1 and MP2 neurons, we utilized the UAS-GFP sim-GAL4 reporter that labels MP1, but not MP2 neurons. Mam·DC mutant embryos also contained 2 MP1 neurons per
segment, similar to wild type (Figure 8A–F; [6]), while \(N^{55e11}\) mutant embryos had approximately 6 Odd positive MP1 neurons per segment (Table S1). Previous studies demonstrated that \(Notch\) mutants contain 2 additional Odd-positive MP2 cells per segment and this was also true in \(mam^{AC}\) mutants (Figure 8E). These results indicate \(mam^{AC}\) mutants resemble wild type embryos and differ...
from Notch mutants in the number of MP1 neurons that form during embryogenesis.

Next, we examined the MP3 lineage which is located just posterior to MP1s within each segment and normally divides asymmetrically to produce 1 H cell (Figure 8G) and 1 iVUM cell sib neuron (Figure 8J) in wild type embryos [6]. In mam\(^{Ac}\) mutant embryos, the H cell sib was not detected as assessed by CG13565 expression (Figure 8K), while approximately 6 H cells that expressed tyrosine hydroxylase (TH) were found in each segment (Table S1; Figure 8L). This was similar to N\(^{55e11}\) mutant embryos in which the H cell sib was absent (Figure 9L) and the number of H cells in each segment increased to 10 (Table S1; Figure 8L). These results indicate both mam\(^{Ac}\) and N\(^{55e11}\) have similar functions in the asymmetric cell division of the MP3 midline lineage and are needed for the formation of the H cell sib. Moreover, the zygotic N\(^{55e11}\) mutation had a significantly larger effect on the number of H cells that formed compared to the mam\(^{Ac}\) mutation (P = 0.0001; Table S1).

Next, we examined lineages derived from MP4-6 found within the posterior of each segment. Each of these divide asymmetrically once to produce an iVUM and a mVUM, resulting in 3 of each per segment (see [6] and Table S1). The number of mVUMs increased from 3 cells per segment in wild type embryos (Figure 8M) to 11 in mam\(^{Ac}\) mutants as assessed with Tyramina β hydroxylase (Thb), a specific marker for these midline cells (Table S1; Figure 8N). In N\(^{55e11}\) mutant embryos, the number of mVUMs also increased to 11 per segment (Table S1; Figure 8O). To follow the iVUMs, which are also derived from MP4-6, as well as the MNs and its progeny, we assayed midline cells for the presence of En which is normally expressed in these midline neural lineages, as well as the PMG (see below). En was undetectable in the midline of N\(^{55e11}\) mutants after stage 10 (data not shown), suggesting the iVUMs and the MNs and its progeny were absent. En protein levels appear relatively normal in mam\(^{Ac}\) mutants (Figure 9E and F) compared to wild type embryos (Figure 9A and B) until mid embryogenesis. During later developmental stages, each midline segment of wild type embryos contains 3 iVUMs and the progeny of the MNB, which divides multiple times after stage 11 to generate approximately 5–6 GABAergic neurons during embryogenesis [6]. However, only PMG express en in stage 13 mam\(^{Ac}\) mutants (Figure 9G) and eventually, these cells also disappear (Figure 9H; also see Figure 3), as they do in wild type embryos (Figure 9D). Moreover, midline cells within mam\(^{Ac}\) mutant embryos remain at the dorsal side of the nerve cord (Figure 9G and H), which was also previously observed in Notch mutant embryos [56]. The results suggest that Notch, like Notch, is needed for the production of iVUMs during the asymmetrical cell divisions of MPs 4, 5 and 6 as well as for the development of the MNB and its progeny. In summary, midline neural phenotypes in mam\(^{Ac}\) mutant embryos are, in some cases, less severe, but consistent with midline phenotypes previously observed in Delta mutants [6] and N\(^{55e11}\) mutants (Table S1), with the exception of the MP1 neurons. The MP1 neurons appear unaffected in mam\(^{Ac}\) mutants, while N\(^{55e11}\) mutants contain additional MP1s. Taken together, these studies of mam\(^{Ac}\) and N\(^{55e11}\) mutants, together with previous experiments with Delta mutants [6], indicate zygotic mutations in all 3 genes produce similar midline phenotypes of most neural subtypes. However, midline glia are eliminated and MP1 neurons expanded in Notch and Delta mutants, but not in mam\(^{Ac}\) mutants.

**Discussion**

*Notch* has been shown to play multiple developmental roles in the CNS of several organisms [4,7–9]. The *Drosophila* midline, with its easy to identify neural and glial lineages, has provided examples of multiple and reiterative roles of the *Notch* pathway within a single CNS lineage [6]. Here, the characterization of mam\(^{Ac}\) mutants indicates how a co-factor within a signaling pathway contributes to the development of different midline cell types and adds to our understanding of Notch signaling complexity.

Initial activation of sim in the mesectoderm depends on maternal *Notch* expression [26,56,57], as N\(^{55e11}\) germine clones

Figure 9. Midline cells that normally express en are absent in late mam\(^{Ac}\) mutant embryos. En (green) and sim (red) expression was monitored in (A–D) wild type and (E–H) mam\(^{Ac}\) mutant embryos during embryogenesis using specific antibodies. Prior to stage 10, 16 midline cells per segment are on the surface of the embryo (A and E), during stages 10 and 11, they delaminate into the developing nerve cord (B and F) and then differentiate into midline neurons and glia (D and H). En expression is indistinguishable in wild type (A and B) and mam\(^{Ac}\) mutant embryos (E and F) during stages 9–12, but diminishes in later stages in mam\(^{Ac}\) mutant embryos (G and H) compared to wild type (C and D). (A, B, E and F) Ventral or (C, D, G and H) lateral views of 2–3 segments are shown with anterior in the top, left-hand corner. Numbers above images indicate the developmental stage shown. At stage 16, iVUM neurons, the MNB and its progeny, and PMG express both sim and en and appear yellow, while the AMG and H cell express sim but not en and appear red. Asterisks indicate lateral CNS neurons that express en, but not sim. doi:10.1371/journal.pone.0026197.g009

Discussion

*Notch* has been shown to play multiple developmental roles in the CNS of several organisms [4,7–9]. The *Drosophila* midline, with its easy to identify neural and glial lineages, has provided examples of multiple and reiterative roles of the *Notch* pathway within a single CNS lineage [6]. Here, the characterization of mam\(^{Ac}\) mutants indicates how a co-factor within a signaling pathway contributes to the development of different midline cell types and adds to our understanding of *Notch* signaling complexity.

Initial activation of sim in the mesectoderm depends on maternal *Notch* expression [26,56,57], as N\(^{55e11}\) germine clones
lack most sim expression and therefore, contain few midline cells. Likewise, mamAC germline clones also show a reduction in sim expression. Thus, maternal contributions of both mam and Notch appear to act in the same pathway to activate sim early in development. Similarly, many midline neural phenotypes in zygotic mamAC mutant embryos are largely consistent with those of N55e11 and DI1 [6], suggesting mam and Notch act together during the development of these neurons. Notch is required for formation of neurons expressing en [6] and may be needed to maintain en expression in midline cells that develop in the posterior compartment of each CNS segment, as first suggested by Bossing and Brand [27]. The results described here suggest mam is also required for the formation of the midline neurons that express en and develop into the iVUMs, the MNB and its progeny (Figure 10). While these cells of the posterior compartment were absent, the H cell and mVUM midline neurons were expanded in mamAC mutants (Figure 10), similar to N55e11 and DI1 mutants, suggesting that mam function is needed within the Notch signaling pathway to obtain the variety of midline neurons found in wild type embryos [6]. The major difference we observed between zygotic mamAC and N55e11 mutants was the presence of midline glia in mamAC, but not N55e11 mutant embryos during mid to late embryogenesis (Figure 10). Not only were AMG present, but additional AMG survived in the mature CNS midline in mamAC mutants compared to wild type embryos (and N55e11 mutants). The presence of AMG in mamAC mutants suggests either 1) the mamAC mutation is hypomorphic, 2) mam is not required within the Notch pathway for midline glial differentiation or 3) maternally deposited mam transcripts are stable and functional during the Notch signaling event needed for midline glial formation. Results with mam deficiency embryos indicated that midline glia formed and persisted in the complete absence of zygotic mam activity, suggesting it is not the hypomorphic nature of the mamAC allele that allows the midline glia to form. Currenty, we cannot distinguish between the other two possibilities, although we favor the last hypothesis due to the timing of midline cell divisions. At gastrulation, each segment contains 8 mesectodermal cells, which each divide, resulting in 16 MPs per segment at stage 10. Cells that give rise to AMG and PMG do not divide again, whereas MPs that develop into neurons each divide once at stage 11. Because MPs that give rise to glia undergo their last division earlier than MPs that give rise to neurons, the Notch signaling event needed for midline glial differentiation may occur prior to Notch events that dictate midline neural fates at stage 11. Maternal Mam protein may linger just long enough to allow midline glia to form, but not long enough to function when MPs divide to give rise to midline neurons slightly later. We think this is the reason N55e11 mutants contain more midline cells per segment than wild type (and mamAC; Table 2). In N55e11 mutants, MPs that would normally form glia and not divide, instead take on neural fates and do divide. Our data are consistent with this hypothesis, but future, additional experiments are required to properly test it.

In addition to this temporal sensitivity, mam may also be sensitive to spatially restricted events within the midline. Existing evidence suggests the 16 MPs fall into 3 equivalence groups at stage 10: the MP1s, MP3s and MP4s [6]. MP1s are in the anterior, MP3s in the middle and MP4s in the posterior of each CNS segment and effects of mamAC vary according to these positions. The results indicate that neurons derived from the anterior MP1s are sensitive to N55e11, but not mamAC; the middle MP3s are more sensitive to N55e11 than mamAC, while the posterior MP4s are equally sensitive to N55e11 and mamAC. In other words, mamAC mutants 1) differ with N55e11 mutants in neurons derived from MP1s (MP1 neurons), 2) have similar, less severe effects compared to N55e11 mutants in cells derived from the MP3s (the H cell and H cell sib) and 3) the same effects as N55e11 mutants in cells derived from the posterior MP4s (mVUMs, iVUMs and MNB). These differences may be due to region specific differences in expression of other midline regulators that combine with Notch and/or Mam to control cell fate specification during embryogenesis [58]. Possible candidates include hedgehog and wingless, which are expressed in the midline, affect cell fate [27] and both interact with mam in a Notch-independent manner in other tissues [48,59,60]. In any case, clear differences in zygotic mam and Notch mutations within the midline exist and demonstrate that variations in different Notch signaling components can alter the cellular composition of the CNS in unique ways.

Close examination of mamAC and N55e11 mutants during mid embryogenesis indicates they also differ in sim expression. After stage 10, sim diminishes in N55e11 mutants, but persists in mamAC mutants. Likewise, midline glia, which are known to require sim expression to differentiate, do not develop in N55e11 mutants, but do develop in mamAC mutants. Our data indicate that all midline lineages that normally express sim are absent in N55e11 mutants, while midline lineages that do not normally express sim are present and expanded in zygotic mutants of N55e11 (Table 2). Therefore, similar to the initiation of sim expression early, the maintenance of sim expression at this later time also appears to require zygotic Notch activity. In contrast, the results suggest sim expression persists in zygotic mamAC mutants.

In the canonical Notch pathway, Mam normally functions as a co-factor and collaborates with both the NCD and Su(H) to

Figure 10. Comparison of CNS midline cell composition in wild type, mamAC and N55e11 mutant embryos. Drosophila midline cells within a single segment at late embryogenesis (stage 16) are compared schematically in (A) wild type, (B) mamAC and (C) N55e11 mutant embryos. (A) Wild type embryos contain 3 AMG and 6 different types of midline neurons. (B) MamAC mutants contain 3 types of midline neurons and 5 AMG, whereas (C) N55e11 mutant embryos contain 3 types of midline neurons and no AMG. (D) Each color corresponds to a particular midline cell type.

doi:10.1371/journal.pone.0026197.g010
activate target genes. Consistent with this role, overexpression of mam alone does not affect the number of AMG generated at mid embryogenesis, whereas the overexpression of the NRCD in wild type embryos increases AMG cell number [6]. Overexpression of the NRCD in a mam AC mutant background still increased the number of AMG during this stage, further supporting the idea that zygotic mam is not needed at this time. During late embryogenesis, mam AC mutants contained extra AMG. Mutations in mam are known to promote neural tissue at the expense of ectoderm and this may result in the production of additional Spi, which inhibits apoptosis and allows extra midline glia to survive.

Altogether, the data suggest a high level of complexity in the regulation of CNS target genes of Notch. Notch likely interacts with additional cell-lineage specific co-activators other than, or in addition to, Mam in certain cells. In this way, combinatorial interactions between components of Notch signaling and other signaling pathways can lead to different outputs in various cell types, increasing cell diversity and function. The results described here indicate mam AC mutants contain AMG and PMG, whereas N55e11 mutants do not. While this report describes major disruptions in mam, less severe mutations, such as small deletions, insertions or polymorphisms could also affect the midline and modify its cellular composition. Because mam mutations have more subtle effects on the midline compared to mutations in Notch or Delta, they may be tolerated more than mutations in major components of the pathway and actually contribute to CNS cellular variation in natural populations. Future experiments are needed to fully explore these functional differences between mam and Notch in the midline, as well as other tissues. Such differences can then be exploited to develop progressively specific research and clinical tools to regulate Notch signaling and the cellular composition of tissues [61,62].

Materials and Methods

Drosophila strains

The Drosophila fly strain used in the genetic screen was homozygous for both the UAS-GFP and sim-GAL4 transgenes which were recombined onto the same second chromosome. This combination labels all Drosophila midline cells beginning at developmental stage 10, through the remainder of embryogenesis and during larval stages. Prior to the mutagenesis screen, this line was isogenized using the yw 67 strain. The deficiency kit DRK2, the 3 small deficiencies of mam, Df(2R)BSC383, Df(2R)50C-38, and Df(2R)BSC18, the mam AC mutant line [34] and the UAS-GFP line were obtained from the Bloomington Stock Center. Additional fly lines used were: N55e11 described in [63], D0 [64], mam AC (this study), sim-GAL4 [65], UAS-NRCD and UAS-Snail, VP16 [53], and UAS-mam [66]. The FLP–DFS technique was used to generate mam AC germline clones [34]. For this, the mam AC mutation was first recombined onto the FRT42B chromosome and then w; P{hs-FLP}/w; P{w +, FRT}42B mam AC/CyO virgin females were crossed to yw 67, P{hs-FLP}; P{w +, FRT}42B, P{Osrop}153D/CyO males. Next, 2–3 days old larvae with the genotype y w; P{hs-FLP}/yw; P{w +, FRT}42B, P{Osrop}153D/P{w +, FRT}, FRT}42B mam AC generated from the cross were incubated at 37°C for 2 hours to induce recombination. Enclosed virgins were then crossed to w; mam AC/CyO males. Embryos collected from this cross were fixed and subjected to fluorescent in situ hybridization and immunohistochemistry. To test the effect of overexpressing the secreted form of Sim in mam AC mutants, the mam AC mutation was recombined onto both the UAS-spi4a chromosome [67] and the sim-GAL4 chromosome.

Isolation of EMS generated mam mutants

To screen for genes on the second chromosome that affect midline development, yw 67; sim-GAL4 UAS-GFP males were mutagenized with ethyl methanesulfonate (EMS) and then mated en mass to yellow (y) white (w) 67; Lobe (L)/CyO Knirpel (ki)-GFP females. Single F1 male progeny were then backcrossed to 3 yw 67, L/E/CyO Ki-GFP virgin females in a single vial. Next, F2 siblings of the genotype yw 67; UAS-GFP sim-GAL4/CyO, Ki-GFP were mated, and the absence of F3 progeny with straight wings indicated a line bearing a lethal second chromosome mutation (Figure 2A). To visually screen the lines bearing a lethal mutation on the second chromosome, embryos were collected every 12 hours, aged for 8 hours at room temperature and then examined for midline defects, first with a Leica MZ FLIII fluorescent stereomicroscope.
and then positives were more closely examined with a Zeiss Axioskop II fluorescent microscope and either a Zeiss Pascal or 710 confocal microscope. Homozygous mutant embryos were identified based on the absence of Ki-GFP fluorescence.

DNA sequence analysis of the mam^{AC} mutant
Genomic DNA was extracted from homozygous mam^{AC} mutant embryos and used as a template to amplify all mam coding exons. After PCR amplification, each coding exon was cloned into the pSTBlue-1 vector (Novagen) and then plasmids were sent to Alpha BioLab, Inc. for sequencing. Sequence analysis was performed using the FinchTV program (Geospiza, Inc.) and indicates the mam^{AC} allele contains a point mutation that creates a premature stop codon. The resulting truncated protein ends at Mam residue 959, eliminating the C-terminal acid cluster and all 3 glycine-valine (GV) runs (Figure 2C). Based on comparison with mam deficiencies, the mam^{AC} mutation behaves as a strong loss of function allele (Figure S2).

Immunohistochemistry and in situ hybridization of embryos
Immunohistochemistry and in situ hybridization of whole mount embryos were performed as previously described [29,60]. The following primary antibodies were used: mouse anti-β-galactosidase (1:1000 Promega); rabbit anti-β-galactosidase (1:2000 Cappel); rabbit anti-En (1:100 Santa Cruz Biotech, Inc.); rat anti-Odd-skipped (1:100), guinea pig anti-Odd-skipped (1:100) and guinea pig anti-Runt (1:100 or 1:200 East Asian Distribution Center; EADC); rabbit anti-GFP (1:500 Molecular Probes, Invitrogen); rat anti-Single-minded (1:100 [69]); and rabbit anti-tyrosine hydroxylase (1:500 [70]) and mouse anti-Wrapper (1:5 Developmental Studies Hybridoma Bank). The anti-guinea pig Alexa 488, anti-guinea pig Alexa 568, anti-mouse Alexa 488, anti-mouse Alexa 568 (Molecular Probes, Invitrogen). Embryos were imaged with a Zeiss Pascal in the Forestry Department and Zeiss 710 laser scanning microscope in the Cellular and Molecular Imaging Facility at NCSU. To determine the number of cells belonging to each lineage, midline cells were labeled with specific markers and at least 8 thoracic segments within several embryos were counted and presented as the mean ± standard error of the mean (SEM) using stacked confocal images. The images shown are projections of multiple focal planes.

Supporting Information
Figure S1 Maternal mam^{AC} mutations have more severe midline glial phenotypes than zygotic mam^{AC} mutations. Midline glial cells were labeled with a wrapper antibody (green; A–D, F, G and I) and either a sim antibody (red; A) or the BP102 monoclonal antibody (red; D, E, G and H). (A) N^{55e11} mutant embryos do not express wrapper. The muscle phenotype characteristic of Notch mutants is indicated with the arrowhead. (B) Most embryos derived from mam^{AC} germline clones did not express wrapper, although (C) low levels were detected in a few embryos. (D–I) Midline glia within mam^{AC} mutant embryos contain extra processes that enwrap lateral axons compared to (D–F) wild type embryos. (D and G) The merge of wrapper and BP102 is shown. (A) Ventral and (B–I) ventrolateral views of whole mount embryos are shown and anterior is toward the top, left corner.

Figure S2 Unlike N^{55e11} mutants, homozygous mam deficiency embryos contain AMG. (A) A schematic map of regions uncovered by the mam deficiencies Df(2R)BSC18, Df(2R)50C-38 and Df(2R)BSC383 is shown. The top bar indicates the cytological bands that include the mam locus. Mam coding exons are indicated by green boxes and deletions are indicated with dotted lines. The entire mam locus is absent in deficiencies Df(2R)BSC18 and Df(2R)50C-38, and the N-terminal region is absent in Df(2R)BSC383. This chromosomal region also contains several genes other than mam that are not shown. (B) In wild type embryos, Wrapper is expressed at a high level in the AMG (arrow) and at a low level in the PMG (arrowhead). (C–E) Wrapper expression was present in all three mam deletions. The midline glia in embryos homozygous for the deficiencies (D) Df(2R)50C-38 and (E) Df(2R)BSC383 appeared more disorganized than in embryos homozygous for deficiency (C) Df(2R)BSC18, which may be due to the absence of additional genes within these deletions. Whole mount embryos were labeled with an anti-Wrapper (red) antibody and ventral views of stage 13 embryos are shown; anterior is toward the top, left corner.

Table S1 Comparison of MP1, H cell, mVUM midline neurons in wild type, mam^{AC} and N^{55e11} mutant embryos.
(DOC)

Table S2 Comparison of PMG in wild type and mam^{AC} mutant embryos during mid and late embryogenesis.
(DOC)

Acknowledgments
We are grateful to Wendy Neckamyer for the anti-TH antibody, Stephen Cohen for the UAS-mam flies, William Saxton, Benny Shiio and Pangkong Moua for fly lines used to generate germline clones, and Scott Wheeler and Stephen Crews for the fly lines and other valuable assistance and discussions. We would like to thank Trudy Mackay, Jim Mahaffey, Coby Schal and Laura Mathies for helpful advice and assistance; Kate Bridges, Kelly Daigle, Frances Haire, Lucy Muhirwa and Warren Perry for technical assistance; and Nadja Long, Laura Mathies and Megan Myrick for critical comments on the manuscript. We also thank the East Asian Distribution Center and Developmental Studies Hybridoma Bank for antibodies and the Bloomington Drosophila Stock Center for fly stocks.

Author Contributions
Conceived and designed the experiments: YZ RW EF PE. Performed the experiments: YZ RW EF AT PE. Analyzed the data: YZ RW AT PE. Contributed reagents/materials/analysis tools: YZ PE. Wrote the paper: YZ RW PE.

References
1. Doe CQ, Skeath JB (1996) Neurogenesis in the insect central nervous system. Current Opinion in Neurobiology 6: 18–24.
2. Guillenot F (2007) Spatial and temporal specification of neural fates by transcription factor codes. Development 134: 3771–3780.
3. Thor S, Thomas JB (2002) Motor neuron specification in worms, flies and mice: conserved and ‘lost’ mechanisms. Current Opinion in Genetics & Development 12: 558–564.
4. Radke F, Schweiguth F, Pear W (2005) The Notch ‘gospel’. Workshop on Notch signalling in development and cancer. Embo Reports 6: 1120–1125.
5. Skeath JB, Thor S (2003) Genetic control of Drosophila nerve cord development. Current Opinion in Neurobiology 13: 14–15.
6. Wheeler SR, Stagg SB, Crews ST (2008) Multiple Notch signaling events control Drosophila CNS midline neurogenesis, gliogenesis and neuronal identity. Development 135: 3071–3079.
36. Fryer CJLE, Turbachova I, Kintner C, Jones KA (2002) Mastermind mediates nuclear entry of Notch in Drosophila central nervous system. Developmental Biology 250: 244–247.

37. Kasaï Y, Nam Y, Weng AP, Aster JC, Blacklow SC (2005) Structural requirements for assembly of the CSL-Intracellular Notch1-Mastermind-like 1 transcriptional activation complex. Journal of Biological Chemistry 278: 21232–21239.

38. Wallberg AE, Pedersen K, Lundahl U, Roeder RG (2002) p300 and PCA act cooperatively to mediate transcriptional activation from chromatin templates by Notch intracellular domains in vitro. Molecular and Cellular Biology 22: 7812–7819.

39. Schulte A, Brand A (1999) Mastermind acts downstream of notch to specify neuronal cell fates in the Drosophila central nervous system. Developmental Biology 205: 287–295.

40. Vlodavsky B, Kumar A, Chaudhury P, Opraseuth J, Mortimer N, et al. (2004) Differential effects of Drosophila mastermind on asymmetric cell fate partitioning and neuroblast migration. Genetics 166: 217–224.

41. Jimenez F, Campos-Ortega JA (1982) Maternal effects of zygotic mutants affecting early neurogenesis in Drosophila. Wilhelm Roux's Archives of Developmental Biology 191: 191–201.

42. Watson JD, Wheeler SR, Stagg SB, Crews ST (2011) Drosophila hedgehog signaling in midline-enlarged mutant repression direct midline glia to alternative ensheathing and non-ensheathing fates. Development 138: 1203–1205.

43. Bergmann A, Tugemun G, Shilo BZ, Steller H (2002) Regulation of cell number by MAPK-dependent control of apoptosis: A mechanism for trophic gene signalling. Developmental Cell 2: 159–170.

44. Stemerding C, Jacobs JR (1997) Argos and Spitz group genes function to regulate midline glial cell number in Drosophila embryos. Development 124: 3770–3776.

45. Kolev V, Mandinova A, Guinea-Viniegra J, Hu B, Lefort K, et al. (2008) EGFR signalling as a negative regulator of Notch1 gene transcription and function in neuroblast differentiation. Nature Cell Biology 10: 902–911.

46. Noordermeer JN, Kopczyński CG, Fetzer RD, Bland KS, Chen WV, et al. (1998) Wrapping, a novel member of the Ig superfamily, is expressed by midline glia and is required for them to ensheathe commissural axons in Drosophila. Neuron 21: 991–1001.

47. Bossing T, Technau GM (2004) Structures of CSL, Notch and Mastermind proteins: piecing together an active transcription complex. Current Opinion in Structural Biology 14: 117–127.

48. Vied C, Kalderon D (2009) Hedgehog-stimulated stem cells depend on non-canonical activity of the Notch co-activator Mastermind. Development 136: 2177–2186.

49. Nambu JR, Franks RG, Hu S, Crews ST (1990) The single-minded gene of Drosophila melanogaster. Genetics 118: 123–133.

50. Nam Y, Sliz P, Song LY, Aster JC, Blacklow SC (2006) Structural basis for cooperativity in recruitment of MAML coactivators to Notch transcription complexes. Genes Dev 15: 143–151.

51. Nam Y, Sliz P, Song LY, Aster JC, Blacklow SC (2006) Structural basis for cooperativity in recruitment of MAML coactivators to Notch transcription complexes. Genes Dev 15: 143–151.

52. Woodhoo A, Alonso MBD, Droggiti A, Turmaine M, D'Antonio M, et al. (2009) Crystal structure of the CSL-Notch-Mastermind ternary complex bound to DNA. Cell 124: 985–996.

53. Stemmer R, Crews ST (1998) Control of cell lineage-specific development and transcription by HD-JH-PAS proteins. Genes & Development 12: 607–620.

54. Smoller D, Friedel C, Schmid A, Bettler B, Lam L, et al. (1990) The Drosophila neurogenic locus mastermind of Drosophila melanogaster. Genetics 118: 123–133.

55. Xu T, Rebay I, Fleming RJ, Scottgale TN, Artavanis-Tsakonas S (1990) The single-minded gene of Drosophila is required for the expression of genes important for the development of Drosophila melanogaster. Genetics 122: 429–438.

56. Xu T, Rebay I, Fleming RJ, Scottgale TN, Artavanis-Tsakonas S (1990) The single-minded gene of Drosophila is required for the expression of genes important for the development of Drosophila melanogaster. Genetics 122: 429–438.

57. Wallberg AE, Pedersen K, Lundahl U, Roeder RG (2002) p300 and PCA act cooperatively to mediate transcriptional activation from chromatin templates by Notch intracellular domains in vitro. Molecular and Cellular Biology 22: 7812–7819.

58. Schulte A, Brand A (1999) Mastermind acts downstream of notch to specify neuronal cell fates in the Drosophila central nervous system. Developmental Biology 205: 287–295.

59. Vlodavsky B, Kumar A, Chaudhury P, Opraseuth J, Mortimer N, et al. (2004) Differential effects of Drosophila mastermind on asymmetric cell fate partitioning and neuroblast migration. Genetics 166: 217–224.

60. Yedvobnick B, Kumar A, Chaudhury P, Opraseuth J, Mortimer N, et al. (2004) Differential effects of Drosophila mastermind on asymmetric cell fate partitioning and neuroblast migration. Genetics 166: 217–224.

61. Jimenez F, Campos-Ortega JA (1982) Maternal effects of zygotic mutants affecting early neurogenesis in Drosophila. Wilhelm Roux's Archives of Developmental Biology 191: 191–201.

62. Watson JD, Wheeler SR, Stagg SB, Crews ST (2011) Drosophila hedgehog signaling in midline-enlarged mutant repression direct midline glia to alternative ensheathing and non-ensheathing fates. Development 138: 1203–1205.

63. Bergmann A, Tugemun G, Shilo BZ, Steller H (2002) Regulation of cell number by MAPK-dependent control of apoptosis: A mechanism for trophic gene signalling. Developmental Cell 2: 159–170.

64. Stemerding C, Jacobs JR (1997) Argos and Spitz group genes function to regulate midline glial cell number in Drosophila embryos. Development 124: 3770–3776.

65. Noordermeer JN, Kopczyński CG, Fetzer RD, Bland KS, Chen WV, et al. (1998) Wrapping, a novel member of the Ig superfamily, is expressed by midline glia and is required for them to ensheathe commissural axons in Drosophila. Neuron 21: 991–1001.

66. Bossing T, Technau GM (2004) Structures of CSL, Notch and Mastermind proteins: piecing together an active transcription complex. Current Opinion in Structural Biology 14: 117–127.

67. Vied C, Kalderon D (2009) Hedgehog-stimulated stem cells depend on non-canonical activity of the Notch co-activator Mastermind. Development 136: 2177–2186.

68. Kolev V, Mandinova A, Guinea-Viniegra J, Hu B, Lefort K, et al. (2008) EGFR signalling as a negative regulator of Notch1 gene transcription and function in neuroblast differentiation. Nature Cell Biology 10: 902–911.

69. Wallberg AE, Pedersen K, Lundahl U, Roeder RG (2002) p300 and PCA act cooperatively to mediate transcriptional activation from chromatin templates by Notch intracellular domains in vitro. Molecular and Cellular Biology 22: 7812–7819.
65. Xiao H, Hedrick LA, Nambu JR (1996) Alternate functions of the single-minded and rhomboid genes in development of the Drosophila ventral neuroectoderm. Mechanisms of Development 58: 65–74.

66. Giraldez AJ, Perez L, Cohen SM (2002) A naturally occurring alternative product of the mastermind locus that represses Notch signaling. Mechanisms of Development 115: 101–105.

67. Schweitzer R, Shilo BZ (1997) A thousand and one roles for the Drosophila EGF receptor. Trends in Genetics 13: 191–196.

68. Patel NH, Snow PM, Goodman CS (1987) Characterization and cloning of fasciclin-III: a glycoprotein expressed on a subset of neurons and axon pathways in Drosophila. Cell 48: 973–988.

69. Ward M, Mosher J, Crews S (1998) Regulation of bHLH-PAS protein subcellular localization during Drosophila embryogenesis. Development 125: 1599–1608.

70. Neckameyer WS, Woodrome S, Holt B, Mayer A (2000) Dopamine and senescence in Drosophila melanogaster. Neurobiology of Aging 21: 145–152.

71. JC R, V C (1998) Evidence for a novel Notch pathway required for muscle precursor selection in Drosophila. Mechanisms of Development 79: 39–50.